Suppression of malignant potentials of A549 human lung cancer cell line by downregulation of the β4-galactosyltransferase 1 gene expression

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Summary

Our previous study demonstrated that downregulation of transcription factor Specificity protein (Sp) 1 suppresses the malignant potentials of A549 human lung cancer cell line with the reduced β4-galactosylation of highly branched N-glycans on cell surface glycoproteins. The reduced β4-galactosylation was brought about by the decreased expression of the β4-galactosyltransferase (β4GalT) 1 gene. Herein, we examined whether the reduced β4-galactosylation by decreasing the β4GalT1 gene expression suppresses the malignant potentials of A549 cells. In the β4GalT1-downregulated cells, the β4-galactosylation of highly branched N-glycans was reduced in several glycoproteins such as lysosome-associated membrane protein-1 and E-cadherin. The anchorage-independent growth and migratory ability of the β4GalT1-downregulated cells decreased when compared with the control cells. Furthermore, the phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) decreased in the β4GalT1-downregulated cells. These results indicate that downregulation of the β4GalT1 gene decreases the β4-galactosylation of highly branched N-glycans and the phosphorylation of p44/42 MAPK, and suppresses the malignant potentials of A549 cells.

Key Words  β4-galactosyltransferase 1; β4-galactosylation; N-glycan; lung cancer; malignant potential, mitogen-activated protein kinase.
INTRODUCTION

Cancer cells show the abnormal social behaviors including altered cell adhesion, uncontrolled cell growth, and metastatic spread.\textsuperscript{1,2} Such behaviors have been shown to be associated with the altered structures of glycans attached to proteins.\textsuperscript{3,4} The changes are brought about by the altered expression of the glycosyltransferase genes at transcriptional level.

The Specificity protein (Sp) transcription factor family regulates the transcription of not only housekeeping molecules but also cancer-associated molecules.\textsuperscript{5} Sp1, one of the Sp transcription factor family, regulates many glycosyltransferases including β4-galactosyltransferase (β4GalT). β4GalT family consists of seven members with different substrate specificity and tissue distribution.\textsuperscript{6} Among β4GalT family, the transcription of the human β4GalT1, β4GalT3, β4GalT4, and β4GalT5 genes was reported to be regulated by Sp1.\textsuperscript{7-10}

Recently, we demonstrated that downregulation of Sp1 suppresses the malignant potentials of A549 human lung cancer cell line with the reduced β4-galactosylation of highly branched N-glycans on cell surface glycoproteins, which is resulted in the decreased expression of the β4GalT1 gene.\textsuperscript{7} The reduced β4-galactosylation was mainly observed for E-cadherin.\textsuperscript{7} Human β4GalT1 can transfer galactose (Gal) from uridine diphosphate-Gal to N-acetylglucosamine (GlcNAc) of highly branched N-glycans.\textsuperscript{11,12} However, direct evidence that the reduced β4-galactosylation of highly branched N-glycans by decreasing the β4GalT1 gene suppresses the malignant potentials of A549 cells remains to be clarified. In this study, the β4GalT1-downregulated cells were established from A549 cells by RNA interference technique, and the relationship between the reduced β4-galactosylation and suppression of the malignant potentials was examined.
MATERIALS AND METHODS

Cell culture

A human lung cancer cell line, A549, was maintained in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin and 50 µg/ml streptomycin.

Lectin and antibody

Horseradish peroxidase-conjugated leuko-agglutinating phytohemagglutinin (L-PHA) and *Ricinus communis* agglutinin-I (RCA-I) were obtained from Seikagaku Kogyo (Tokyo). Antibodies against p44/42 mitogen-activated protein kinase (MAPK) and phospho-p44/42 MAPK (T202Y204) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Antibodies against lysosome-associated membrane protein (LAMP)-1 and E-cadherin were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Plasmid construction

Downregulation of the β4GalT1 gene was conducted by RNA interference technique as described previously.7) The target of the nucleotide sequences was obtained from the human β4GalT1 gene (GenBank™ accession number: NM_001497) by siRNA Target Finder (Ambion, Austin, TX, U.S.A.). The target sequence to the β4GalT1 mRNA was confirmed to be unique by BLAST analysis, and then the following oligonucleotides, TS23-49 (5’-GATCCCTGGTCGGAGTCTCCACACTTCAAGAGAGTGTGGAGAC- TCCGACCAGTTA-3’) and TS23-50 (5’-AGCTTAACTGGTCGGAGTCTC- CACACTCTCTTTGAAGTGTGGAGACTCCGACCAGG-3’), were designed. The double-stranded DNA fragment was prepared by annealing the oligonucleotides, and then
inserted into the BamH I-Hind III sites of the pSilencer4.1-hygro vector (Ambion), to construct pSilencer/siβ4GalT1. pSilencer/Negative control vector (Ambion) was utilized as a control.

Transfection

In order to generate the control and β4GalT1-downregulated cell clones, pSilencer/Negative control vector and pSilencer/siβ4GalT1 were transfected into A549 cells, respectively, as described previously. They were cultured for 72 hours, and then the culture medium was exchanged to DMEM supplemented with 10% FCS and 0.5 mg/ml hygromycin B. The cells were maintained continuously for two weeks. The hygromycin-resistant clones were isolated by limited dilution.

Real-time quantitative PCR analysis

Real-time quantitative PCR analysis was carried out as described previously. As an internal control, the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcript was used.

Western blotting

The membrane glycoprotein samples were prepared from the control and β4GalT1-downregulated cells by acetone precipitation, and analyzed by lectin blotting using L-PHA and RCA-I. Immunoblotting was performed with antibodies against p44/42 MAPK and phospho-p44/42 MAPK as described previously. The band intensity of p44/42 MAPK and phospho-p44/42 MAPK was measured by ImageJ analysis (version 1.50i), and ratios between those of control and β4GalT1-downregulated cells were evaluated.
Immunoprecipitation

The cell lysates of A549 cells were immunoprecipitated by incubating with antibody against LAMP-1 or E-cadherin, and μMACS Protein G MicroBeads (Miltenyi Biotec Inc., Auburn, CA, U.S.A.) according to the manufacturer’s instructions. The immunoprecipitated samples were subjected to lectin blotting with L-PHA.

Growth curve analysis

Growth curve analysis was performed with a Celltiter 96 aqueous one solution cell proliferation assay (Promega Corp., Madison, WI, U.S.A.).

Soft agar assay

Anchorage-independent cell growth in soft agar was conducted using 12-well tissue culture plates as described previously. The tissue culture plates were maintained for two weeks, and then the numbers of colonies larger than 60 μm in five fields were counted under a microscope.

Cell migration assay

Cell migratory ability was measured by wound healing assay. Briefly, the confluent cell monolayers in tissue culture dishes (ϕ100 mm) were scratched with 1000 μl blue plastic tip, and then extensively washed with 10 mM phosphate-buffered saline (pH 7.4). The cells were maintained in DMEM supplemented with 2% FCS and 50 ng/ml epidermal growth factor for 24 hours. Migration into wound area was observed with a phase-contrast inverted microscope and chronologically photographed. Cell migration area was evaluated by ImageJ (version 1.50i), and then the relative wound area was expressed by taking the area at the start
time as 100%.

Statistical analysis

To analyze the significant differences, the data were subjected to Student’s \( t \)-test for the comparison of the means of two independent groups, and one-way ANOVA followed by Bonferroni’s test for the comparison of the means of more than two independent groups.
RESULTS AND DISCUSSION

Preparation of β4GalT1-downregulated cell clones

Our previous study showed that the expression level of the β4GalT1 gene decreases to 45% of control by downregulation of Sp1. Two of the β4GalT1-downregulated cell clones showed that the expression levels of the β4GalT1 gene decrease to 58% in clone 1 and to 37% in clone 2 as compared with the control clone (Fig. 1). The clones 1 and 2 were named as GT1-D1 and GT1-D2, respectively. In order to elucidate whether or not the reduced β4-galactosylation suppresses the malignant potentials of A549 cells, we used GT1-D2 as the β4GalT1-downregulated cells for the further study.

β4GalT1-downregulation decreases β4-galactosylation of N-glycans

The staining with Coomassie Brilliant Blue (CBB) exhibited that the membrane protein components appear similar between the control clone and GT1-D2 (Fig. 2-CBB). To show the changes in the β4-galactosylation of N-glycans by decreasing the β4GalT1 gene expression, lectin blotting was carried out using the sulfuric acid-treated blots, in which sialic acid attached to galactose residues was removed. When the blots were incubated with RCA-I, the decreased binding of RCA-I was observed for several glycoproteins including 90, 94, 96, and 120 kDa of glycoproteins in GT1-D2 (Fig. 2-RCA-I). Similarly, the decreased binding of L-PHA in GT1-D2 was observed for several glycoproteins, which correspond to those reacted with RCA-I (Fig. 2-L-PHA). As the carbohydrate-binding specificities of lectins showed that RCA-I interacts with the Galβ1→4GlcNAc group, while L-PHA interacts with highly branched N-glycans containing the Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man branch, the amounts of the β4-galactosylated and highly branched N-glycans decreased in GT1-D2. The results indicate that downregulation of the β4GalT1 gene inhibits the β4-galactosylation of highly branched N-glycans, since the galactosylation of highly branched N-glycans is required for
L-PHA-binding. Our previous study demonstrated that the 90 kDa and 94 kDa of glycoproteins are LAMP-1, and the 120 kDa of glycoprotein is E-cadherin, as revealed by immunoblotting of A549 cells. To confirm that LAMP-1 and E-cadherin contain highly branched N-glycans, we performed immunoprecipitation with antibodies against LAMP-1 and E-cadherin followed by lectin blotting with L-PHA. The results showed that the immunoprecipitated LAMP-1 and E-cadherin interact with L-PHA (Fig. 3), indicating that LAMP-1 and E-cadherin contain highly branched N-glycans. However, the 96 kDa of glycoprotein is unknown, and has to be characterized by the further study. These results suggest that downregulation of the β4GalT1 gene decreases the β4-galactosylation of highly branched N-glycans on several glycoproteins including LAMP-1 and E-cadherin.

β4GalT1-downregulation suppresses malignant potentials

Our previous study revealed that downregulation of Sp1 suppresses the growth rate, anchorage-independent growth, and migratory ability of A549 cells with the reduced β4-galactosylation of highly branched N-glycans. Next, whether or not these potentials of A549 cells were suppressed by decreasing the β4GalT1 gene expression was analyzed. Growth curve analysis showed that the growth rate of GT1-D2 decreases by 18-22% in a growth phase (Fig. 4). Soft agar assays exhibited that the average numbers of colonies formed with the control clone and GT1-D2 were $21.3 \pm 4.0$ and $5.3 \pm 1.7$, respectively (Fig. 5). Furthermore, cell migration assay showed that the wound area filled with the control clone and GT1-D2 at 24 hours were 58% and 32%, respectively (Fig. 6). These results indicate that downregulation of the β4GalT1 gene suppresses the growth rate, anchorage-independent growth, and migratory ability of A549 cells, which is similar to the Sp1-downregulated cells.

β4GalT1-downregulation decreases phosphorylation of p44/42 MAPK

The epidermal growth factor receptor (EGFR)-MAPK signaling has been shown to be
critical for the cell growth, anchorage-independent cell growth, and migration of several human cancer cell lines by using MAPK kinase inhibitors such as PD98059 and U0126.\textsuperscript{18,19)} Since the downregulation of the $\beta_4$GalT1 gene expression suppresses the cell growth, anchorage-independent cell growth, and migration of A549 cells, the phosphorylation of p44/42 MAPK was analyzed. In GT1-D1 and GT1-D2, the phosphorylation of p44/42 MAPK decreased depending on the reduced expression levels of the $\beta_4$GalT1 gene (Fig. 7). The results indicate that the MAPK signaling is suppressed according to the reduced expression levels of the $\beta_4$GalT1 gene.

In the present study, we revealed that by decreasing the $\beta_4$GalT1 gene expression, the reduced $\beta_4$-galactosylation of highly branched $N$-glycans is occurred on several glycoproteins including LAMP-1 and E-cadherin, and the malignant potentials of A549 cells are suppressed. The reduced $\beta_4$-galactosylation of highly branched $N$-glycans is considered to be common between the Sp1- and $\beta_4$GalT1-downregulated cells. Since highly branched $N$-glycans are crucial for the malignant potentials of cancer cells,\textsuperscript{20,21)} downregulation of the $\beta_4$GalT1 gene contributed to inhibit the biosynthesis of the highly branched $N$-glycans and suppressed the malignant potentials of A549 cells. Alternatively, the sialylation of glycans is also important for the malignant potentials of cancer cells.\textsuperscript{22)} The reduction of the terminal galactose residues by downregulation of the $\beta_4$GalT1 gene may result in the decreased amounts of sialic acid linked to galactose residues, which is considered to suppress the malignant potentials of A549 cells. In fact, the desialylation of cell surface glycoproteins by overexpression of sialidase NEU1 decreased the cell growth, anchorage-independent cell growth, and metastasis of B16-BL6 mouse melanoma cell line.\textsuperscript{23)} Moreover, the reduced sialylation of integrin $\beta_4$ in HT29 human colon cancer cell line by overexpression of NEU1 suppressed the cell migration, metastasis, and the phosphorylation of p44/p42 MAPK.\textsuperscript{24)} From these reports, it could be possible that the reduced sialylation of cell surface glycoproteins brought about by downregulation of the $\beta_4$GalT1 gene suppresses the cell growth, anchorage-independent cell growth, and migration of A549 cells.

LAMP-1 has been shown to contain heavily sialylated glycans, and enhance the malignant
potentials such as migration and invasion. As the reduced β4-galactosylation of highly branched N-glycans was observed for LAMP-1 in the β4GalT1-downregulated cells, the sialylation of LAMP-1 may decrease in the β4GalT1-downregulated cells, which leads to the suppression of the migration of A549 cells. With regard to the cell growth, the reduction in the cell number was observed for the β4GalT1-downregulated cells. The neuraminidase of H5N1 influenza A virus has been shown to cleave sialic acid from LAMP-1 at an early stage of viral infection, thereby inducing lysosomal rapture and cell death of A549 cells. The study suggests that the decreased cell number of the β4GalT1-downregulated cells is ascribed to cell death by the reduced sialylation of LAMP-1.

In the β4GalT1-downregulated cells, the decreased phosphorylation of p44/42 MAPK was observed. The decreased phosphorylation of p44/p42 MAPK by MAPK kinase inhibitors suppressed the cell growth, anchorage-independent cell growth, and cell migration. Therefore, it is considered that the suppression of the malignant potentials of A549 cells by downregulation of the β4GalT1 gene is due to the decreased phosphorylation of p44/42 MAPK. Since EGFR and E-cadherin have been shown to be co-localized on the cell surface, the reduced β4-galactosylation of highly branched N-glycans on E-cadherin may modulate the function of EGFR and decrease the phosphorylation of p44/42 MAPK. On the other hand, the majority of LAMP-1 are present in lysosomal membrane but a small portion is also expressed on cell surface. Although there is no report on the relationship between LAMP-1 and MAPK, the possibility that the reduced β4-galactosylation of highly branched N-glycans on LAMP-1 decreases the phosphorylation of MAPK can not be excluded. It is of interest to analyze whether LAMP-1 is involved in the EGFR-MAPK signaling.

In conclusion, the present study demonstrates that downregulation of the β4GalT1 gene decreases the β4-galactosylation of highly branched N-glycans and the phosphorylation of p44/42 MAPK, and suppresses the malignant potentials of A549 cells. Analysis of the detailed molecular mechanism of the relationship between the reduced β4-galactosylation and signaling molecules will provide a new strategy for cancer therapy.
Acknowledgments

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (23590070 to TS) and Presidential Research Grant from Nagaoka University of Technology.
Conflict of Interest

The authors declare no conflict of interest.
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Fig. 1. Expression levels of the β4GalT1 gene. Real-time quantitative PCR was carried out for the control and β4GalT1-downregulated cells (GT1-D1 and GT1-D2). The data are presented as mean ± standard deviation from three independent experiments. *p < 0.05 and **p < 0.01 referred to that of control cells.
Fig. 2. Lectin blotting of membrane glycoprotein samples from the control clone (a) and GT1-D2 (b). The blots were stained with CBB or incubated with RCA-I or L-PHA. The arrows and arrowhead indicate the migratory positions of LAMP-1 and E-cadherin, respectively, as reported by our previous study (ref. 7).
Fig. 3. L-PHA-lectin blotting of immunoprecipitated LAMP-1 and E-cadherin. The cell lysates of A549 cells were subjected to immunoprecipitation (IP) with antibody against LAMP-1 (A) or E-cadherin (B). The blots of immunoprecipitates were probed with anti-LAMP-1 and -E-cadherin antibodies for immunoblotting (IB) and with L-PHA for lectin blotting (LB).
Fig. 4. Growth curves of GT1-D2. The values indicate mean ± standard deviation from three independent experiments. Closed and open circles show the control clone and GT1-D2, respectively.
Fig. 5. Anchorage-independent growth of GT1-D2. Numbers of colonies formed with the control clone and GT1-D2 in soft agar. The data are presented as mean ± standard deviation from three independent experiments. *p < 0.05 referred to that of control cells.
Fig. 6. Migratory ability of GT1-D2. The values indicate mean ± standard deviation from three independent experiments. Closed and open circles show the control clone and GT1-D2, respectively.
Fig. 7. Phosphorylation of p44/42 MAPK in GT1-D1 and GT1-D2. The blots were incubated with antibodies against p44/42 MAPK and aphospho-p44/42 MAPK. The ratio of phosphorylated MAPK against MAPK between the control and β4GalT1-downregulated cells is shown by taking control as 1.0.