The regulatory roles of cell surface sialylation and N-glycans in human B cell lymphoma cell adhesion to galectin-1

OSAMU SUZUKI, YOSHIHIRO NOZAWA and MASAFUMI ABE

First Department of Pathology, School of Medicine, Fukushima Medical University, Fukushima, Japan

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Abstract. Surface sialylation and glycosylation of tumor cells is known to affect various biological phenomena. In the present study, we analyzed the regulatory roles of cell surface sialylation in cell adhesion to galectin-1 in the human diffuse large B cell lymphoma (DLBCL) cell line, HBL-2, and Burkitt's lymphoma cell line, HBL-8. Vibrio cholerae neuraminidase treatment enhanced HBL-2 cell adhesion to galectin-1, suggesting that sialic acid inhibits HBL-2 cell adhesion to galectin-1. The data from employing two different neuraminidases, Vibrio cholerae and Newcastle disease virus neuraminidase, showed that α2,6-linked sialic acid plays an important role in the inhibition of HBL-2 cell adhesion to galectin-1. In addition, neuraminidase treatment enhanced the cell adhesion to galectin-1 much more with the highly sialylated HBL-8 3G3 clone than with the hyposialylated HBL-8 3D2 clone. Flow cytometric analysis revealed the expression of partially sialylated L-PHA reactive oligosaccharides on the surfaces of HBL-2 cells. Swainsonine (SW) treatment also enhanced HBL-2 cell adhesion to galectin-1. These data indicate that SW treatment decreased sialylated L-PHA reactive oligosaccharides resulting in cell surface desialylation and leading to enhancement of cell adhesion to galectin-1. In conclusion, alteration of cell surface sialylation or N-glycan expression regulates cell adhesion to galectin-1 in human malignant lymphoma.

Introduction

Galectin-1 is one of the endogenous lectins which react with galactose residues of lactosamine structures (1-5). The biological functions of galectins are reported to be the regulation of tumor cell adhesion and immune reactions (6,7). Galectin-1 plays an important role in cell adhesion to the extracellular matrix (ECM) and ligands for galectin-1 are lactosamine structures of cell surface oligosaccharides in various tumor cells (6,7). Several reports have suggested that galectin-1 plays an important role in growth regulation through its interaction with CD45 N-glycans or O-glycans in immune reactions or tumor cell growth (8-15). Dennis *et al* reported that T cell growth is regulated by the interaction between galectin-3 and N-glycans of TCR (16), and galectin-1 was reported to induce cell death in B cell lymphoma (17,18) or T cell lymphoma (19).

Sialic acid plays a significant role in various biological phenomena, such as cell adhesion to the ECM and apoptosis (20,21) and is associated with patients' clinical outcomes (22,23).

We reported previously that expression of L-PHA reactive oligosaccharide, one of the N-glycans, is a useful marker for predicting the survival of patients with DLBCL (22,23). L-PHA reactive oligosaccharides are synthesized by N-acetylglucosaminyl-transferase-V (GnT-V) and the transcription of GnT-V is regulated by proto-oncogenes, such as the Ets family (24). GnT-V cDNA was cloned by Saito *et al* (25). Several previous reports suggested that high expression of GnT-V is correlated with poor prognosis in colon carcinoma (26), and that increased expression of L-PHA reactive oligosaccharides is correlated with a high clinical stage of colon carcinoma and malignant transformation in breast neoplasia (27). In addition, other reports showed a relationship between GnT-V activity in human hepatocellular carcinoma tissue and metastasis (28-30), and between increased GnT-V activity in pancreatic carcinoma and metastasis (31). On the other hand, loss of L-PHA reactive oligosaccharides is closely related with a poor prognosis in non-small cell lung cancer (32). The expression of N-glycosylation is closely associated with the clinical outcome of patients with various tumors. N-glycosylation affects the adhesive properties of $ß_1$ integrin to ECM (33) and also affects lysosomal associated membrane protein-1 (LAMP-1) which is an N-glycan carrier protein to ECM (34). Cell surface N-glycosylation might regulate adhesion or migration of tumor cells to ECM.

In the present study, we determined whether alterations in cell surface sialylation or N-glycan expression affect cell adhesive properties to galectin-1 in human lymphoma cell lines, HBL-2 (diffuse large B cell lymphoma cell line) and HBL-8 (Burkitt lymphoma cell line).

Materials and methods

Cell lines. The HBL-2 cell line was established in our laboratory from a patient who had diffuse large B cell lymphoma.

Correspondence to: Dr Osamu Suzuki, Department of Pathology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan
E-mail: osuzuki@fmu.ac.jp

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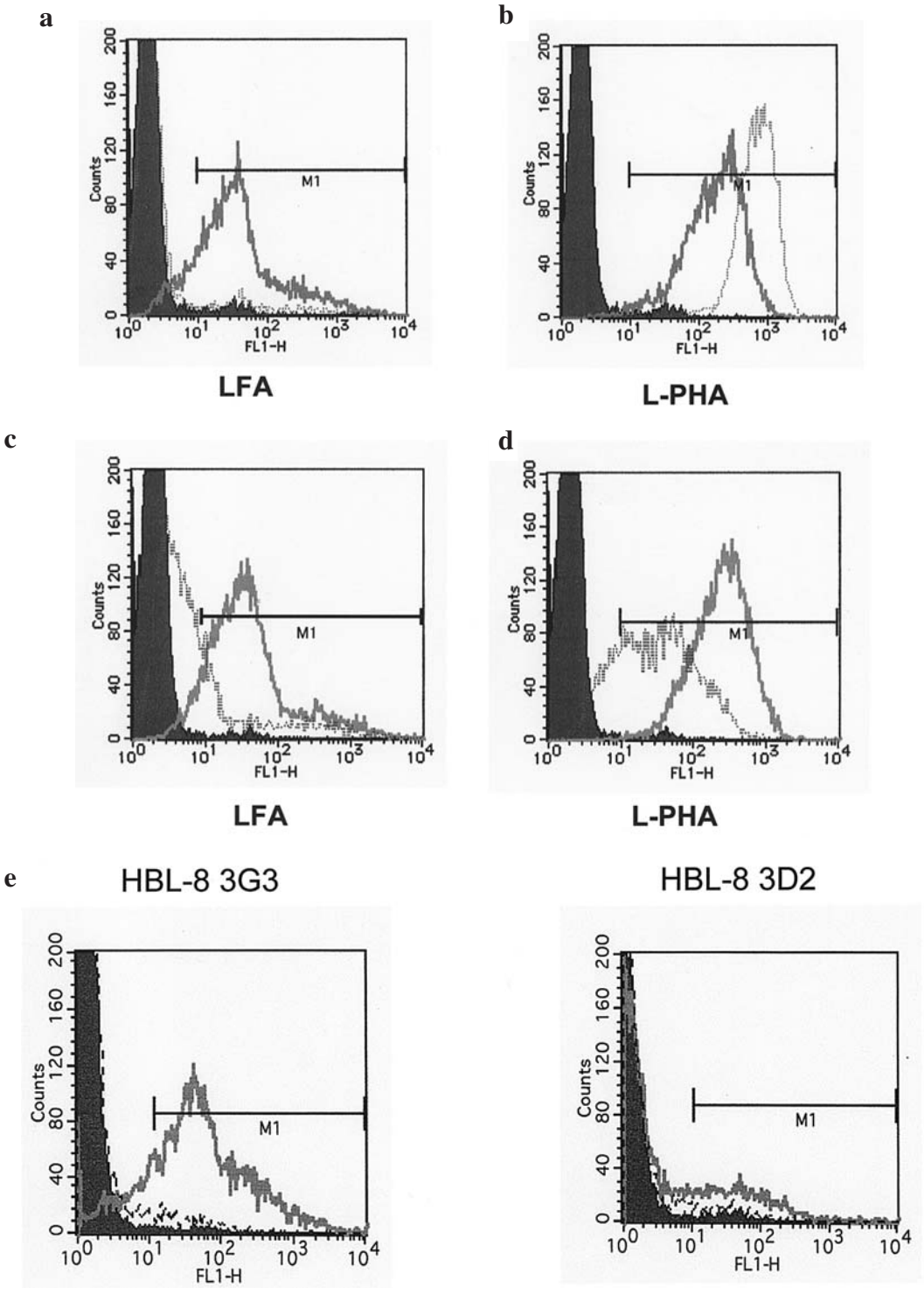

Figure 1. Flow cytometric analysis of alteration of cell surface sialylation and N-glycosylation. Thick line represents lectin reactivity without neuraminidase treatment and dotted line with Vibrio cholerae neuraminidase treatment. (a) LFA lectin reactivity was decreased by neuraminidase treatment of HBL-2. Neuraminidase treatment removed cell surface sialic acids. (b) L-PHA lectin reactivity was increased by neuraminidase treatment of HBL-2. L-PHA reactive oligosaccharides were partially sialylated. (c) LFA lectin reactivity was decreased by swainsonine treatment of HBL-2. Swainsonine treatment resulted in reduction of cell surface sialic acid. (d) L-PHA lectin reactivity was decreased by swainsonine treatment of HBL-2. Swainsonine treatment resulted in reduction of N-linked oligosaccharides. (e) LFA lectin reactivity was found in the 3G3 clone but not in the 3D2 clone of HBL-8. 3G3 clone cell surfaces were sialylated but the 3D2 clone cells were not.

The HBL-2 cells were grown at 37˚C in 5% CO_2 in RPMI-1640 containing 15% fetal calf serum (35).

HBL-8, a human Burkitt's lymphoma cell line which was established in the Department of Pathology of Fukushima Medical University (36), was cultured in RPMI-1640 containing 15% fetal calf serum (FCS). Two clones of HBL-8 (3G3 and 3D2) which showed different reactivities to SBA or VVA lectins were established and they showed different metastatic capacities in the severe combined immunodeficiency (SCID) mouse model (36). Their amounts of cell surface

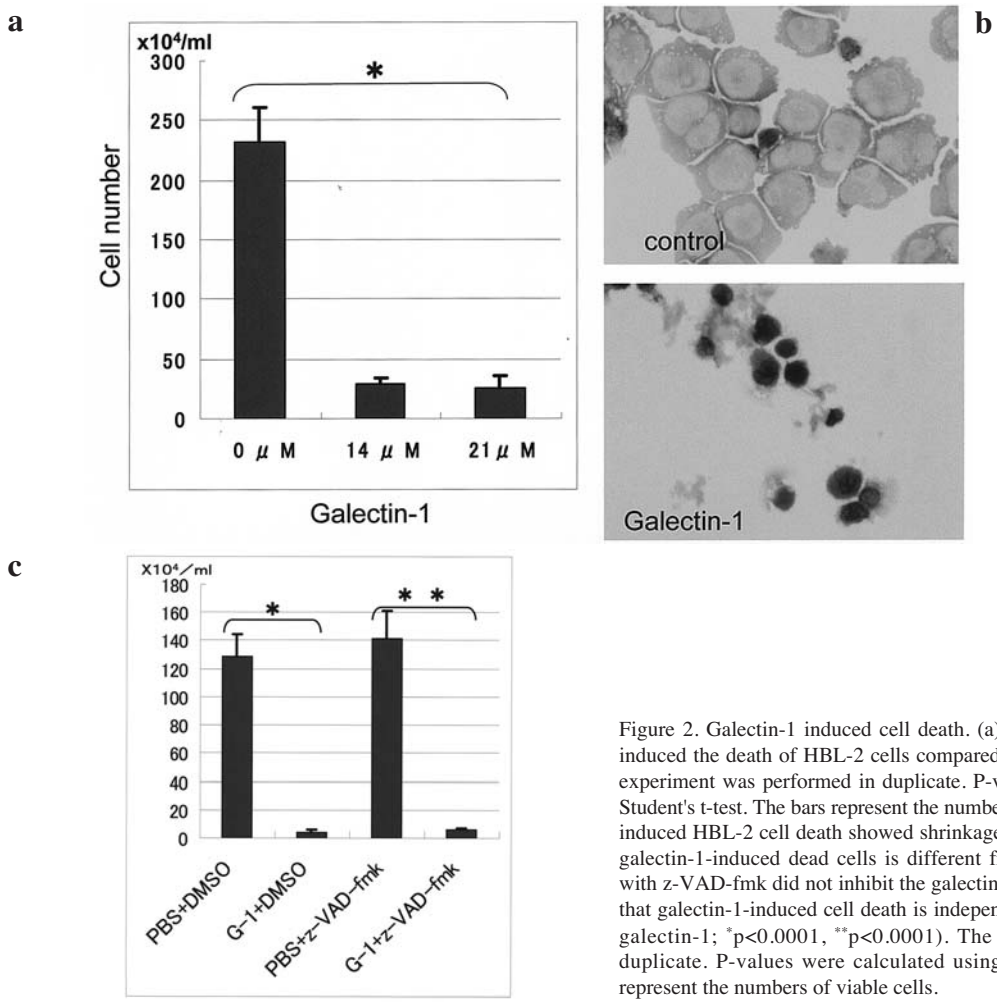

Figure 2. Galectin-1 induced cell death. (a) Galectin-1 treatment markedly induced the death of HBL-2 cells compared to PBS alone ($^{*}p<0.0001$). The experiment was performed in duplicate. P-values were calculated using the Student's t-test. The bars represent the numbers of viable cells. (b) Galectin-1-induced HBL-2 cell death showed shrinkage of cell volume. Morphology of galectin-1-induced dead cells is different from apoptosis. (c) Pretreatment with z-VAD-fmk did not inhibit the galectin-1-induced cell death, indicating that galectin-1-induced cell death is independent of caspase activation (G-1, galectin-1; $^{*}p<0.0001$, $^{**}p<0.0001$). The experiment was performed in duplicate. P-values were calculated using the Student's t-test. The bars represent the numbers of viable cells.

sialic acid are different due to different expressions of mRNA of uridine diphosphate-N-acetylglucosamine2-epimerase (UDP-GlcNAc2- epimerase), which is a key enzyme in sialic acid biosynthesis (20).

Reagents. Biotinylated lectins, Phaseolus vulgaris leuko-aggrutinating lectin (L-PHA) and Limax flavus (LFA), were purchased from E.Y. Laboratories (San Mateo, CA, USA). Swainsonine (SW) (S9263, Sigma), and the amino-acid sequences of galaptin (G8777, Sigma), which is the same as bovine galectin-1, were confirmed by Internet NBCI Sequence Viewer. Vibrio cholerae neuraminidase was from Roche, Germany, and Newcastle disease virus neuraminidase was from Boehringer Mannheim, Germany. Caspase inhibitor [lyo-philized carbobenzoxy-L-valyl-ß-methyl-L-aspart-1-fluoromethane (z-VAD-fmk)] was from Peptide Institute Inc., Japan, and was dissolved in dimethyl sulfoxide (DMSO).

Flow cytometry. Briefly, $5x10^5$ HBL-2 cells were suspended in 100 μl phosphate-buffered saline (PBS), incubated at 4˚C for 20 min with 5 μl biotinylated lectins, L-PHA or LFA, and washed twice with PBS. Then, cells were incubated at 4˚C for 20 min with 5 μl avidin-FITC (Vector Laboratories Inc., Burlingame, CA, USA) and washed twice with PBS. Fluorescent intensities were analyzed on a FACScan (Becton-Dickinson, Mountain View, CA, USA). To analyze cell surface sialylation, $6x10^6$ cells were incubated at 37˚C for 30 min in 200 μl RPMI-1640 containing 15% FCS and 40 μl of 1U/ml Vibrio Cholerae neuraminidase before incubation with biotinylated L-PHA or LFA lectins.

Alteration of N-glycans by swainsonine. HBL-2 cells ($5x10^5$) were incubated at 37˚C for 24 h in 20 ml SW containing medium [4 μl of SW solution (0.5 μg/μl in ethanol) was added to 20 ml 15% FCS RPMI-1640, at a final concentration of 0.1 μg/ml]. Then L-PHA or LFA reactivities to HBL-2 cells were analyzed by flow cytometry as described above.

Galectin-1-induced cell death. HBL-2 cells were grown in RPMI-1640 containing 15% FCS. Then, 100 μl of $5x10^5$/ml cells was added to each well of a 96-well microculture plate, and the cells were cultured for 24 or 48 h, with or without 14 or 21 μM bovine galectin-1 with or without the broad caspase inhibitor, z-VAD-fmk (120 μM). Then, the cells in each well were counted using the trypan blue exclusion method. All experiments were performed in triplicate. Giemsa-stained cytospin cell preparations were evaluated for morphological changes in galectin-1 induced cell death.

Cell adhesion assay. Tissue culture plates with 96 wells were coated with galectin-1 (5, or 10 μg/well) and dried at room

temperature overnight. Each well was washed with 100 μl PBS and was filled with RPMI-1640 containing 15% bovine serum albumin (BSA) 15% FCS, and the plates were incubated at 37˚C for 60 min. After aspiration of the medium, HBL-2 or HBL-8 cells, with or without SW treatment (final concentration 1 μg/ml at 37˚C for 24 h), and with or without neuraminidase [from Vibrio cholerae (VC) or Newcastle disease virus (NDV): final concentration 0.2 U/ml at 37˚C for 30 min] treatment as described above, were added to each well and incubated at 37˚C for 2 h. After aspiration of the medium, PBS was added to each well and then aspirated to remove non-adhered cells. Then, 100 μl of 3.7% formaldehyde was added to each well to fix the adhesive cells at RT for 40 min. After aspiration of formaldehyde, 100 μl of 0.1% crystal violet was added to each well and the plates were incubated at RT for 40 min. After washing twice, 100 μl of 10% acetic acid was added to each well and the absorbance at 570-655 nm was determined using an ELISA plate reader (37). The adhesive capacity to galectin-1 was evaluated by subtracting the absorbance in non-coated wells from that in coated wells.

Results

Flow cytometric analysis of alteration of cell surface sialylation and N-glycosylation. LFA lectin reactivity was decreased by neuraminidase treatment (Fig. 1a), but L-PHA lectin reactivity was increased by neuraminidase treatment (Fig. 1b). LFA lectin (Fig. 1c) and L-PHA lectin (Fig. 1d) reactivities were decreased by swainsonine treatment. LFA lectin reactivity was found in the 3G3 clone but not in the 3D2 clone of HBL-8 (Fig. 1e).

Galectin-1-induced cell death. Galectin-1 treatment markedly induced the death of HBL-2 cells compared to PBS alone. (Fig. 2a, $^{*}p<0.0001$). The galectin-1-induced death of HBL-2 cells showed shrinkage of cell volume (Fig. 2b). Cell death by galectin-1 was not inhibited by the broad caspase inhibitor, z-VAD-fmk, suggesting that galectin-1-induced cell death is independent of caspase activation (Fig. 2c, $^{*}p<0.0001$).

Adhesion of HBL-2 cells to galectin-1. Vibrio cholerae neuraminidase treatment increased the number of cells adherent to galectin-1 compared to untreated cells (Fig. 3a, $^{*}p=0.006$, $^{**}p=0.0139$). The number of cells adherent to galectin-1 was increased by Vibrio cholerae (VC) neuraminidase treatment but not by treatment with Newcastle disease virus (NDV) neuraminidase (Fig. 3b, $^{*}p=0.0266$). The number of adherent cells was also increased by SW treatment (Fig. 3c, $^{*}p=0.0284$).

Adhesion of HBL-8 clone cells to galectin-1. VC neuraminidase treatment tended to enhance the adhesion of HBL-8 3G3 clone cells more than HBL-8 3D2 clone cells (Fig. 4, $^{*}p=0.0641$, $^{**}p=0.2102$).

Discussion

The biological functions of galectins are reported to be regulation of immune reactions, tumor cell growth and cell

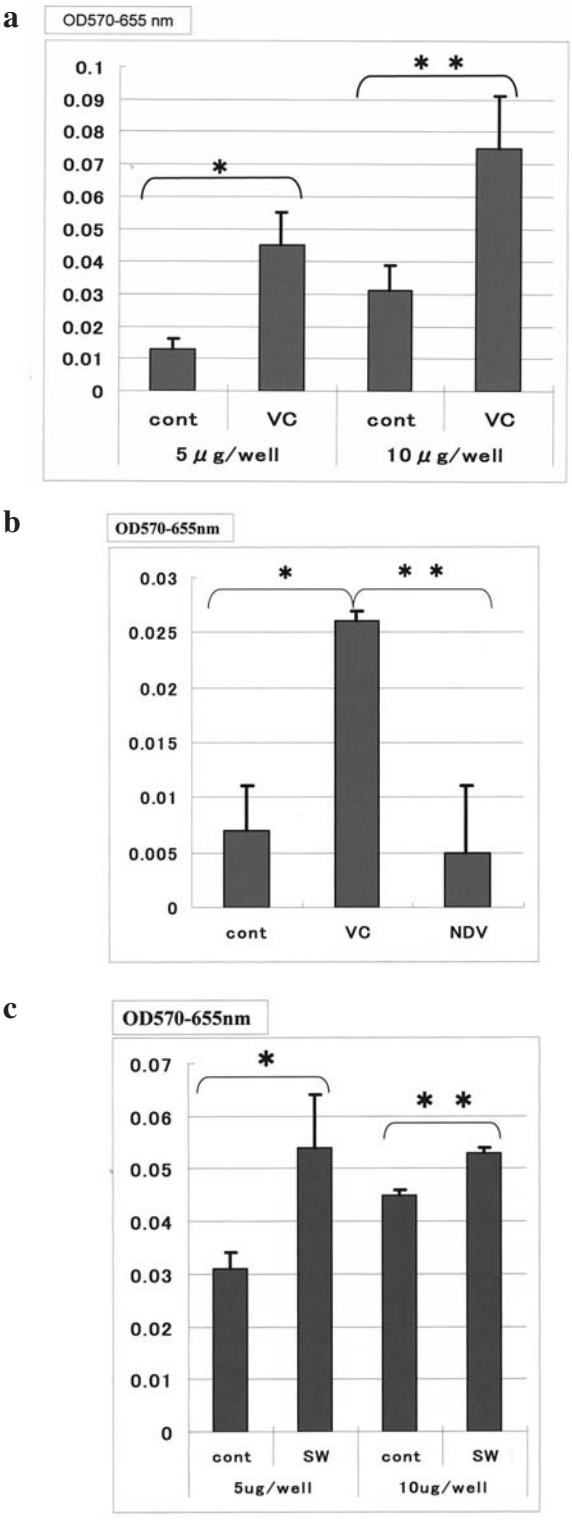

Figure 3. HBL-2 cell adhesion to galectin-1. The experiment was performed in triplicate. P-values were calculated using the Student's t-test. The bars represent the numbers of adherent cells. (a) Cell adhesion to galectin-1 with or without neuraminidase treatment. Neuraminidase treatment increased the number of cells adherent to galectin-1 compared to untreated cells ($^{*}p=0.006$, $^{**}p=0.0139$). VC, treatment with Vibrio cholerae neuraminidase; cont, not treated with neuraminidase. Concentration represents that of galectin-1 coated in 96-well plate. (b) Comparison of two different neuraminidases, the number of adherent cells to galectin-1 was increased by Vibrio cholerae (VC) neuraminidase treatment but not by Newcastle disease virus (NDV) neuraminidase treatment ($^{*}p=0.0266$, $^{**}p=0.0430$, galectin-1:10 μg/well). Cont, not treated with neuraminidase. (c) Cell adhesion to galectin-1 with or without SW treatment. The number of adherent cells was increased by SW treatment ($^{*}p=0.0213$, $^{**}p=0.010$, galectin-1). Cont, not treated with SW. Concentration represents that of galectin-1 coated in 96-well plate.

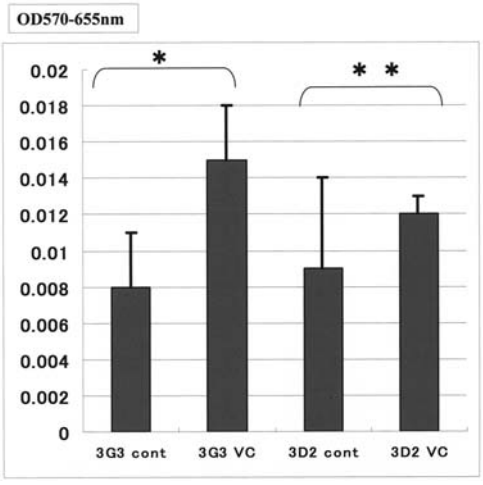

Figure 4. Adhesion to galectin-1 of cells from HBL-8 clones. The number of adherent cells to galectin-1 was increased by neuraminidase treatment. Cell adhesion to galectin-1 tended to be more enhanced by neuraminidase treatment of HBL-8 3G3 clone cells than of HBL-8 3D2 clone cells (*p=0.0641, **p=0.2102, galectin-1:10 μg/well). The experiment was performed in triplicate. P, p-values were calculated based on Student's t-test. VC, treatment with Vibrio cholerae neuraminidase. Cont, not treated with neuraminidase. The bars represent the numbers of adherent cells.

adhesion (1-15,17-19). Among the various galectins, galectin-1 plays an important role in cell adhesion to the ECM (6,7). For instance, human ovarian cancer cells can adhere to galaptin (galectin-1) through its interaction with cell surface lactosamine structures, and cell surface sialylation inhibits the interaction between cell surface lactosamine and galaptin (6). In the present study, cell surface sialylation of HBL-2 cells inhibited their adhesion to galectin-1 (Fig. 3a) and galectin-1 induced the death of HBL-2 cells (Fig. 2). Therefore, in human B cell lymphoma, sialylation of cell surface oligosaccharides appears to inhibit cell adhesion to galectin-1 and protect lymphoma cells from galectin-1-induced cell death, as shown in Fig. 5. The oligosaccharide ligands for galectin-1 are reported to be CD45 N-glycans (15). Further study is needed to clarify whether or not the oligosaccharide ligands for galectin-1 are CD45 N-glycans in HBL-2 and HBL-8 cell lines. The number of adherent cells to galectin-1 increased with VC neuraminidase treatment but not with NDV neuraminidase treatment (Fig. 3b). These data suggest that α2,6-linked sialic acid on their cell surfaces prevents HBL-2 cells from adhesion to galectin-1. α2,6-linked sialic acid inhibits galectin-1 binding to T cells (15,19), and galectin-1 binds α2,3-sialylated and non-sialylated terminal N-acetyllactosamine units, but not α2,6-sialylated ones (38). These data suggest that the type of sialylation linkage affects the binding ability of galectin-1 to terminal residues of oligosaccharides. Furthermore, we reported previously that α2,6-linked sialylation of terminal residues of L-PHA reactive oligosaccharides was closely associated with a worse prognosis for patients with DLBCL. It appears that the modulation of cell adhesion to galectin-1 by cell surface α2,6-linked sialylation may affect the malignant behavior of human DLBCL.

We reported recently that SW treatment could prevent the galectin-1-induced death of HBL-2 cells (39). In the present study, SW treatment enhanced cell adhesion to galectin-1 (Fig. 3c) and flow cytometric analysis showed that L-PHA reactive oligosaccharides were partially sialylated (Fig. 1b). This suggests that SW treatment reduces sialylated L-PHA reactive oligosaccharides resulting in cell surface desialylation, which enhances cell adhesion to galectin-1.

There were significant differences in the amounts of cell surface sialic acids in the 3G3 and 3D2 clones of HBL-8 cells, which could be attributed to differences in the expression of UDP-GlcNAc2-epimerase, which is a key enzyme in sialic acid biosynthesis (20). In the present study, there appeared to be differences in the enhancement of cell adhesion to galectin-1 by neuraminidase treatment between the highly sialylated clone, 3G3, and the hyposialylated clone, 3D2. UDP-GlcNAc2-epimerase may regulate cell adhesion to galectin-1 through the regulation of cell surface sialylation in HBL-8 lymphoma cells.

In conclusion, cell surface sialylation may inhibit galectin-1-induced cell death through inhibition of interactions between HBL-2 lymphoma cells and galectin-1. This may be associated with clinical outcome since DLBCL patients who had the sialylated type of L-PHA reactive oligosaccharides had a worse prognosis.

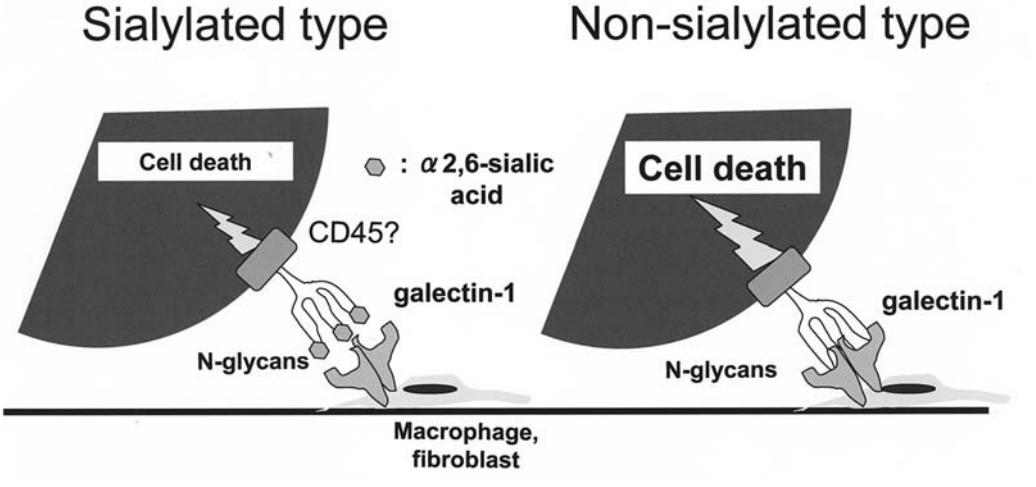

Figure 5. Schematic representation of regulatory roles of sialic acid in lymphoma cell adhesion to galectin-1.

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