

Cell surface N-glycosylation and sialylation regulate galectin-3-induced apoptosis in human diffuse large B cell lymphoma

OSAMU SUZUKI and MASAFUMI ABE

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

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Abstract. Galectin-3 is a soluble endogenous lectin in vertebrates and is implicated in a variety of biological functions, including tumor cell adhesion, proliferation, differentiation, apoptosis, cancer progression and metastasis. In the present study, we analyzed the role of galectin-3 in apoptosis in human malignant lymphoma. Galectin-3 induced cell death in the HBL-2 human diffuse large B cell lymphoma cell line. A morphological examination and annexin V assays revealed that galectin-3-induced cell death is consistent with apoptosis and swainsonine, a potent inhibitor of α -mannosidase II, which catalyzes the synthesis of complex type N-linked oligosaccharides, inhibited galectin-3-induced apoptosis in HBL-2 cells. These results suggest that galectin-3 induces apoptosis in HBL-2 cells by interacting with cell surface N-linked oligosaccharides. Furthermore, treatment of cells with *Vibrio Cholerae* neuraminidase enhanced galectin-3-induced apoptosis, suggesting that cell surface sialylation regulates galectin-3-induced apoptosis in human B cell lymphoma. In conclusion, our results indicate that galectin-3-induced apoptosis is regulated by cell surface expression of N-glycans and sialic acid in human diffuse large B cell lymphoma. This mechanism may be involved in the malignant behavior of human lymphoma cells.

Introduction

Galectin is a family of endogenous mammalian β -galactoside-binding lectins that appears to be involved in a variety of biological functions (1-4). Galectin also regulates cell growth (5), cell adhesion, differentiation and cell death (6-11). It has been revealed that some galectins are involved in apoptosis.

For example, galectin-1 and galectin-9 induce apoptosis of T and B lymphoma and melanoma cells (6-14).

Galectin-3 is expressed in anaplastic large B cell lymphoma and diffuse large B cell lymphoma (DLBCL) (15,16). Intracellular galectin-3, a novel anti-apoptotic molecule containing a bcl-2 family BH1 (NWGR) domain, inhibits cytochrome c release from mitochondria (17,18). In contrast, secreted extracellular galectin-3 can induce apoptosis by interacting with terminal galactose residues on cell surface glycans (19). In addition, N-glycans on CD45 are reported to regulate galectin-1-induced death of T cells (13) and an alteration in N-glycans on the cell surface can regulate cell death in DLBCL by interacting with galectin-1 (20,21).

Unlike galectin-1, the relationship of galectin-3 with cell surface N-glycans in human B cell lymphoma has not been elucidated. In the present study, we therefore examined the role of N-glycans and cell surface sialylation in galectin-3-induced cell death in HBL-2 human DLBCL cells.

Materials and methods

Cell line. The HBL-2 cell line was established in our laboratory from a patient with DLBCL (22). HBL-2 cells were grown in RPMI-1640 containing 15% fetal calf serum at 37°C in 5% CO₂.

Reagents. Human recombinant galectin-3 was obtained from R&D Systems, USA. Swainsonine (SW) was from Sigma, (St. Louis, MO, USA). The following biotinylated lectins were purchased from EY Laboratories (San Mateo, CA, USA): *Phaseolus vulgaris* leucoagglutinating lectin (L-PHA), peanut agglutinin (PNA) and *Limax flavus* agglutinin (LFA), a sialic acid-specific lectin. Avidin-FITC was from Vector Laboratories, Inc. (Burlingame, CA, USA). Neuraminidase from *Vibrio Cholerae* was from Roche, Germany.

Lectin flow cytometry. HBL-2 cells (1×10^7) were incubated at 37°C for 24 h in 20 ml RPMI/15% fetal calf serum containing 4 μ l of 0.5 μ g/ μ l SW in ethanol (final concentration 0.1 μ g/ml). Controls were incubated under the same conditions except with 4 μ l of ethanol in place of the SW solution. Then, the cells were suspended in 100 μ l phosphate-buffered saline (PBS) and incubated at 4°C for 20 min with 5 μ l biotinylated

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

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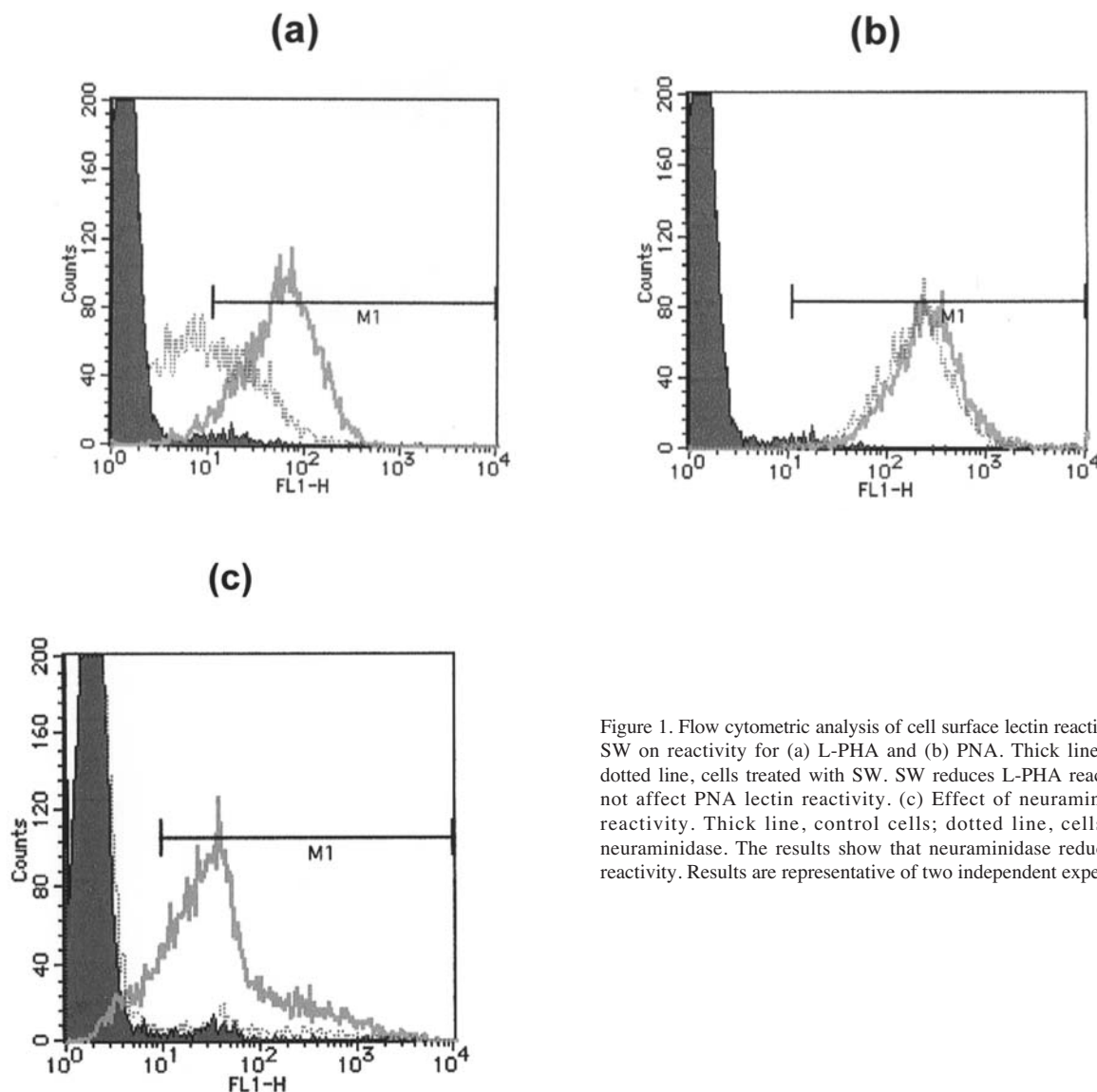


Figure 1. Flow cytometric analysis of cell surface lectin reactivities. Effect of SW on reactivity for (a) L-PHA and (b) PNA. Thick line, control cells; dotted line, cells treated with SW. SW reduces L-PHA reactivity but does not affect PNA lectin reactivity. (c) Effect of neuraminidase on LFA reactivity. Thick line, control cells; dotted line, cells treated with neuraminidase. The results show that neuraminidase reduces LFA lectin reactivity. Results are representative of two independent experiments.

lectin, L-PHA, or PNA, washed twice with PBS, incubated at 4°C for 20 min with 5 μ l avidin-FITC, and washed twice with PBS. Fluorescence intensities were analyzed with a FACS Calibur (Becton-Dickinson, Mountain View, CA, USA) (20).

To analyze cell surface sialylation, 5×10^6 cells were incubated at 37°C for 30 min in 200 μ l of RPMI-1640 containing 15% fetal calf serum and 40 μ l of 1 U/ml *Vibrio Cholerae* neuraminidase (Boehringer Mannheim, Germany; final concentration 0.2 U/ml) before incubation with biotinylated LFA. Then, the cells were suspended in 100 μ l PBS, incubated at 4°C for 20 min with 5 μ l biotinylated LFA lectin, washed twice with PBS, incubated at 4°C for 20 min with 5 μ l avidin-FITC and washed twice with PBS. Fluorescence intensities were analyzed with a FACS Calibur.

Galectin-3-induced death of HBL-2 cells. HBL-2 cells (5×10^5 /ml) were grown in 96-well plates containing 100 μ l of RPMI-1640/15% fetal calf serum and with or without 15 μ M human galectin-3. After 48 h, the number of viable cells remaining in each well was assessed by Trypan blue dye exclusion. All experiments were performed in duplicate or

triplicate. To evaluate the effect of inhibiting cell surface N-glycans on galectin-3-induced cell death, HBL-2 cells (1×10^7) were incubated in 20 ml of RPMI-1640/15% fetal calf serum containing 8 μ l of 2.5 μ g/ μ l SW in ethanol (final concentration 1 μ g/ml) at 37°C for 24 h before incubation with galectin-3. Controls were incubated under the same conditions except with 8 μ l of ethanol in place of the SW solution.

To evaluate the effects of cell surface desialylation in galectin-3-induced cell death, 5×10^6 cells were preincubated at 37°C for 30 min in 200 μ l of RPMI-1640/15% fetal calf serum and 40 μ l of 1 U/ml *Vibrio Cholerae* neuraminidase (final concentration 0.2 U/ml) before incubation with galectin-3.

Morphological evaluation in galectin-3-induced cell death. The extent of cell death induced by galectin-3 was morphologically evaluated by Giemsa staining.

Annexin V assay. HBL-2 cells (5×10^6) were treated with galectin-3 (30 μ M) in 2.5 ml of RPMI-1640/15% fetal calf serum. After 48 h, the cells were incubated in 100 μ l reaction

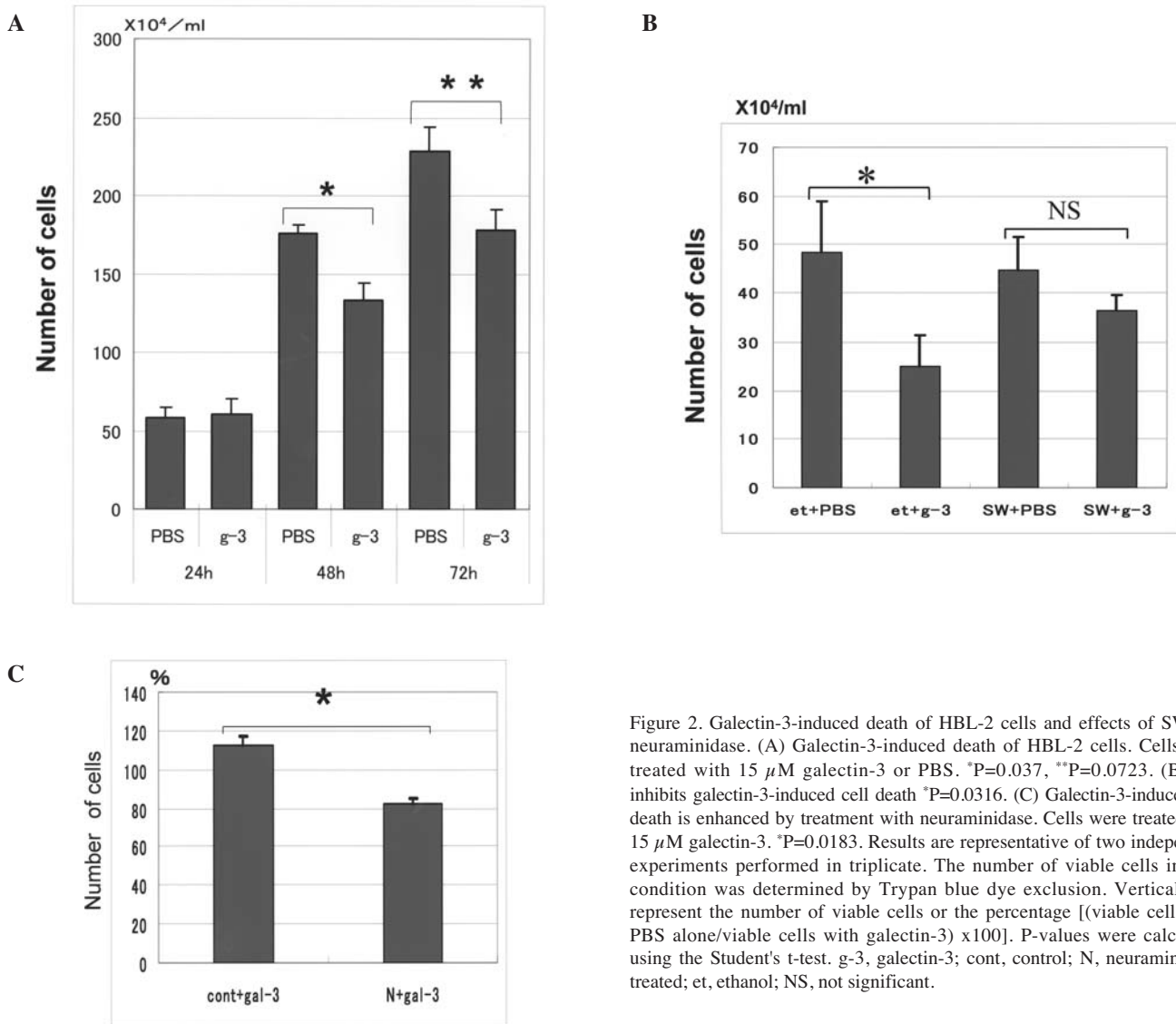


Figure 2. Galectin-3-induced death of HBL-2 cells and effects of SW and neuraminidase. (A) Galectin-3-induced death of HBL-2 cells. Cells were treated with 15 μ M galectin-3 or PBS. * $P=0.037$, ** $P=0.0723$. (B) SW inhibits galectin-3-induced cell death * $P=0.0316$. (C) Galectin-3-induced cell death is enhanced by treatment with neuraminidase. Cells were treated with 15 μ M galectin-3. * $P=0.0183$. Results are representative of two independent experiments performed in triplicate. The number of viable cells in each condition was determined by Trypan blue dye exclusion. Vertical lines represent the number of viable cells or the percentage [(viable cells with PBS alone/viable cells with galectin-3) $\times 100$]. P-values were calculated using the Student's t-test. g-3, galectin-3; cont, control; N, neuraminidase-treated; et, ethanol; NS, not significant.

buffer containing 2 μ l annexin V-FITC according to the manufacturer's instructions (23). The annexin V reactivity was then analyzed with a FACS Calibur.

Results

Expression of N-glycans and sialic acids on HBL-2 cells. A flow cytometric analysis showed a reduction of L-PHA reactivity in HBL-2 cells treated with SW (Fig. 1a). A reduction in the expression of L-PHA-reactive oligosaccharides continued for 24 h (data not shown). In contrast, SW did not affect PNA reactivity (Fig. 1b). Furthermore, LFA reactivity was reduced after neuraminidase treatment (Fig. 1c).

Galectin-3-induced death of HBL-2 cells. Galectin-3 induced the death of HBL-2 cells (Fig. 2A). This galectin-3-induced cell death was inhibited by treatment with 1 μ g/ml SW (Fig. 2B) and enhanced by treatment with neuraminidase (Fig. 2C).

Morphology of galectin-3-induced cell death. A morphological examination revealed chromatin condensation and nuclear

fragmentation during galectin-3-induced cell death. This is consistent with the induction of apoptosis (Fig. 3).

Annexin V assay of galectin-3-induced cell death. Apoptosis was examined in HBL-2 cells treated for 48 h with 30 μ M of galectin-3. To analyze the early stages of apoptosis, gate analysis was performed on non-necrotic cell fractions (monotonous cell population in the gate) (Fig. 4a). There were more annexin V-positive cells in the galectin-3-treated condition (Fig. 4c; 50%) than in the control (Fig. 4b; 33%).

Discussion

Galectin-3 is a novel anti-apoptotic molecule containing a functional bcl-2 family BH1 (NWGR) domain (17) that protects cells from apoptosis by inhibiting cytochrome c release from mitochondria (18). When added to the exterior of cells, however, galectin-3 induces apoptosis by interacting with terminal galactose residues on cell surface glycans (19). In the present study, we showed that exogenous galectin-3 induces the death of HBL-2 human DLBCL cells. Annexin V assays and a morphological examination revealed that

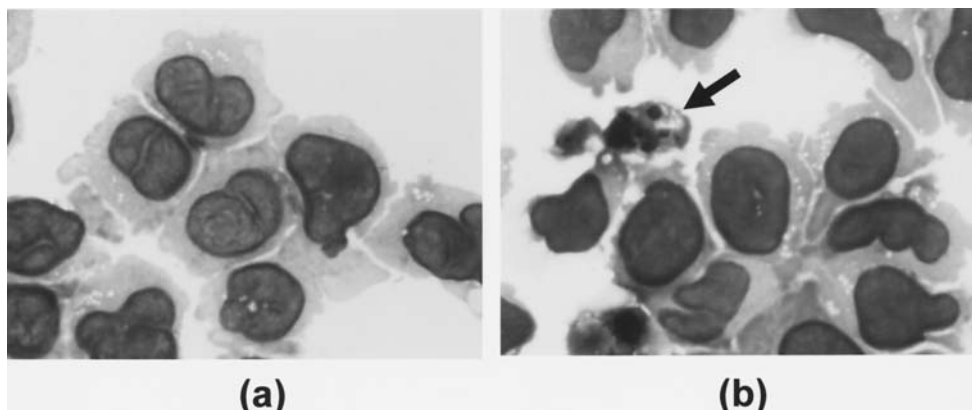


Figure 3. Morphology of cells undergoing galectin-3-induced apoptosis. Cells were treated for 48 h without (a) or with (b) 15 μ M galectin-3. Cells undergoing galectin-3-induced death show chromatin condensation and nuclear fragmentation, consistent with apoptosis.

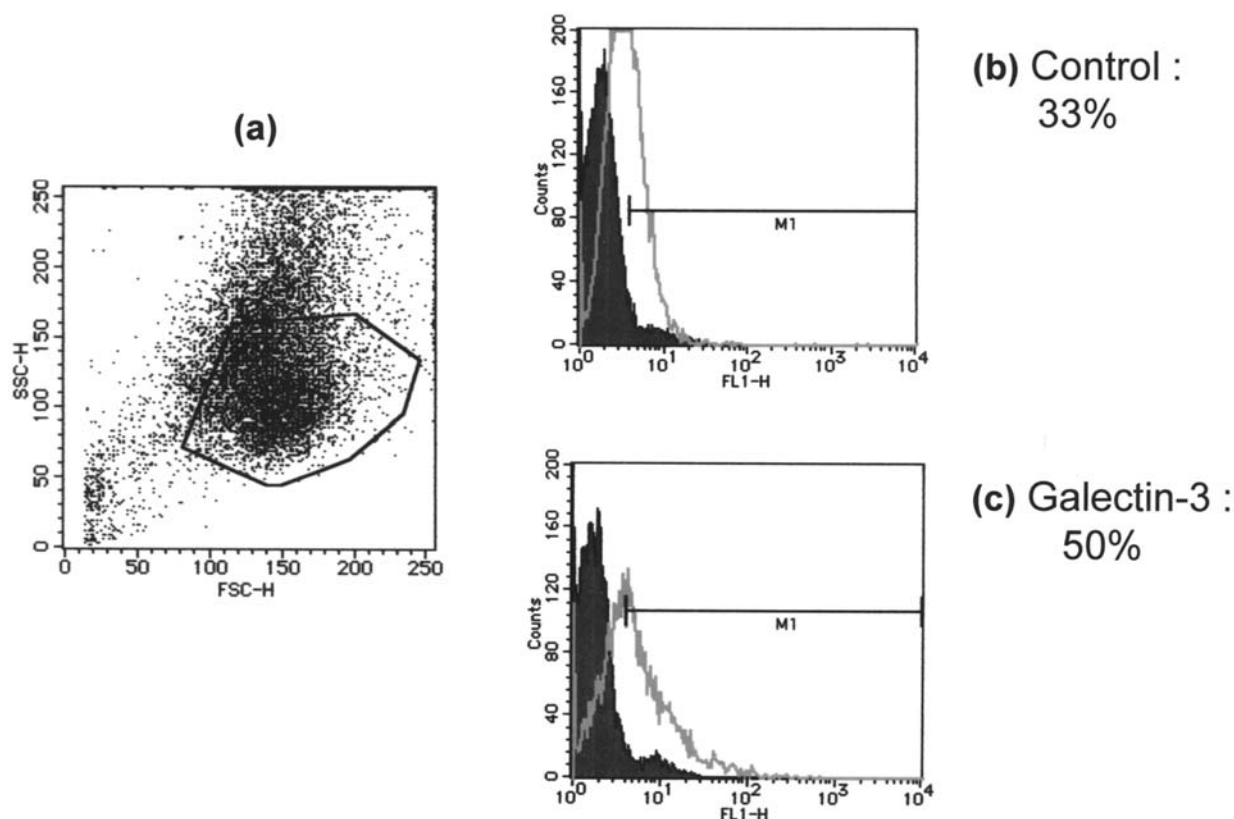


Figure 4. Analysis of galectin-3-induced cell death by annexin V assay. HBL-2 cells were incubated for 48 h with 30 μ M galectin-3. (a) To analyze early-stage apoptosis, gate analysis was performed on non-necrotic cell fractions (monotonous cell population in the gate). The number of annexin V-positive cells was higher in the galectin-3 treated condition (c; 50%) than in the control condition (b; 33%). Results are representative of two independent experiments.

extracellular galectin-3 causes cell death by inducing apoptosis. Previous reports have shown that galectin-3 interacts with lysosome-associated membrane proteins or with the glycans of CD45 (24,25). Further investigations will be needed to elucidate the interaction between galectin-3 and its ligand proteins in galectin-3-induced apoptosis.

L-PHA-reactive oligosaccharide is one of the complex-type N-linked oligosaccharides and is synthesized by N-acetylglucosaminyltransferase V (26). Studies in murine lymphoma cell lines suggest that elevated levels of β 1-6

branching in L-PHA-reactive oligosaccharides correspond with high metastatic potential (27). This branching is catalyzed by N-acetylglucosaminyltransferase V, whose transcription is regulated by proto-oncogenes such as those of the Ets family (28). High expression of N-acetylglucosaminyltransferase V correlates with a poor prognosis in colonic carcinoma (29) and an increased expression of L-PHA-reactive oligosaccharides is associated with an advanced clinical stage in colonic carcinoma as well as with malignant transformation in breast neoplasia (30). On the other hand, loss of L-PHA reactive

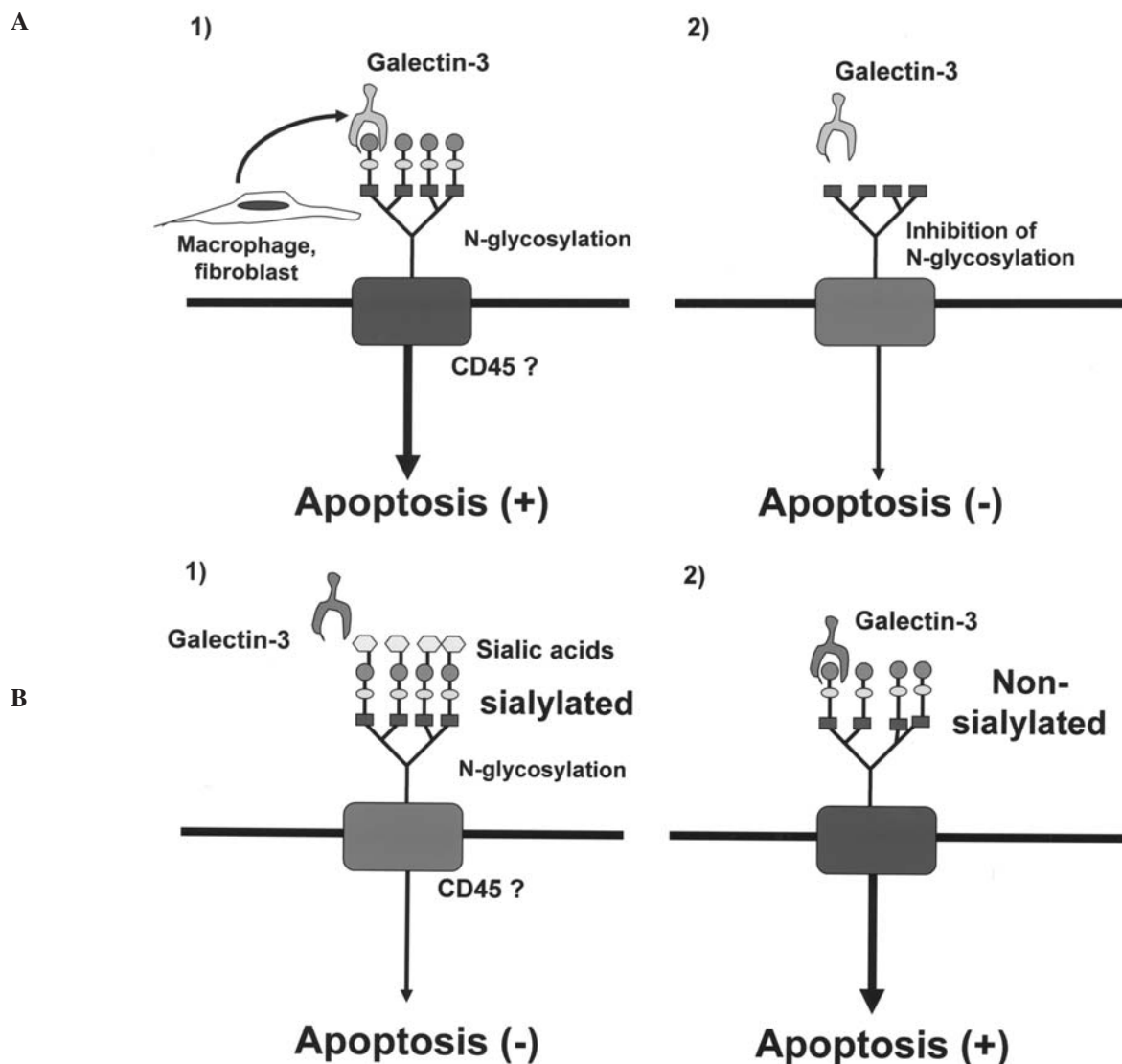


Figure 5. Schematic representation of the regulatory roles of N-glycans and cell surface sialylation in galectin-3-induced apoptosis of lymphoma cells. (A) Schematic representation of the regulatory role of the interaction of galectin-3 with N-glycans in galectin-3-induced apoptosis of lymphoma cells. 1) Galectin-3 produced by macrophages or fibroblasts reacts with the terminal galactose residues of N-glycans on the surface of lymphoma cells, inducing apoptosis. 2) Inhibition of elongation of N-glycans by treatment with SW protects lymphoma cells from galectin-3-induced apoptosis. Thus, N-glycans may regulate galectin-3-induced apoptosis in lymphoma cells. (B) Schematic representation of the regulatory role of cell surface sialylation in galectin-3-induced apoptosis of lymphoma cells. 1) Sialic acids are linked to the terminal galactose residues of N-glycans. As a result, galectin-3 cannot react with terminal galactose residues of N-glycans on the surface of lymphoma cells. 2) Neuraminidase removes the sialic acids from the terminal galactose residues of N-glycans, enhancing the interaction between terminal galactose residues and galectin-3, which promotes galectin-3-induced apoptosis of lymphoma cells. Thus, sialylation of cell surface oligosaccharides may inhibit galectin-3-induced apoptosis.

oligosaccharides is closely associated with a worse prognosis in patients with DLBCL (31,32).

The present study showed that SW treatment inhibits galectin-3-induced apoptosis in HBL-2 cells. The data suggest that N-linked oligosaccharides such as L-PHA-reactive oligosaccharides are responsible for the induction of lymphoma cell apoptosis by galectin-3. Therefore, the loss of N-linked oligosaccharides may inhibit galectin-3-induced apoptosis, leading to more aggressive behavior in DLBCL as illustrated in Fig. 5A. In addition, neuraminidase treatment enhanced galectin-3-induced apoptosis in HBL-2 cells. These results suggest that cell surface sialylation can protect lymphoma cells from galectin-3-induced apoptosis. In agreement with this,

sialylation of L-PHA reactive oligosaccharides has been found to be closely associated with a worse prognosis for patients with DLBCL (31,32). As summarized in 5B, these findings suggest that protection of lymphoma cells from galectin-3-induced apoptosis by cell surface sialylation results in more aggressive tumorigenic behavior, at least in part, by enhancing tumor cell survival and proliferation.

In conclusion, our results show that cell surface N-glycans and sialylation play an important role in galectin-3-induced apoptosis. The regulatory mechanisms mediating galectin-3-induced apoptosis contribute to tumor cell biology of human malignant lymphoma.

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References

- Rabinovich GA, Rubinstein N and Toscano MA: Role of galectins in inflammatory and immunomodulatory processes. *Biochim Biophys Acta* 1572: 274-284, 2002.
- Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K, Leffler H, Liu FT, Lotan R, Mercurio AM, Monsigny M, Pillai S, Poirer F, Raz A, Rigby PWJ, Rini JM and Wang J: Galectins: a family of animal beta-galactoside-binding lectins. *Cell* 76: 597-598, 1994.
- Allen HJ, Gottstine S, Sharma A, DiCioccio RA, Swank RT and Li H: Synthesis, isolation and characterization of endogenous beta-galactoside-binding lectins in human leukocytes. *Biochemistry* 30: 8904-8910, 1991.
- Barondes SH: Soluble lectins: a new class of extracellular proteins. *Science* 223: 1259-1264, 1984.
- Adams L, Scott GK and Weinberg CS: Biphasic modulation of cell growth by recombinant human galectin-1. *Biochim Biophys Acta* 1312: 137-144, 1996.
- Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM and Hirabayashi J and Chernajovsky Y: Recombinant galectin-1 and genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190: 385-397, 1999.
- Rabinovich GA, Alonso CR, Sotomayor CE, Durand S, Bocco JL and Riera CM: Molecular mechanisms implicated in galectin-1-induced apoptosis: activation of the AP-1 transcription factor and down regulation of bcl-2. *Cell Death Differ* 7: 747-753, 2000.
- Perillo NL, Pace KE, Seilhamer JJ and Baum LG: Apoptosis of T cells mediated by galectin-1. *Nature* 378: 736-739, 1995.
- Nguyen JT, Evans DP, Galvan M, Pace KE, Leitenberg D, Bui TN and Baum LG: CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 O-glycans. *J Immunol* 167: 5697-5707, 2001.
- Pace KE, Lee C, Stewart PL and Baum LG: Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* 163: 3801-3811, 1999.
- Murphy JJ, Stevanin T, Schoendorf DS, Wells V and Mallucci L: β -galactoside binding protein inhibits B cell growth and induces cell apoptosis. *Biochem Soc Trans* 25: 248S, 1997.
- Foullit M, Joubert-Caron R, Poirier F, Bourin P, Monostori E, Levi-Strauss M, Raphael M, Bladier D and Caron M: Regulation of CD45-induced signaling by galectin-1 in Burkitt lymphoma B cells. *Glycobiology* 10: 413-419, 2000.
- Amano M, Galvan M, He J and Baum LG: The ST6Gal I sialyltransferase selectively modifies N-glycans on CD45 to negatively regulate galectin-1-induced CD45 clustering, phosphatase modulation and T cell death. *J Biol Chem* 278: 7469-7475, 2003.
- Kageshita T, Kashio Y, Yamauchi A, Seki M, Abedin MJ, Nishi N, Shoji H, Nakamura T, Ono T and Hirashima M: Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. *Int J Cancer* 99: 809-816, 2002.
- Konstantinov KN, Robbins BA and Liu FT: Galectin-3, a beta-galactoside-binding animal lectin, is a marker of anaplastic large-cell lymphoma. *Am J Pathol* 148: 25-30, 1996.
- Hoyer KK, Pang M, Gui D, Shintaku IP, Kuwabara I, Liu FT, Said JW, Baum LG and Teitel MA: An anti-apoptotic role for galectin-3 in diffuse large B-cell lymphomas. *Am J Pathol* 164: 893-902, 2004.
- Akahani S, Nangia-Makker P, Inohara H, Kim HR and Raz A: Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res* 57: 5272-5276, 1997.
- Yu F, Finery Jr RL, Raz A and Kim HRC: Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. *J Biol Chem* 277: 15819-15827, 2002.
- Fukumori T, Takenaka Y, Yoshii T, Kim HRC, Hogan V, Inohara H, Kagawa S and Raz A: CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. *Cancer Res* 63: 8302-8311, 2003.
- Suzuki O, Nozawa Y and Abe M: Altered N-glycosylation in CD45 and regulatory roles of altered N-glycosylation in galectin-1-induced growth inhibition in human diffuse large B cell lymphoma. *Oncol Rep* 13: 109-114, 2005.
- Suzuki O, Nozawa Y and Abe M: Regulatory roles of altered N- and O-glycosylation of CD45 in galectin-1-induced cell death in human diffuse large B cell lymphoma. *Int J Oncol* 26: 1063-1068, 2005.
- Abe M, Nozawa Y, Wakasa H, Ohno H and Fukuhara S: Characterization and comparison of two newly established Epstein-Barr virus-negative lymphoma B-cell lines. *Cancer* 61: 483-490, 1988.
- Suzuki O, Nozawa Y and Abe M: Regulatory roles of N-glycosylation of immunoglobulin M in CD40-CD40L mediated cell survival of human diffuse large B cell lymphoma. *Oncol Rep* 11: 1031-1039, 2004.
- Sarafian V, Jadot M, Foidart JM, Letesson JJ, Van den Brule F, Castronovo V, Wattiaux R and Coninck SW: Expression of Lamp-1 and Lamp-2 and their interactions with galectin-3 in human tumor cells. *Int J Cancer* 75: 105-111, 1998.
- Stillman BN, Hsu DK, Pang M, Brewer CF, Johnson P, Liu FT and Baum LG: Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol* 176: 778-789, 2006.
- Saito H, Nishikawa A, Gu J, Ihara Y, Soejima H, Wada Y, Sekiya C, Niikawa N and Taniguchi N: cDNA cloning and chromosomal mapping of human N-acetylglucosaminyltransferase V. *Biochem Biophys Res Commun* 198: 318-327, 1994.
- Dennis JW, Laferte S, Waghorne C, Breitman ML and Kerbel RS: Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 236: 582-585, 1987.
- Kang R, Saito H, Ihara Y, Miyoshi E, Koyama N, Sheng Y and Taniguchi N: Transcriptional regulation of the N-acetylglucosaminyltransferase V gene in human bile duct carcinoma cells (HuCC-T1) is mediated by Ets-1. *J Biol Chem* 271: 26706-26712, 1996.
- Murata K, Miyoshi E, Kameyama M, Ishikawa O, Kabuto T, Sasaki Y, Hiratsuka M, Ohigashi H, Ishiguro S, Ito S, Honda H, Takemura F, Taniguchi N and Imaoka S: Expression of N-acetylglucosaminyltransferase V in colorectal cancer correlates with metastasis and poor prognosis. *Clin Cancer Res* 6: 1772-1777, 2000.
- Fernandes B, Sagman M, Auger M, Demetrio M and Dennis JW: β 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res* 51: 718-723, 1991.
- Suzuki O, Nozawa Y, Kawaguchi T and Abe M: *Phaseolus vulgaris* leucoagglutinating lectin-binding reactivity in human diffuse large B cell lymphoma and its relevance to the patient's clinical outcome: lectin histochemistry and lectin blot analysis. *Pathol Int* 49: 874-880, 1999.
- Suzuki O, Nozawa Y, Kawaguchi T and Abe M: Alpha-2,6-sialylation of L-PHA reactive oligosaccharides and expression of N-acetyl glucosaminyltransferase V in human diffuse large B cell lymphoma. *Oncol Rep* 10: 1759-1764, 2003.