Regulatory roles of cell surface sialylation in sphingolipid-induced cell death in human B cell lymphoma

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Received May 16, 2006; Accepted June 26, 2006

Abstract. Sphingolipid metabolites are important regulators of cell growth and death. In the present study, we examined the function of cell surface sialic acid in exogenous sphingosine-1-phosphate (S-1-P) or sphingosine-induced cell death. HBL-2 human diffuse large B cell lymphoma cells were incubated with or without *Vibrio Cholerae* neuraminidase followed by S-1-P or sphingosine. Flow cytometric analysis using *Limax flavus* agglutinin, a sialic acid-specific lectin, showed that sialylated glycoconjugates are present on the surface of HBL-2 cells and that they were removed by neuraminidase. In addition, the pretreatment with neuraminidase enhanced S-1-P- and sphingosine-induced cell death, an effect that was not dependent on caspase activation. Furthermore, the cell death induced by S-1-P and sphingosine was morphologically distinct from apoptosis. We further examined S-1-P-induced cell death in two clones of HBL-8 Burkitt lymphoma cells with different amounts of cell surface sialic acid. Clone 3G3, which is hypersialylated, was less sensitive to S-1-P than the 3D2 clone, which is hyposialylated, suggesting that the extent of surface sialylation influences the sensitivity to S-1-P. In conclusion, S-1-P and sphingosine induce cell death, and the sensitivity of human B lymphoma cells to these agents appears to depend on the amount of sialic acid on their cell surfaces.

Introduction

Sialic acid plays an important role in tumor cell behavior by affecting adhesion, invasiveness and metastasis (1-11), thereby influencing clinical outcome (12,13). The role of sialic acid as a biological mask of surface structures is well documented (14), and cell surface sialylation of thymocytes and myeloid cells is closely associated with their maturation and differentiation (15,16). Cell surface sialylation regulates Fas-induced apoptosis in lymphoma cells (17,18), and we previously showed that this occurs in human T cell lymphoma in a caspase-dependent manner (19). Uridine diphosphate-N-acetylglucosamine 2-epimerase (UDP-GlcNAc2-epimerase) is a key enzyme in sialic acid biosynthesis (20), and expression of UDP-GlcNAc2-epimerase mRNA was found to correlate with sialic acid content in two clones of HBL-8 Burkitt lymphoma cells (21). Furthermore, expression of UDP-GlcNAc2-epimerase mRNA may regulate the expression of sialoglycoconjugates, which affect the adhesion of Burkitt's lymphoma cells to collagen type IV and fibronectin (21).

Sphingolipids are structural components of the cell membrane that play a key role in cell signaling (22-27). Sphingosine-1-phosphate (S-1-P) is a bioactive sphingolipid metabolite and is present in serum (28,29). S-1-P plays an important role in the regulation of a variety of biological processes through intracellular or extracellular mechanisms (23). The endothelial differentiation gene (EDG) family of G-protein-coupled receptors act as receptors for S-1-P (24,25,31). S-1-P is released from activated platelets and in allergically stimulated mast cells, and it is secreted by erythrocytes, neutrophils, and mononuclear cells (30). S-1-P is a novel inhibitor of T cell proliferation and maintains the homeostatic immune system (31,32). Previous reports indicate that S-1-P triggers apoptotic signals in human hepatic myofibroblasts (24,25) and induces cell death in a hepatoma cell line (26).

Sphingosine is another bioactive sphingolipid that has various cellular roles, including inhibition of protein kinase C (33) and induction of apoptosis in HL-60 cells (34). Sphingosine also induces necrotic cell death (35) and is involved in tumor necrosis factor (TNF)-α-induced apoptosis in neutrophils (36).

In the present study we show that alteration in cell surface sialylation modulates S-1-P or sphingosine-induced cell death. This suggests a novel role of sialic acid in the regulation of sphingolipid-induced cell death.

Materials and methods

Cell lines. HBL-2 and HBL-8 are human diffuse large B cell lymphoma and human Burkitt lymphoma cell lines, respectively (10,37). HBL-2 and HBL-8 cells were grown at 37˚C in RPMI-1640 containing 15% fetal calf serum and in an atmosphere containing 5% CO_2. Two clones of HBL-8 (3G3 and 3D2) show different reactivities to Soybean agglutinin (SBA) and *Vicia villosa* agglutinin (VVA) lectins as well as different metastatic capacities in the SCID mouse model (10). These clones have different amounts of cell surface sialic

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Key words: human malignant lymphoma, sialic acid, sphingolipids, cell death

acid due to differences in the expression of UDP-GlcNAc2-epimerase, which is a key enzyme in sialic acid biosynthesis (21).

Antibodies and reagents. Biotinylated *Limax flavus* agglutinin (LFA), which is a sialic acid-specific lectin, was from EY Laboratories (San Mateo, CA, USA). Neuraminidase from *Vibrio Cholerae* was purchased from Roche (Germany). The caspase inhibitor carbobenzoxy-L-valyl-ß-methyl-L-aspart-1-fluoromethane (z-VAD-fmk) was obtained from Peptide Institute, Inc. (Japan) and was dissolved in dimethyl sulfoxide. Anti-CDw76 monoclonal antibody (clone BL-B8), which reacts with sialic acid-linked gangliosides, was purchased from Monosan (The Netherlands). Mouse monoclonal antibody G155-228 (BD PharMingen, USA) was used for isotype control studies.

Flow cytometry. HBL-2 cells ($5x10^5$ cells) were suspended in 100 μl of phosphate-buffered saline (PBS) and incubated at 4˚C for 20 min with 5 μl of biotinylated lectins or anti-CDw76 monoclonal antibody and then washed twice with PBS. Next, the cells were incubated at 4˚C for 20 min with 5 μl of avidin-fluorescein isothiocyanate (FITC; Vector Laboratories, Inc., Burlingame, CA, USA) or FITC-conjugated anti-mouse immunoglobulins (Biosource, 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 of RPMI-1640 containing 15% fetal calf serum and 40 μl of 1 U/ml *Vibrio Cholerae* neuraminidase (Boehringer Mannheim, Germany) before incubation with biotinylated lectins (19).

Effects of neuraminidase on S-1-P- and sphingosine-induced cell death. Cell viability was assessed using a WST-1 assay kit (Boehringer Manheim) (19). HBL-2 cells or HBL-8 clones 3G3 and 3D2 were grown for two days and then seeded for 30 min at 37˚C in 96-well microtiter plates at $5x10^4$ cells/well in 120 μl phenol red- and serum-free culture medium containing 7.5 μM S-1-P or 10 μM sphingosine (Biomol Research Laboratories, Inc., USA) in the presence or absence of 120 μM z-VAD-fmk. Next, 10 μl of WST-1 reagent was added to each well, and the plates were incubated for 2 h at 37˚C. The plates were then shaken for 1 min at room temperature, and the absorbance at 450-655 nm was measured using a model 550 microplate reader (Bio-Rad, CA, USA). To evaluate the effects of cell surface desialylation, $6x10^6$ cells were incubated at 37˚C for 30 min in 200 μl of RPMI-1640 containing 15% fetal calf serum and 40 μl of 1 U/ml *Vibrio Cholerae* neuraminidase (Boehringer Mannheim) before incubation with S-1-P or sphingosine.

Morphology of cell death. Giemsa-stained Cytospin® HBL-2 cell preparations were evaluated for morphological changes during S-1-P- or sphingosine-induced cell death.

Annexin-V assay. HBL-2 cells ($2x10^6$) were treated with serum-free culture medium containing S-1-P (Biomol Research Laboratories, Inc.) at a final concentration of 3.75 μM. After 30 min, the cells were incubated with 20 μl of Annexin V-FITC and 20 μl of propidium iodide (PI) according to the manufacturer's instructions (Roche, Tokyo, Japan). The fluorescence intensity from fluorescein and PI intensity were analyzed with a FACScan (Becton-Dickinson).

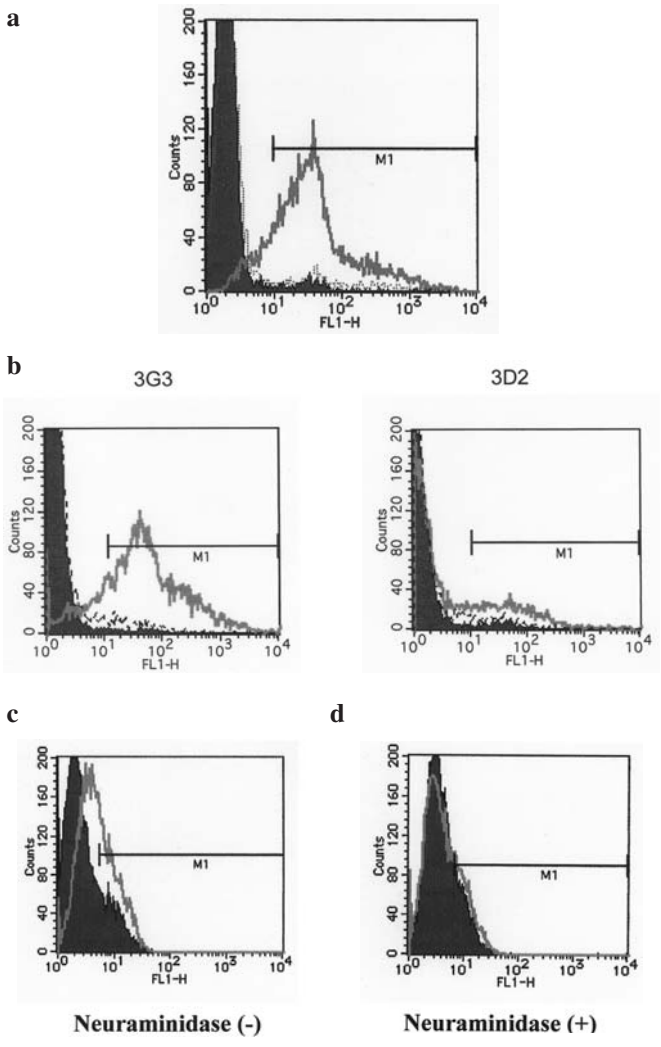

Figure 1. Analysis of cell surface sialylation by flow cytometry. (a) Cell surface sialylation in HBL-2 cells. The thick and dotted lines show LFA binding without and with neuraminidase treatment, respectively. Stippled areas indicate the negative controls. LFA lectin binding was eliminated by neuraminidase treatment, indicating the removal of cell surface sialic acids. (b) Cell surface sialylation in the 3G3 and 3D2 clones of HBL-8 cells. Solid and dotted lines show LFA binding without or with neuraminidase treatment. Stippled areas indicate the negative controls. Substantial LFA lectin binding was found in the 3G3 clone, but only weak binding was found in the 3D2 clone. (c) Detection of gangliosides on HBL-2 cells by an anti-CDw76 monoclonal antibody. The anti-CDw76 monoclonal antibody bound in the absence of neuraminidase treatment. (d) Binding of the anti-CDw76 monoclonal antibody to the HBL-2 cell line was eliminated by neuraminidase treatment.

Results

Cell surface sialylation on HBL-2 and HBL-8 clones. Flow cytometry analysis showed that LFA, a sialic acid-specific lectin, reacted with the surface of HBL-2 cells. LFA lectin reactivity was completely eliminated by treatment with neuraminidase, indicating removal of cell surface sialic acids (Fig. 1a). The 3G3 clone of HBL-8 cells showed substantial cell surface binding of LFA lectin, but little binding was exhibited by the 3D2 clone (Fig. 1b). Furthermore, binding of

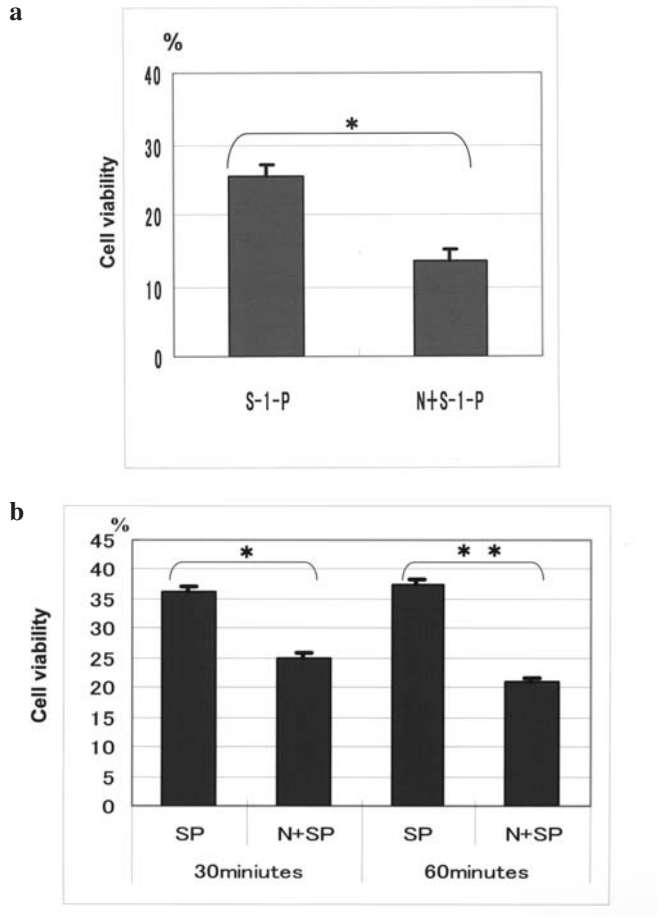

Figure 2. Effects of neuraminidase on S-1-P or sphingosine-induced cell death on the HBL-2 cell line. (a) Effect of S-1-P on cell viability as assessed with a WST-1 assay. Cells were incubated with 7.5 μM S-1-P. Cell viability of HBL-2 cells was decreased following incubation with S-1-P without or with neuraminidase treatment (25% and 13%, respectively). This shows that S-1-P-induced cell death was markedly enhanced by neuraminidase treatment. N, with neuraminidase treatment. *P=0.0009 as determined by Student's t-test. Values represent the mean cell viability ± SD. The experiment was performed in triplicate. (b) Effect of sphingosine on cell viability was assessed using a WST-1 assay. Cells were incubated with 10 μM sphingosine. The viability of HBL-2 cells was decreased by sphingosine without or with neuraminidase treatment (35% and 25%, respectively). This shows that sphingosine-induced cell death was markedly enhanced by neuraminidase treatment. SP, sphingosine; N, with neuraminidase treatment. *P<0.0001, **P<0.0001 as determined by Student's t-test. Values represent the mean cell viability ± SD. The experiment was performed in triplicate.

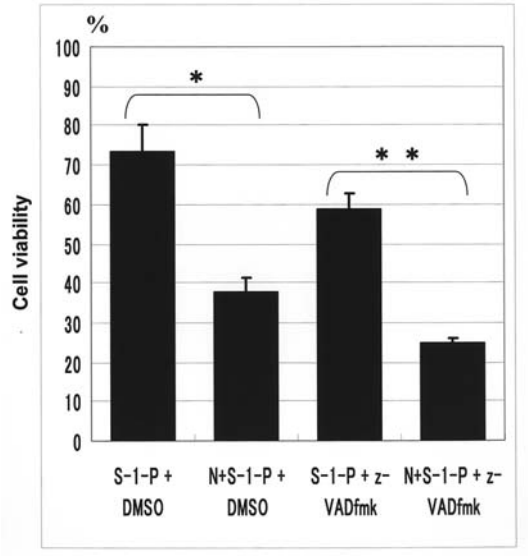

Figure 3. Effects of caspase inhibitors on S-1-P-induced cell death in HBL-2 cells. The effect of S-1-P on cell viability in the presence or absence of the pan-caspase inhibitor z-vad-FMK and with or without neuraminidase treatment was assessed using a WST-1 assay. The induction of cell death by S-1-P with or without neuraminidase treatment was not blocked by z-VAD-fmk. DMSO, dimethyl sulfoxide (control); N, with neuraminidase treatment. *P=0.0014, **P=0.0001 by Student's t-test. Values represent the mean cell viability ± SD. The experiment was performed in triplicate.

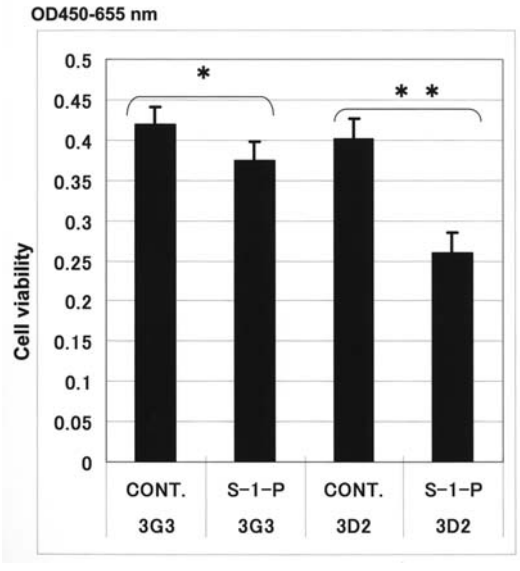

Figure 4. S-1-P-induced cell death in HBL-8 clones. Effect of S-1-P on the viability of HBL-8 clones was assessed by a WST-1 assay. S-1-P was less effective at inducing cell death in the 3G3 clone than the 3D2 clone. *P=0.0683, **P=0.0023 as determined by Student's t-test. Values represent the mean cell viability ± SD. The experiment was performed in triplicate. CONT, control experiment (without S-1-P).

the anti-CDw76 monoclonal antibody was detected without neuraminidase treatment (Fig. 1c), but it was completely eliminated with neuraminidase pretreatment (Fig. 1d). This finding suggests that CDw76 is α2,6-linked ganglioside and CDw76 ganglioside is converted to the asialo-form with neuraminidase treatment.

Effect of neuraminidase treatment on S-1-P- or sphingosine-induced cell death. Serum-deprived HBL-2 cells or HBL-8 clones were incubated with or without S-1-P or sphingosine. The viability of HBL-2 cells decreased when they were incubated with S-1-P in the absence or presence of neuraminidase treatment (25% and 13%, respectively; Fig. 2a). Also, the viability of HBL-2 cells decreased when they were incubated for 30 min with sphingosine in the absence or presence of neuraminidase treatment (35% and 25%, respectively; Fig. 2b). Cell death induced by S-1-P with or without neuraminidase treatment was not blocked by z-VAD-fmk (Fig. 3). In contrast, cell death by S-1-P was slightly enhanced by z-VAD-fmk. Also, S-1-P significantly reduced the viability of both the 3D2 and 3G3 clones of HBL-8 cells, although the 3G3 cells were less sensitive than the 3D2 cells (Fig. 4).

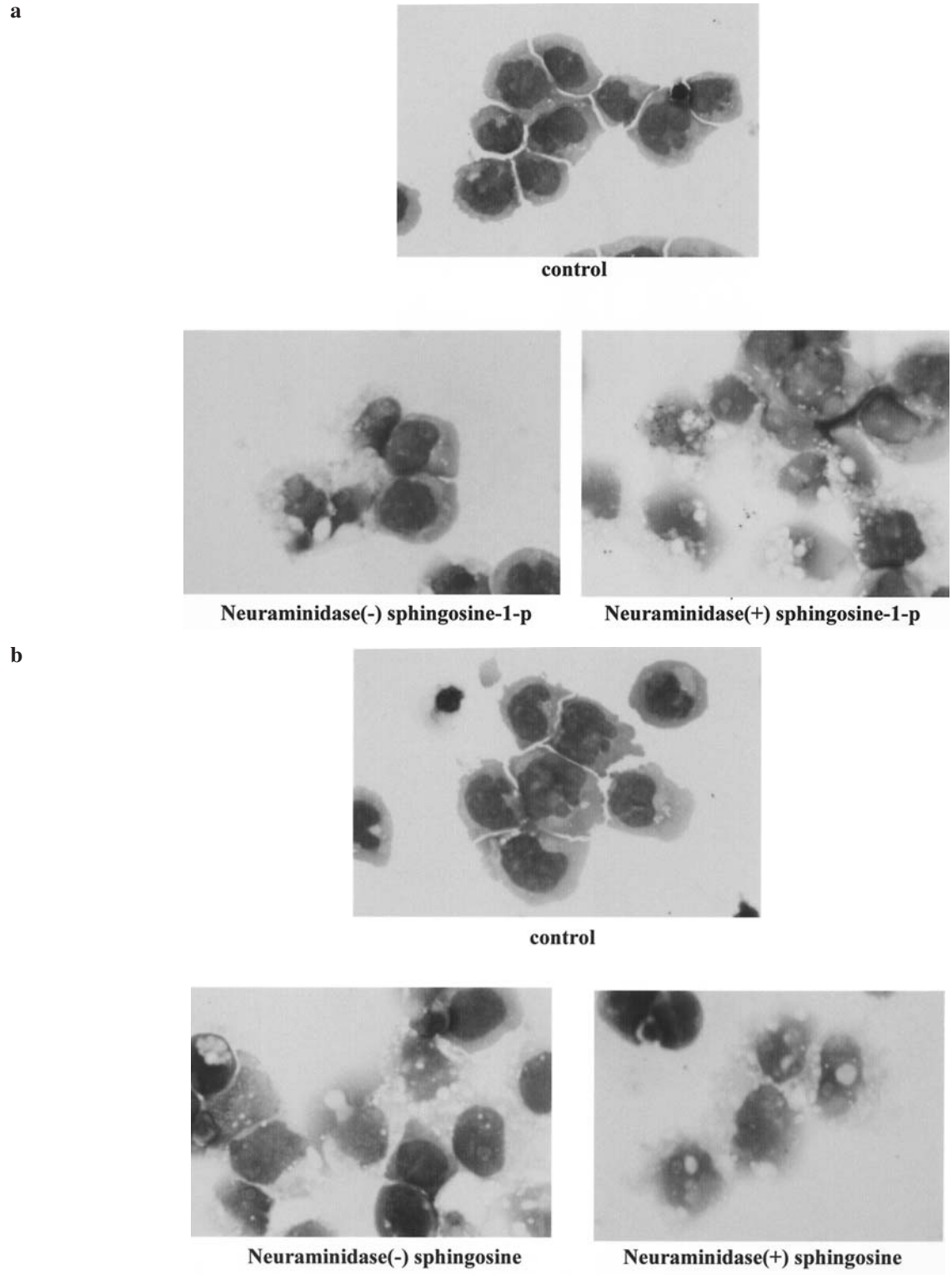

Figure 5. Morphology of sphingolipid-treated HBL-2 cells. HBL-2 cells were incubated with S-1-P (a) or sphingosine (b) for 60 min. The cells showed swelling and cytoplasmic vacuoles in both conditions with or without neuraminidase treatment.

Morphologic changes during sphingolipid-induced cell death. HBL-2 lymphoma cells incubated with S-1-P or sphingosine showed swelling and the appearance of cytoplasmic vacuoles (Fig. 5) both with or without neuraminidase treatment.

Annexin-V assay. Treatment of HBL-2 cells with S-1-P caused an increase in the number of PI-positive cells compared to controls. This increase was enhanced by neuraminidase pre-treatment (Fig. 6).

Discussion

S-1-P is a novel inhibitor of T cell proliferation and maintains the homeostatic immune systems (31). S-1-P also triggers

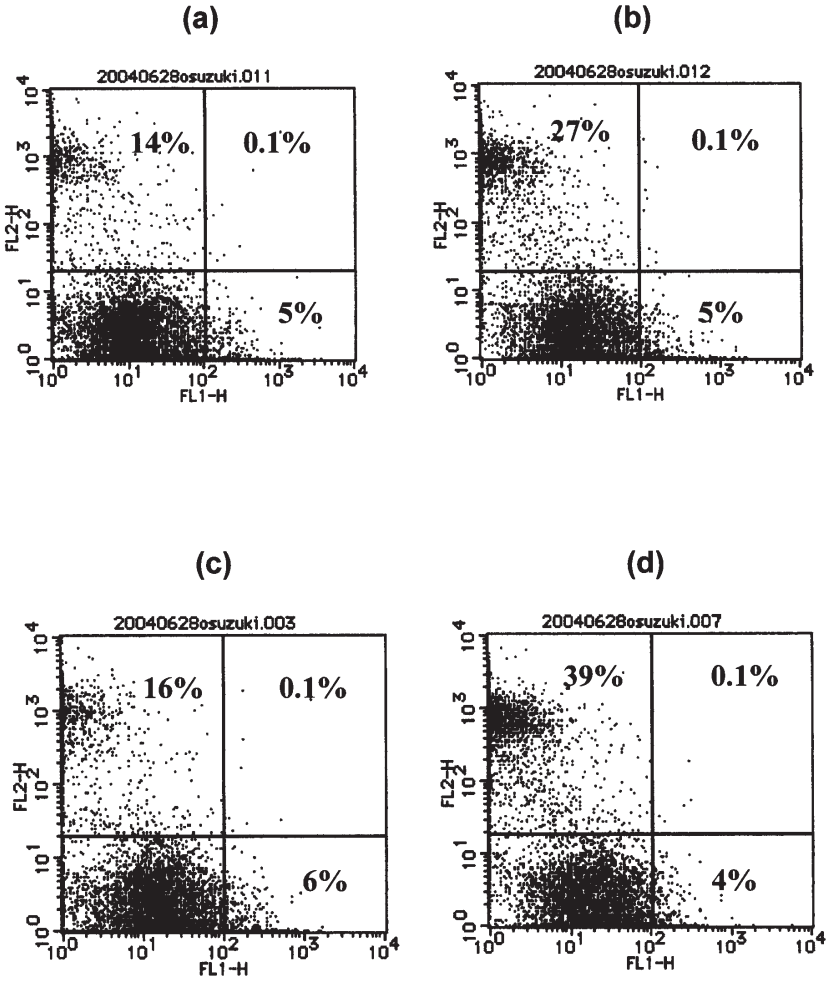

Figure 6. Annexin-V assay on the HBL-2 cell line. Annex-V-FITC binding was assessed following treatment with (a) control, (b) S-1-P alone, (c) neuraminidase alone or (d) S-1-P with neuraminidase pretreatment. The fluorescein fluorescence was plotted on the X-axis, and the PI fluorescence was plotted on the Y-axis. Neuraminidase enhanced the ability of S-1-P to increase the number of PI-positive cells.

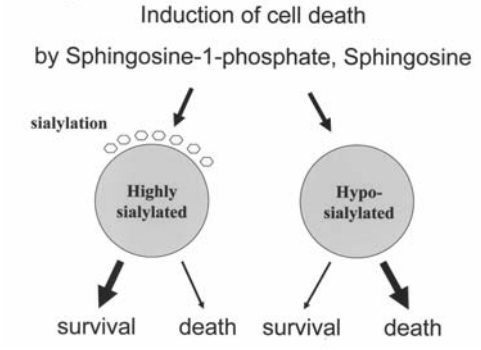

Figure 7. Schematic representation of the relationship between cell surface sialylation and sphingolipid-induced cell death. A highly sialylated cell surface protects lymphoma cells from sphingolipid-induced cell death, whereas hyposialylation enhances the sensitivity of cells to sphingolipid-induced cell death.

apoptotic signals in human hepatic myofibroblasts (24,25) and induces the death of several types of cells, including hepatoma cells (26). In the present study, we showed that a highly sialylated cell surface can protect lymphoma cells from S-1-P- or sphingosine-induced cell death and that hyposialylation increases the sensitivity of the cells to these agents (summarized in Fig. 7). In addition, results from the Annexin-V assay and morphological evaluation indicate that S-1-P-induced cell death is due to necrosis rather than apoptosis. These data suggest that cell surface sialylation plays an important role in S-1-P-induced necrotic cell death of human B cell lymphomas.

CDw76 is reported to be an α2,6-sialylated ganglioside (38). Cell surface desialylation with neuraminidase reduced the binding of an anti-CDw76 monoclonal antibody that recognizes α2,6-sialylated ganglioside. Therefore, alteration of gangliosides to asialo-gangliosides may be associated with a perturbation of the cell membrane leading to enhanced susceptibility of lymphoma cells to S-1-P and sphingosine.

There was a significant difference in the susceptibility of the HBL-8 clones to S-1-P. Our results suggest that this may be due to different extents of cell surface sialylation; specifically, increased cell surface sialylation (clone 3G3) corresponds to a reduced susceptibility of the cells to S-1-P-induced cell death. When injected into SCID mice, the highly sialylated 3G3 clone is more metastatic than the hyposialylated 3D2 clone (10). Together with our findings, this suggests that the higher cell surface sialylation in the 3G3 clone protects

them from S-1-P-induced cell death in the bloodstream or at distant target organs, leading to a higher metastatic rate.

The EDG family G-protein-coupled receptors have been shown to be receptors for S-1-P (24,25,31). EDG receptor-1 was not expressed on the surface of HBL-2 or HBL-8 cells (data not shown). Therefore, the induction of cell death by S-1-P is not mediated by an EDG receptor, and S-1-P acts directly on cell death of HBL-2 and HBL-8 cells. Further study is needed to determine the signaling mechanisms mediating S-1-P-induced cell death.

In conclusion, cell death induced in human B cell lymphoma by S-1-P or sphingosine appeared to be influenced by cell surface sialylation. Investigation of the relationship between cell surface sialylation and sphingolipid-induced cell signaling may provide new insight into the treatment of hematological malignancies.

Acknowledgements

We would like to thank Mrs. M. Satoh for her technical assistance and advice.

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