Protein Sialylation by Sialyltransferase Involves Radiation Resistance

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Abstract

Previously, we identified β-galactoside α(2,6)-sialyltransferase (ST6Gal I) as a candidate biomarker for ionizing radiation. The expression of ST6Gal I and the level of protein sialylation increased following radiation exposure in a dose-dependent manner. Radiation induced ST6Gal I cleavage and the cleaved form of ST6Gal I was soluble and secreted. Sialylation of integrin β1, a glycosylated cell surface protein, was stimulated by radiation exposure and this increased its stability. Overexpression of ST6Gal I in SW480 colon cancer cells that initially showed a low level of ST6Gal I expression increased the sialylation of integrin β1 and also increased the stability of the protein. Inhibition of sialylation by transfection with neuraminidase 2 or neuraminidase 3 or by treatment with short interfering RNA targeting ST6Gal I reversed the effects of ST6Gal I overexpression. In addition, ST6Gal I overexpression increased clonogenic survival following radiation exposure and reduced radiation-induced cell death and caspase 3 activation. However, removal of sialic acids by neuraminidase 2 or knockdown of expression by short interfering RNA targeting ST6Gal I restored radiation-induced cell death phenotypes. In conclusion, radiation exposure was found to increase the sialylation of glycoproteins such as integrin β1 by inducing the expression of ST6Gal I, and increased protein sialylation contributed to cellular radiation resistance. (Mol Cancer Res 2008;6(8):1316–25)

Introduction

The oligosaccharide structures present at the surface of mammalian cells are involved in highly specific recognition events (1) and are frequently terminated by sialic acids, nine carbon sugars bearing a negative electric charge at physiologic pH (2). Sialic acids are known to be ubiquitously expressed on the nonreducing ends of the sugar chains of glycoproteins and glycolipids in tissues and are known to be key determinants for a large variety of biological processes, including cell-cell communication, immune defense, tumor cell metastasis, and inflammation (3, 4).

The disaccharide Gal β(1,4)GlcNAc (N-acetylgalactosamine) is a common constituent of glycoconjugate N-linked oligosaccharide chains that can be sialylated by α(2,3)- or α(2,6)-sialyltransferase (ST3 or ST6; refs. 5-7). The β-galactoside α(2,6)-sialyltransferase (ST6Gal I) has been identified as being able to catalyze the α(2,6)-sialylation of N-acetylgalactosamine (7). Recently, another type of human β-galactoside α(2,6)-sialyltransferase, ST6Gal II, has been cloned but it showed lower enzymatic activity toward several glycoproteins and glycolipids as compared with ST6Gal I. Moreover, ST6Gal I was expressed ubiquitously, particularly in tumors, whereas ST6Gal II was mainly expressed in fetal brain and intestine (8, 9). The up-regulation of ST6Gal I is probably the basis for the increased α(2,6)-sialylation seen in cancer cells (10, 11). Mice genetically engineered to be deficient in ST6Gal I are essentially unable to generate Sia-α2,6Galβ1,4GlcNAc structures on glycoproteins (12) and ST6Gal I shares remarkable structural conservation between human, rat, and mouse homologues (13, 14). The level of ST6Gal I expression differs dramatically from tissue to tissue (15, 16) and it is particularly high in liver (16, 17), B-lymphocytes (13, 18), and lactating mammary glands (19). Overexpression of ST6Gal I has been reported in a number of human malignancies. Several clinical and experimental studies suggest a positive correlation between high ST6Gal I levels and the invasive behavior of cancer cells (20), but other studies have reported opposite conclusions (21, 22). Furthermore, there have been no reports of ST6Gal I expression changes in response to radiation exposure.

In our previous study, we showed that mRNA expression levels of ST6Gal I are increased in C57BL6 mice by exposure to a low dose (0.2 Gy) of radiation, especially in the spleen and intestine (23). Here, for the first time, we show that radiation exposure increases ST6Gal I expression and sialylation of proteins such as integrin β1 in both cell lines in vitro and in the mouse spleen in vivo. It was previously reported that integrin β1 is a substrate for ST6Gal I (24, 25) and increased sialylation of integrin β1 in colon epithelial cells alters the cell preference for certain extracellular matrix milieus and stimulates cell migration (25). Therefore, in this study, we examined the link between radiation-induced ST6Gal I expression and integrin β1 sialylation. ST6Gal I overexpression increased integrin β1 protein stability and cellular radiation resistance.
FIGURE 1. Ionizing radiation induces the expression of ST6Gal I. One day following whole-body irradiation with a dose of 1 Gy, spleens were extracted and subjected to reverse transcription-PCR (A), immunoblotting (B) or immunohistochemical analysis (C) for detection of ST6Gal I expression. The expression of ST6Gal I, ST8Sia I, ST3Gal I, ST3Gal II, ST3Gal III, and ST3Gal IV was also analyzed by reverse transcription-PCR following whole-body irradiation with a single dose of 1 Gy or regional irradiation (D). Daudi and U937 cells were exposed to the indicated dose of radiation and mRNA expression of sialyltransferases was analyzed by reverse transcription-PCR. Raw 264.7, U937, and Daudi cells were exposed to the indicated dose of radiation and mRNA (E) and protein (F) levels of ST6Gal I were analyzed. Densitometric scanning quantitated the relative intensity of the mRNA or protein bands. The reverse transcription-PCR and Western blotting data are each representative of one out of three independent experiments. Columns, mean of three independent experiments; bars, SD (*, $P < 0.05$, when compared with the corresponding control cells).
Results
Radiation Exposure Increases ST6Gal I Expression

Because our previous study indicated that mRNA expression levels of ST6Gal I in the mouse spleen and intestine were increased by whole-body irradiation, we reconfirmed these findings in the mouse spleen system. Induction of ST6Gal I at both the mRNA (Fig. 1A) and protein levels (Fig. 1B) was observed following whole-body radiation with a dose of 1 Gy, and immunohistochemical analysis of the spleen after radiation exposure indicated that ST6Gal I was mainly expressed in the marginal zone of the white pulp of the spleen (Fig. 1C). Western blotting analysis showed that the soluble form of ST6Gal I (15 kDa, with a molecular weight of ~41 kDa (versus 47 kDa for the proform) was also observed after radiation exposure, suggesting that post-translational modification of ST6Gal I was induced by radiation.

Additionally, induction of other sialyltransferase mRNAs such as ST8Sia I, ST3Gal I, ST3Gal II, ST3Gal III, and ST3Gal IV were also observed in the spleen following whole-body irradiation and local irradiation of the mice (Fig. 1D). The expression of these mRNAs was also increased by radiation exposure in cell lines such as Daudi and U937 cells (Supplementary Data Fig. S1), suggesting that radiation exposure increased the expression of a variety of sialyltransferase genes. When we examined the mRNA and protein expression levels of ST6Gal I in monocyte/macrophage or lymphoid cell lines such as Raw 264.7, U937, and Daudi, expression of the gene was up-regulated in all of the cell lines in a dose-dependent pattern (Fig. 1E and F).

Radiation Exposure Increases Protein Sialylation

To elucidate whether increased expression of ST6Gal I following radiation exposure affects protein sialylation, fluorescence-activated cell sorting (FACS) analysis using a fluorescent lectin from *Sambucus negra* agglutinin (SNA),
which is an α2,6 sialylation–specific lectin or *Maackia amurensis* agglutinin (MAA), which is specific for α2,3 sialylation, was done. A total-body irradiation dose of 1 Gy resulted in increased binding of SNA and MAA to splenocytes (Fig. 2A), suggesting that radiation exposure increased the sialylation of cellular proteins.

When we examined these phenomena in the cell lines, similar effects were observed (Fig. 2B). To elucidate whether increased protein sialylation by radiation was dependent on ST6Gal I expression, RNA interference of ST6Gal I (Si-ST6Gal I) was carried out on control and ST6Gal I–overexpressing SW480 cells (SW480 colon cancer cells showed lower endogenous expression of ST6Gal I as compared with B lymphoma Daudi and weak enzymatic activity of ST6Gal I; Supplementary Data Fig. S3A; ref. 26). Radiation-induced SNA binding was inhibited by either Si-ST6Gal I treatment on SW480 cells (Fig. 2C) or neuraminidase treatment on Raw264.7 macrophage (Supplementary Data Fig. S2), suggesting that the radiation-induced increase in sialylation of glycoproteins was dependent on ST6Gal I activity.

The Soluble Form of ST6Gal I Has Protein Sialylation Activity

Radiation exposure induced ST6Gal I expression and cleavage of ST6Gal I, producing the soluble form (Fig. 1). We next tested whether the cleaved form of ST6Gal I affects protein sialylation. When wild-type Myc-tagged ST6Gal I (the Myc tag was added to the COOH terminus of ST6Gal I; the COOH-terminal fragment is soluble and is secreted into the medium) was overexpressed in SW480 cells, the secreted soluble form of ST6Gal I with a molecular weight of ~37 to 41 kDa was observed in the culture medium and its levels were increased by exposure to radiation (Fig. 3A). Following transfection of HA-ST6Gal I wild-type or HA-ST6Gal I ΔN (a truncated form of ST6Gal I with hemagglutinin added to the NH2 terminus; ref. 27), HA-ST6Gal I ΔN transfection resulted in detectable hemagglutinin fusion protein in the medium. However, following HA-ST6Gal I wild-type transfection, there was no detectable soluble HA-ST5Gal I (Fig. 3B), suggesting that the COOH-terminal soluble fragment was a cleaved form of ST6Gal I.

Expression of the soluble form of ST6Gal I, ST6Gal I ΔN, resulted in an increase in cellular lectin binding activity similar to that induced by ST6Gal I wild-type expression when analyzed by FACS (Fig. 3C), even though the transfection efficiency was higher for HA-ST6Gal I ΔN than for the ST6Gal I wild-type control vector. From these results, ionizing radiation significantly induced the production of the soluble form of ST6Gal I, which also had sialyltransferase enzyme activity and was secreted into the culture medium.

**Radiation Exposure Increases Sialylation of Integrin β1**

When we examined total protein sialylation patterns, proteins of ~100 kDa molecular weight showed increased sialylation following radiation exposure (Fig. 4A). Moreover, there have been reports that integrin β1 (120 kDa) and the transferrin receptor (98 kDa) are sialylated proteins. Indeed, these proteins were sialylated following radiation exposure either *in vivo* or *in vitro* with a slight increase in their protein levels (Fig. 4B; Supplementary Data Fig. S3). Flow cytometry analysis also indicated that radiation increased integrin β1 protein expression in spleen and Raw 264.7 cells (Fig. 4C), suggesting that radiation exposure increased the levels of integrin β1 in both *in vitro* and *in vivo* systems.

**Overexpression of ST6Gal I Increases Both Sialylation and Protein Stability of Integrin β1**

To elucidate the exact mechanisms of integrin β1 sialylation in response to radiation exposure, SW480 cells were transfected
with ST6Gal I (Myc-ST6Gal I). Increased SNA affinity for integrin $\beta_1$ was observed by FACS analysis (Fig. 5A). Radiation exposure increased integrin $\beta_1$ sialylation, as well as its protein stability in both control and ST6Gal I–overexpressing cells (Fig. 5B). Treatment with an interfering RNA targeting ST6Gal I reversed these effects (Fig. 5C). As the mRNA level of integrin $\beta_1$ was not altered by ST6Gal I overexpression (Supplementary Data Fig. S4B), a direct connection between sialylation by ST6Gal I and protein stability was examined. When neuraminidase 2 (cytosolic form, Neu2) or neuraminidase 3 (membrane form, Neu3), both of which have the ability to remove sialic acid from proteins, was cotransfected into ST6Gal I–overexpressing cells, the increased sialylation and protein stability of integrin $\beta_1$ was inhibited (Fig. 5D), suggesting that sialylation of integrin $\beta_1$ affects its protein stability. Moreover, treatment with cycloheximide to inhibit new protein synthesis increased integrin $\beta_1$ protein stability in ST6Gal I–overexpressing cells when compared with the control vector–transfected cells (Fig. 5E), suggesting that ST6Gal I overexpression leads to increased integrin $\beta_1$ sialylation and stability.

**Overexpression of ST6Gal I Induces Radiation Resistance**

To examine the role of ST6Gal I in the radiation response, Raw264.7 cells were treated with Si-RNA targeting ST6Gal I prior to radiation exposure. Radiation-induced cell death was significantly increased by Si-ST6Gal I treatment, as were PARP cleavage and caspase 3 activation (Fig. 6A-C). SW480 cell clones stably overexpressing ST6Gal I (nos. 6 and 22) also showed increased clonogenic survival following radiation and reduced radiation-induced cell death (Fig. 6D and E). Caspase 3 activation and PARP cleavage following exposure to a dose of 10 Gy of gamma radiation were also inhibited by ST6Gal I overexpression (Fig. 6F). Furthermore, the additional transfection of ST6Gal I overexpression clones with Si-ST6Gal I inhibited ST6Gal I–mediated radioresistance (data not shown).

To elucidate whether the radioresistance by ST6Gal I was related to sialylation by ST6Gal I, cells were cotransfected with Neu2 and cell death was examined. Following cotransfection with Neu2, inhibition of radiation-induced cell death and caspase 3 activation by ST6Gal I were reversed (Fig. 6G and H), suggesting that the ST6Gal I–induced radioresistance was mediated by protein sialylation.

**Discussion**

In the present study, we observed that radiation exposure increased the expression of ST6Gal I and its major cleavage form. Cleavage at a lysine residue produced a soluble form of ST6Gal I. Both forms were responsible for protein sialylation even though the soluble form showed less protein sialylation activity than its proform. Sialylation of integrin $\beta_1$, a protein known to be sialylated by ST6Gal I, was detected after radiation and this affected its protein stability. Moreover, ST6Gal I–mediated protein sialylation is involved in the radiation resistance of cells.

Our previous study suggested that mRNA expression of ST6Gal I was induced by exposure to a low dose of radiation, specifically in the spleen and intestine, both radiation-sensitive organs (23). Here, we elucidated the mechanisms of ST6Gal I in the radiation response. Radiation exposure increased the expression of ST6Gal I mRNA as well as protein in the mouse spleen and monocyte/macrophage or lymphoid cell lines. Expression of other sialyltransferases, such as ST8Sia I and ST3Gal I, ST3Gal II, ST3Gal III, and ST3Gal IV were also increased following radiation exposure (Fig. 1), suggesting that radiation affected the expression of a variety of sialyltransferases, which may be involved in protein sialylation. Indeed, when we examined sialylation patterns, increased sialylation...
after radiation exposure was shown in proteins isolated from splenocytes or from the cell line system (Fig. 2), suggesting that radiation-induced sialyltransferases promoted protein sialylation. Sialylation is a complex process initiated by the Golgi type II transmembrane glycosyltransferases. These enzymes have been identified in both the human and murine genomes (7). Sialic acids influence cellular enzymatic and receptor functions during tissue proliferation and differentiation. Likewise, sialic acids regulate intercellular communication and cell-to-extracellular matrix interactions.

Changes in the expression of specific sialic acids during differentiation may be useful markers for the degree of cell maturation (28, 29). In particular, changes in glycosylation have been observed in some congenital diseases (30-32) and pathologic processes, such as Alzheimer’s disease (33), prion protein disease (34), and cancer (35). Therefore, radiation-induced sialyltransferase expression and glycoprotein sialylation may be involved in late, inflammatory-type effects after exposure to ionizing radiation.

Because radiation exposure induced ST6Gal I cleavage in tissue and cell systems similar to Alzheimer disease (Fig. 1; ref. 36), we examined the soluble, secreted forms of ST6Gal I that were involved in protein sialylation, and found that both parental and soluble forms of ST6Gal I exhibited protein sialyltransferase activity that could be detected in the medium. We are now trying to find the candidate enzymes that are responsible for the radiation-induced cleavage of ST6Gal I. Recently, it was shown that the production of the cleaved form of ST6Gal I by β-site amyloid precursor protein-cleaving enzyme 1 enhanced the sialylation of soluble glycoproteins, but did not affect cell surface sialylation levels (37). Therefore, we hypothesized that wild-type ST6Gal I may affect cell surface sialylation and the cleaved form may function specifically in the sialylation of soluble proteins, even though it is not clear

FIGURE 5. Integrin β1 sialylation by ST6Gal I increases its protein stability. A. SW480 cells were transiently transfected with ST6Gal I-Myc. The level of integrin β1 expression and sialylation were analyzed by immunoblotting or FACS. B. SW480 cells were stably transfected with HA-ST6Gal I and exposed to 10 Gy of radiation for 24 and 48 h. Cellular proteins were subjected to immunoblotting and the lectin affinity assay. A representative immunoblot analysis shows the expression level and sialylation pattern of integrin β1 in the control vector and ST6Gal I stably transfected cell line with or without cotransfection of ST6Gal I (C), and of Neu2 or Neu3 (D). Protein extracts were prepared at the indicated time points following cycloheximide (20 μg/mL) treatment of control and ST6Gal I—overexpressing cells, and an immunoblot was done using an integrin β1 antibody (E). The results represent one of three independent experiments.
whether radiation-induced ST6Gal I cleavage and secretion are dependent on β-site amyloid precursor protein-cleaving enzyme I activity or not.

The soluble form of ST6Gal I showed lower sialyltransferase activity than the full-length wild-type control in transfected cells. We do not know exactly why high levels of the soluble form exhibited weak sialyltransferase activity (Fig. 3); however, there has been one report in which the soluble form of ST6Gal I showed weak enzymatic activity when compared with its proform (27).

We wished to determine whether individual proteins were sialylated after radiation exposure. We made stably expressed clones of ST6Gal I in SW480 colon cancer cells (line nos. 6 and 22). A prominent characteristic of cell lines stably expressing ST6Gal I is their strong attachment to culture dishes (it was hard to detach from the culture dish using trypsin-EDTA as compared with control cells). Therefore, we are now investigating the adhesion of these cells to the extracellular matrix and to adhesion-related proteins. Additionally, when the sialylation of total protein extracts was examined, many candidate sialylated proteins with approximate molecular weights of 100 kDa were detected (Fig. 4). Among candidate sialylated glycoproteins, the transferrin receptor (98 kDa) and integrin β1 (120 kDa) have been reported to be sialylated (25, 38). Because sialylation of integrin β1 following radiation exposure was predominant, we examined radiation-mediated integrin β1 sialylation.

Integrin β1 proteins, recently reported as having increased levels in patients involved in radiation accidents (39), are substrates of several glycosyltransferases including β1,6,
N-acetylgalcosaminyltransferase V (40, 41), sialyltransferase ST6GalNAc 1 (42, 43), and ST6Gal 1 (25, 44). Various phenotypic changes have been shown to be the direct results of altered glycosylation of integrin molecules, but in some cases, they have also been related to the differential glycosylation of integrin-associated cell surface molecules (45, 46). In our system, integrin β1 was sialylated following radiation exposure and sialylated integrin β1 exhibited increased protein stability. Moreover, neureaminidase treatment inhibited the radiation-induced increase in protein stability (Fig. 5), indicating that sialylation of integrin β1 was directly related to the stability of the protein. Several studies support a role for integrin carbohydrate groups in regulating the association between integrins and ligands. Akiyama et al. (47) reported that the treatment of human foreskin fibroblasts with glycosylation inhibitors blocked cell adhesion to fibronectin. Similarly, Zheng et al. (40) showed that ligand binding was altered when N-linked carbohydrates were enzymatically cleaved from cell surface α5β1 integrins, suggesting that the presence of sialic acids could directly modulate ligand/receptor interactions. We do not know exactly why sialylation of integrin β1 affected its protein stability. However, one possibility is that adhesion to a solid substrate can stabilize integrin β1 on the cell surface through a ST6Gal 1–dependent mechanism (48).

ST6Gal I has been suggested to have an important role in oncogenic transformation and metastasis (49-51). Increased expression of ST6Gal I has been observed in colorectal cancer (52, 53), breast cancer (54), cervical cancer (55), and choriocarcinoma (56). However, elevated ST6Gal I inhibited the formation of a glioma in vivo (21). Therefore, expression of ST6Gal I may have different effects in different cancer types. However, an altered radiation response by ST6Gal I was never suggested. In this study, ST6Gal I induced radioresistance and when Si-ST6Gal I or Neu2 was cotransfected, the increased radioresistance was abolished (Fig. 6), suggesting that ST6Gal I mediated protein sialylation is involved in the radiation resistance response and protein sialylation enables the cell to resist radiation-induced damage through the inhibition of apoptosis. Ionizing radiation causes cancer and metastasis (57, 58). Therefore, we are now examining the effects of radiation-induced increases in protein sialylation on adhesion and metastasis using sialylation site mutants of integrin β1.

In conclusion, radiation-induced expression of sialyltransferases includes ST6Gal I. Protein sialylation by ST6Gal I has been frequently shown to be higher in cancer cells, is involved in the protein stability of integrin β1, and provides cellular radioresistance, suggesting that protein sialylation might be a novel target to overcome radioresistance in radiation therapy.

Materials and Methods

Cell Culture
Daudi human B-lymphoma and U937 human monocytic leukemia were cultured in RPMI supplemented with heat-inactivated 10% fetal bovine serum and antibiotics at 37°C in a humidified incubator with 5% CO2. The SW480 human colorectal carcinoma cell line and Raw 264.7–transformed macrophages were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Life Technologies).

Plasmids and Transfection
Wild-type ST6Gal I (GenBank accession no. X17247) and ΔN-ST6Gal I (amino acids 43-403; ref. 27) were cloned into pcDNA3 containing a hemagglutinin tag (59). ST6Gal I was cloned into the pCMV vector, which contains an NH2-terminal Flag tag and COOH-terminal Myc tag (Sigma). Predesign small interfering RNA for ST6Gal I was purchased from Dharmacon. Neu2 and Neu3 were generously provided by Dr. Eugenio Monti (Department of Biomedical Science and Biotecnology, University of Brescia, viale Europa, Italy) and Dr. Taeko Miyagi (Miyagi Cancer Center Research Institute, Miyagi, Japan). Neu2 and Neu3 were each subcloned into new vectors containing a hemagglutinin tag and a Flag tag. Cells were transfected with plasmids using LipofectAMINE 2000 (Invitrogen). Primers for amplification of ST6Gal, Neu2, and Neu3 are listed in Supplementary Table S1.

Animals
Female C57BL/6 mice, 6 to 7 weeks old, were purchased from Charles River. The housing conditions were 22 ± 2°C, 50 ± 10% humidity, and a 12-h light-dark cycle. Studies were conducted under the guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences.

Irradiation
Cells were exposed to γ-rays with a 137Cs γ-ray source (Atomic Energy of Canada, Mississauga, Ontario, Canada) at a dose rate of 3.81 Gy/min.

Immunoblot, Lectin Affinity Assay, and Immunoprecipitation
Protein levels were detected using the following commercial antibodies: mouse monoclonal anti-integrin 1β (BD Biosciences), anti-hemagglutinin (Cell Signaling Technology), anti-Myc (Santa Cruz Biotechnology), and anti-Flag (Sigma). Sialyltransferase was detected using a polyclonal rabbit antiserum that had been raised against the peptide GYLPKENFRTKAGP (amino acids 164-177 of mouse sialyltransferase). Splenocyte suspensions were prepared by disrupting the spleens over a 200-μm mesh (Cadisch Precision Meshes Limited) into 3 mL of RPMI 1640 (Dutch modification; Invitrogen) containing 10% heat-inactivated fetal bovine serum and antibiotics. Akiyama et al. (47) reported that the treatment of human foreskin fibroblasts with glycosylation inhibitors blocked cell adhesion to fibronectin. Similarly, Zheng et al. (40) showed that ligand binding was altered when N-linked carbohydrates were enzymatically cleaved from cell surface α5β1 integrins, suggesting that the presence of sialic acids could directly modulate ligand/receptor interactions. We do not know exactly why sialylation of integrin β1 affected its protein stability. However, one possibility is that adhesion to a solid substrate can stabilize integrin β1 on the cell surface through a ST6Gal 1–dependent mechanism (48).

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the detection of sialylated proteins, cell lysates were incubated with SNA-biotin and protein-lectin complexes were precipitated with avidin-coated protein A–agarose (Sigma). The protein complexes were then analyzed by SDS-PAGE. After electrophoresis, immunoblotting was carried out as previously described (60).

Clonogenic Survival Assay

Clonogenicity was examined by use of the colony-forming assay as previously described (61).

Immunohistochemistry

Spleen tissues were fixed in 4% formaldehyde solution, dehydrated in ethanol followed by xylene, and then finally embedded in paraffin. Sections were prepared at 3 µm in thickness, de waxed, and then rehydrated. Sections were stained with ST6Gal I antibody and immunoperoxidase labeling was done as previously described (62).

Reverse Transcription-PCR

Total RNA was isolated with TRI reagents (Molecular Research Center) and reverse transcription was done using Omniscript transcriptase (Qiagen). PCRs were processed by heating the reaction mixtures at 95°C for 5 min, annealing at 58°C for 30 s, and extension at 72°C for 30 s, for 30 cycles. Primer sequences are listed in Supplementary Table S1.

FACS Analysis

Cells were detached with trypsin-EDTA at the indicated times, washed with PBS and stained with propidium iodide (1 µg/mL) according to the manufacturer’s protocol for cell death analysis or with FITC-conjugated lectins (FITC-SNA and FITC-MAL; Vector Laboratories) for sialylation detection as previously described (63, 64). Stained cells were then analyzed using a FACSScan flow cytometer (Becton Dickinson). Cell surface integrin β1 was detected with FITC-conjugated integrin β1 antibody (Santa Cruz Biotechnology) as previously described (65).

Neuraminidase Treatment

Cells were treated with 6 mU/10^6 cells of neuraminidase (Roche Diagnostics) in serum-free DMEM for 90 min at 37°C. After treatment, cells were incubated with FITC-conjugated lectins (FITC-SNA) for sialylation detection, and then analyzed by FACS.

Statistical Analysis

Statistical significance was determined using Student’s t test for comparison between the means. Mean values were given as mean ± SD. A null hypothesis was rejected whenever a P ≤ 0.05 value was found.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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