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3′-Sulfo-Le^x Is Important for Regulation of Integrin Subunit αV†

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ABSTRACT: Carbohydrate structures with a 3′-sulfo βGal linkage, such as 3′-sulfo-Le^x, can be synthesized by Gal:3-*O*-sulfotransferase-2 (Gal3ST-2) catalysis, but little is known about their roles in many biological processes. To investigate the role of Gal3ST-2 and its product 3′-sulfo-Le^x, we depleted Gal3ST-2 via siRNA and added exogenous Lewis-x trisaccharide 3′-sulfate sodium salt in human SMMC7721 hepatoma cells. After siRNA transfection, a striking morphological change in SMMC7721 hepatoma cells from polygon to shuttle shape and a significant decrease in the level of adhesion to sL-selectin, HUVEC, fibronectin, vitronectin, and fibrinogen were observed. The expression of integrin subunit αV was markedly downregulated, and 3′-sulfated subunit αV almost disappeared in the transfectants. The level of cell surface integrin αVβ3 was reduced simultaneously, although total subunit β3 underwent almost no change. After treatment with exogenous Lewis-x 3′-sulfate, cellular integrin subunit αV was upregulated and the level of cell surface integrin αVβ3 was elevated. Interestingly, knockdown of Gal3ST-2 expression effectively inhibited cell proliferation, and the result was significantly correlated with the decrease in the levels of ILK, phosphorylated AKT, and ERK. On the other hand, treatment with Lewis-x trisaccharide 3′-sulfate sodium salt greatly upregulated the phosphorylation of AKT and ERK. Our results also indicated that downregulation of Gal3ST-2 via siRNA transfection was associated with the decrease in the level of expression of anti-apoptotic protein, Bcl-2, with a consequent decrease in the ratios for Bcl-2 to Bax. By exposure to Lewis-x trisaccharide 3′-sulfate sodium salt, the apoptotic response of cells was inhibited. Therefore, Gal3ST-2 and its product, 3′-sulfo-Le^x, were involved in regulation of integrin subunit αV and might be associated with cancer cell regulation.

Sulfated glycoconjugates occur in a wide range of biological compounds, including glycoproteins, proteoglycans, glycolipids, and polysaccharides (*1*). A considerable body of evidence has accumulated relative to the biological importance of sulfation of carbohydrate chains (*2*−*5*). The sulfate group may be attached to positions 3 and 6 of Gal, and the 3-sulfo-βGal linkage is found in both *N*-glycans and *O*-glycans. Among these are sulfo-3Galβ1−4(Fucα1−3)GlcNAc-R (3′-sulfo-Le^x) and sulfo-3Galβ1−3(Fucα1−4)GlcNAc-R (3′-sulfo-Le^a) (*6*−*8*), which recently attracted special attention because they serve as ligands for the adhesion molecule selectin and are thought to play significant roles in cell adhesion, inflammation, and cancer metastasis (*9*, *10*). It has been determined that the 3′-sulfo-Lewis epitopes have a stronger affinity for E-selectin and are more potent ligands for L-selectin than the corresponding 3′-sialylated Lewis epitopes as evidenced by a binding assay using chemically synthesized oligosaccharides (*10*, *11*). In a human colon carcinoma LS174T-HM7 cell line with a high metastasis potential, the 3′-sulfo-Le^x epitope is shown to be a major carbohydrate motif (*7*, *12*). However, the biosynthesis of these determinants might need Gal:3-*O*-sulfotransferase (Gal3ST). The sulfate group linked to the Gal C-3 position is transferred by Gal3ST from adenosine 3′-phosphate 5′-phosphosulfate (PAPS), and subsequently, the fucosyl residue may be transferred to the 3′-sulfated Galβ1−3/1−4GlcNAc-R by α1,3-fucosyltransferase (*13*, *14*) to biosynthesize 3′-sulfo-Lewis antigens (*15*). To date, four human Gal3STs have been cloned. The first, Gal3ST-1, is a cerebroside 3-*O*-sulfotransferase (CST), which acts on GalCer, LacCer, and GalDG (*16*). Gal3ST-2[1] and Gal3ST-3 could act on both type 1 (Galβ1−3GlcNAc-R) and type 2 (Galβ1−4GlcNAc-R) oligosaccharides and were thought to be the key enzymes in the biosynthesis of 3′-sulfo-Lewis antigens. Gal3ST-2 is expressed in various tissues, including colonic epithelial cells, which had a relatively high level in human colon and colonic mucinous adenocarcinoma (*15*), but Gal3ST-3 exhibits rather limited expression, which is mainly expressed in the human thyroid, with moderate expression in the brain and kidney (*17*, *18*). Gal3ST-4 (*19*) is highly specific for core 1 (Galβ1→3GalNAc) structures.

Gal3ST-2 was strongly expressed in tumor cells with high metastatic potential compared with those cells with low metastatic potential (*20*). Thus, the change in 3-sulfated Lewis structure was mainly due to the abnormal expression of Gal3ST-2 in tumor

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[1]Abbreviations: Gal3ST-2, Gal:3-*O*-sulfotransferase-2; HUVEC, human umbilical vein endothelial cells; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; CST, cerebroside 3-*O*-sulfotransferase; GalCer, galactosylceramide; LacCer, lactosylceramide; GalDG, galactosyldiacylglycerol; FCS, fetal calf serum; PBS, phosphate-buffered saline; EB, ethidium bromide; PVDF, polyvinylidene difluoride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ECL, enhanced chemiluminescence; ECM, extracellular matrix components; FN, fibronectin; ILK, integrin-linked kinase; PKB/AKT, protein kinase B/AKT; Mek, MAPK kinase; PI-3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase.

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cells. Our previous studies (*20*) revealed that there was a correlation between the overexpression of Gal3ST-2 and tumor metastasis, and the sulfated galactose may play a vital role in the process of metastasis. However, the problem of how Gal3ST-2 and its products, sulfated glycans, in cancer cells influence tumor cell metastasis behaviors remained unclear. In this study, we investigated the effect of Gal3ST-2 siRNA and Lewis-x trisaccharide 3′-sulfate sodium salt, a synthesized 3′-sulfated Le^x-type trisaccharide, on tumor cell adhesion, proliferation, and regulation of integrin. Our aim was to determine the roles of sulfated Le^x, the product of Gal3ST-2, in SMMC7721 cancer cells, which may be able to serve as a therapeutic target.

MATERIALS AND METHODS

Reagents. Lewis-x trisaccharide 3′-sulfate sodium salt was purchased from Sigma (St. Louis, MO). Lewis-x trisaccharide was from Merck (Darmstadt, Germany). Polyclonal anti-ILK (rabbit anti-human), anti-phospho-p44/42 ERK1/2 (rabbit anti-human), anti-p44/42 ERK1/2 (rabbit anti-human), anti-phospho-AKT (rabbit anti-human), and anti-AKT (rabbit anti-human) antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-integrin αV (rabbit anti-human), anti-integrin $\beta3$ (rabbit anti-human), anti-Bcl-2 (rabbit anti-human), and anti-Bax (rabbit anti-human) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FITC anti-human CD51, CD104, and CD61 antibodies were from BioLegend (San Diego, CA). FITC anti-human integrin $\beta1$ and $\beta5$ antibodies were from eBioscience (San Diego, CA). Fibrinogen was from Sigma-Aldrich (St. Louis, MO). The antibody against integrin αV$\beta3$ was from Dako (Carpinteria, CA).

Gal3ST-2 siRNA Transfection. The sequence 5′-atgtggttcgacttcggct-3′ that targeted Gal3ST-2 (AF048727) was designed, as it excluded the similar sequence with other genes, especially its homologous family genes by BLAST. Then, the 55 bp synthesized forward and reverse primers containing the siRNA sequence were annealed and ligated to the pSilencer 4.1 vector digested by BamHI and HindIII, and the recombinant plasmid was sequenced. The negative control vector was from Ambion (Austin, TX). Transient transfection of SMMC7721 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The pSilencer vector encoding Gal3ST-2 siRNA was transfected to 80% confluent SMMC7721 cells. At different times (12, 24, or 48 h) after transfection, cells were used for detection.

Cell Culture. Hepatoma cells (SMMC7721) were obtained from the Institute of Cell and Biochemistry Research of the Chinese Academy of Science. Hep3B cells were from ATCC (Rockville, MD). The cells were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% newborn bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a 5% CO_2 atmosphere.

Lewis-x trisaccharide and Lewis-x trisaccharide 3′-sulfate sodium salt were diluted in phosphate-buffered saline (PBS) at the desired concentrations. SMMC7721 cells were treated with Lewis-x trisaccharide or Lewis-x trisaccharide 3′-sulfate sodium salt at a final concentration of 40 μM in culture medium, and cells were harvested after being incubated for 12, 24, or 48 h. The cells without treatment were used as a negative control.

Reverse Transcription Polymerase Chain Reaction (*RT-PCR*). Isolation of total cellular RNA and RT were described previously (*21*). Primer sequences for PCR were as follows: AGCACCGTCTATGTCACCAT (sense) and GAAGTACTCGGCGATCATGA (antisense) for Gal3ST-2, ACTGGGAGCACAAGGAGAACC (sense) and CCGCTTAGTGATGAGATGGTC (antisense) for αV subunit, CCTACATGACCGAAAATACCT (sense) and AATCCCTCCCCACAAATACTG (antisense) for subunit $\beta3$, and GAGCTACGAGCTGCCTGACG (sense) and CCTAGAAGCATTTGCGGTGG (antisense) for β-actin. PCR amplification was performed by initial denaturation at 94 °C for 2 min, followed by 25 cycles of 94 °C for 0.5 min, 55 °C for 1 min, and 72 C for 1.5 min with a further extension of 72 °C for 10 min. After amplification, the PCR product was analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide (EB) staining on an image system (Pharmacia Biotech). The intensity of the DNA bands was analyzed with Glyco Bandscan Analysis.

Cell Adhesion to Soluble L-Selectin, Vitronectin, Fibronectin, and Fibrinogen. The adhesion assay was based on our previous report (*22*). Briefly, 96-well plates were incubated with 100 μL of sL-selectin per well (5 μg/mL) produced by the baculovirus expression system (kindly provided by Dr. Stott, GlaxoSmithKline), 200 μL of vitronectin per well (2 μg/mL), 200 μL fibronectin per well (10 μg/mL), or 200 μL of fibrinogen per well (20 μg/mL) at 4 °C overnight. The negative control groups were incubated with 1% BSA instead, and the positive groups were coated with polylysine. After the samples had been blocked with 1% BSA for 1 h at 37 °C and washed three times with PBS, 200 μL of the cell suspension (2×10^4 per milliliter) was added to each well. All the cells were harvested by trypsin/EDTA treatment, washed with PBS, resuspended in 10% serum-containing medium, and allowed to recover from the trypsinization for 1 h at 37 °C. After the samples had been added to the wells and incubated at 37 °C for 1 h, all wells were washed with PBS twice and stained with MTT, and absorbance at 490 nm was measured.

Adhesion of Cells to HUVECs. HUVECs in 150 μL of complete growth medium were seeded onto 96-well plates at a density of 1×10^4 cells per well and incubated overnight at 37 °C to confluence. Then, the HUVEC monolayer was stimulated with 10 ng/mL TNF-α (Sigma) for 4 h prior to the addition of the transfected SMMC7721 (1×10^5 per milliliter) cells in 200 μL of RPMI 1640 and 1% BSA. After incubation at 37 °C for 1 h, unattached cells were vigorously washed off with PBS, and the attached cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, adherent cells were counted under a phase contrast microscope (Olympus) in five random fields in each well. The percentage of adherent cells in total was calculated to show the affinity of cells for HUVECs as described previously (*20*). Data are reported as means ± the standard deviation (SD) of at least five wells, and the experiment was repeated three times independently.

Flow Cytometry Analysis. SMMC-7721 cells were detached by treatment with 0.02% EDTA, washed with PBS, and maintained in suspension for 1 h in DMEM with 10% FBS. Cells were then incubated with 5 μg/mL fluorescein isothiocyanate (FITC)-labeled primary antibody for 1 h at 4 °C. After the samples had been washed in PBS, cell surface immunofluorescence was analyzed by a flow cytometer fluorescence-activated cell sorting (FACS) scan (Becton Dickinson, Mountain View, CA).

Western Blotting Analysis. Cells were rinsed twice with PBS and then lysed with RIPA buffer (100 mM Tris, 0.15 M NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1% aprotinin, 2 mM PMSF, 10 mg/mL leupeptin, 5 mM EDTA, 1 mM sodium vanadate, and 50 mM NaF) for 1 h. For analysis of protein expression, equal amounts of cell extracts (50 μg of

protein) were determined using the BCA protein assay reagent (Pierce, Rockford, IL). Samples were analyzed by SDS−PAGE and then transferred onto a PVDF membrane. Anti-human rat polyclonal antibodies against integrin αV and β3 were used at a dilution of 1:200. To detect ILK, phospho-AKT, and phospho-ERKs, the ILK, phospho-AKT, and phospho-ERK antibodies were used at a 1:1000 dilution; to probe total AKT and ERK, the AKT and ERK polyclonal antibodies were used at a 1:1000 dilution. Anti-human rat polyclonal antibody against Bcl-2 and Bax was used at a dilution of 1:200. To check the amount of proteins transferred to the nitrocellulose membranes, β-actin was used as a control and detected by an anti-actin polyclonal antibody at a 1:5000 dilution. Immunoreactive bands were visualized using horseradish peroxidase-coupled goat anti-rabbit immunoglobulin and the ECL detection system (Amersham Biosciences, Fairfield, CT).

Immunoprecipitation. Cells were grown to confluence on 100 mm dishes, rinsed twice in ice-cold PBS, harvested, and then hydrolyzed with 125 milliunits/mL neuraminidase at 37 °C for 1 h, followed by lysis in immunoprecipitation (IP) buffer containing 0.5% Triton X-100, 120 mM NaCl, 15 mM Tris-HCl (pH 7.5), 1 mM PMSF, 0.1 mM DTT, 10 μM aprotinin, and 10 μM leupeptin. After incubation in lysis buffer on ice for 30 min, the extracts from cells were centrifuged at 4 °C and 14000*g* for 30 min and the supernatants were transferred to new tubes. The supernatants with an equal amount of protein were mixed with 100 μL of Streptavdin-agarose (Vector) and 5 μL of Bio-MALII (Vector) followed by rotation at 4 °C overnight. The bound beads was then washed four times with lysis buffer, and 50 μL of the elution solution (100 mM lactose) (*23*) was added and votexed for 10 min. Aliquots of the supernatants were added into SDS loading buffer, heated at 100 °C for 5 min, and then applied for Western blot analysis. The sample lacking Bio-MALII was regarded as the negative control.

Proliferation Assay. The effect of Gal3ST-2 siRNA or exogenous Lewis-x trisaccharide 3′-sulfate odium salt upon proliferation in SMMC7721 cells was measured using the MTT assay. Cells were seeded in 96-well plates and incubated for 12, 24, or 48 h in complete medium with plasmids or drugs, and then 20 μL of the MTT labeling solution (5 mg/mL) per well was added. After incubation at 37 °C for 4 h, culture medium was discarded and 150 μL of DMSO was added to each well. The samples were quantified spectrophotometrically by measuring the absorbance of the formazan product at 540 nm with an ELISA plate reader.

Apoptosis Assay. Forty-eight hours after treatment, SMMC7721 cells grown on the cover glass were fixed with methanol and glacial acetic acid (3:1) at 4 °C for 5 min. After being washed with triply distilled water, the cells were stained with Hoechst 33258 (emitting blue fluorescence) at room temperature for 10 min. Then the cover glasses were observed under a phase microscope with fluorescence attachment after being fixed with citrate buffer [20 mM citrate, 50 mM monosodium phosphate, and 50% glycerol (pH 5.5)]. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin bodies in intact but shrunken cells and were counted among 1000 cells randomly. The apoptotic index was the mean of three independent experiments.

Statistical Analysis. Quantitative data are expressed as the means ± SD of at least three independent experiments. The Student's *t* test was used to evaluate experimental data with SPSS version 11.0. *P* values of < 0.05 were considered significant.

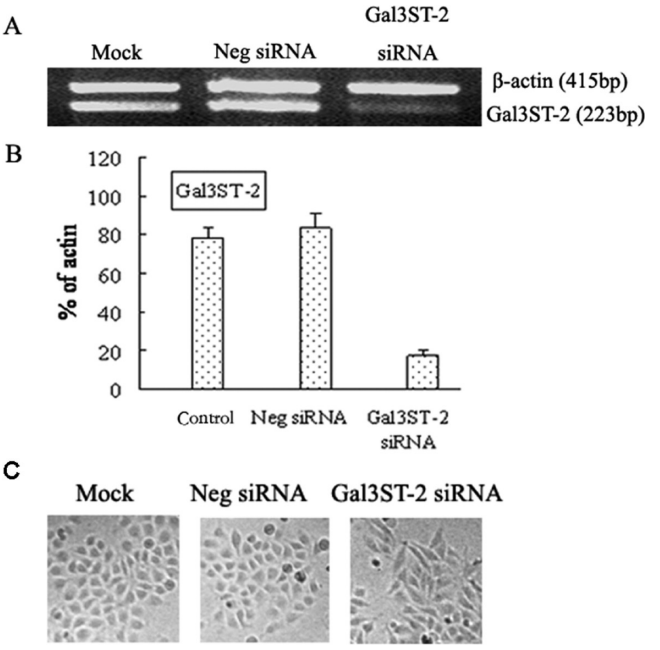

FIGURE 1: Observation of Gal3ST-2 expression in transfected SMCC7721 cells with Gal3ST-2 siRNA. Cells were transfected without (Mock) or with a control vector (Neg siRNA) or Gal3ST-2 siRNA vector (Gal3ST-2 siRNA) for 48 h. (A) Detection of Gal3ST-2 mRNA expression by RT-PCR. (B) Relative Gal3ST-2 mRNA levels using β-actin as a system reference. (C) Morphological comparison between SMMC7721 cells transfected with Gal3ST-2 siRNA and control cells, at 400× magnification.

RESULTS

Gal3ST-2 Gene Target by siRNA. The sequence targeting Gal3ST-2 was designed, and the 55 bp synthesized hairpin sequence was inserted into the pSilencer vector and subsequently transfected to hepatoma SMMC7721 cells in which the level of expression of Gal3ST-2 was high. Forty-eight hours post-transfection, a significantly lower Gal3ST-2 expression level was confirmed in the Gal3ST-2 siRNA transfectants than in mock and negative siRNA-transfected cells determined by semiquantitative RT-PCR (Figure 1A,B). Moreover, Gal3ST-2 siRNA-transfected cells exhibited a striking morphological change from polygon to shuttle shape (Figure 1C). Compared with negative siRNA-transfected cells, Gal3ST-2 siRNA-transfected cells exhibited weaker adhesion to the culture bottle and were trypsinized easily.

Adhesion Alteration. Because 3′-sulfo-Lewis epitopes have a strong affinity for E-selectin and are potent ligands for L-selectin that could participate in cell adhesion and cancer cell metastasis, the transfected SMMC7721 cells were assayed for adhesion to HUVECs induced by TNF-α and sL-selectin. As shown in panels A and B of Figure 2, adhesion to sL-selectin and HUVECs was all significantly attenuated in Gal3ST-2 siRNA-transfected cells compared with the control cells. In contrast, exogenous Lewis-x trisaccharide 3′-sulfate sodium salt treatment could enhance the adhesion of both sL-selectin and HUVECs (Figure 2C,D). With vitronectin, fibronectin, or fibrinogen, the adhesion of the cells transfected with Gal3ST-2 siRNA was significantly inhibited (Figure 2E), while the adhesion of negative siRNA control remained at a similar rate with Mock cells. With exogenous Lewis-x trisaccharide 3′-sulfate sodium salt, the cells were treated for 48 h and became more adhesive to vitronectin, fibronectin, or fibrinogen than the negative controls (Figure 2F). In Hep3B,

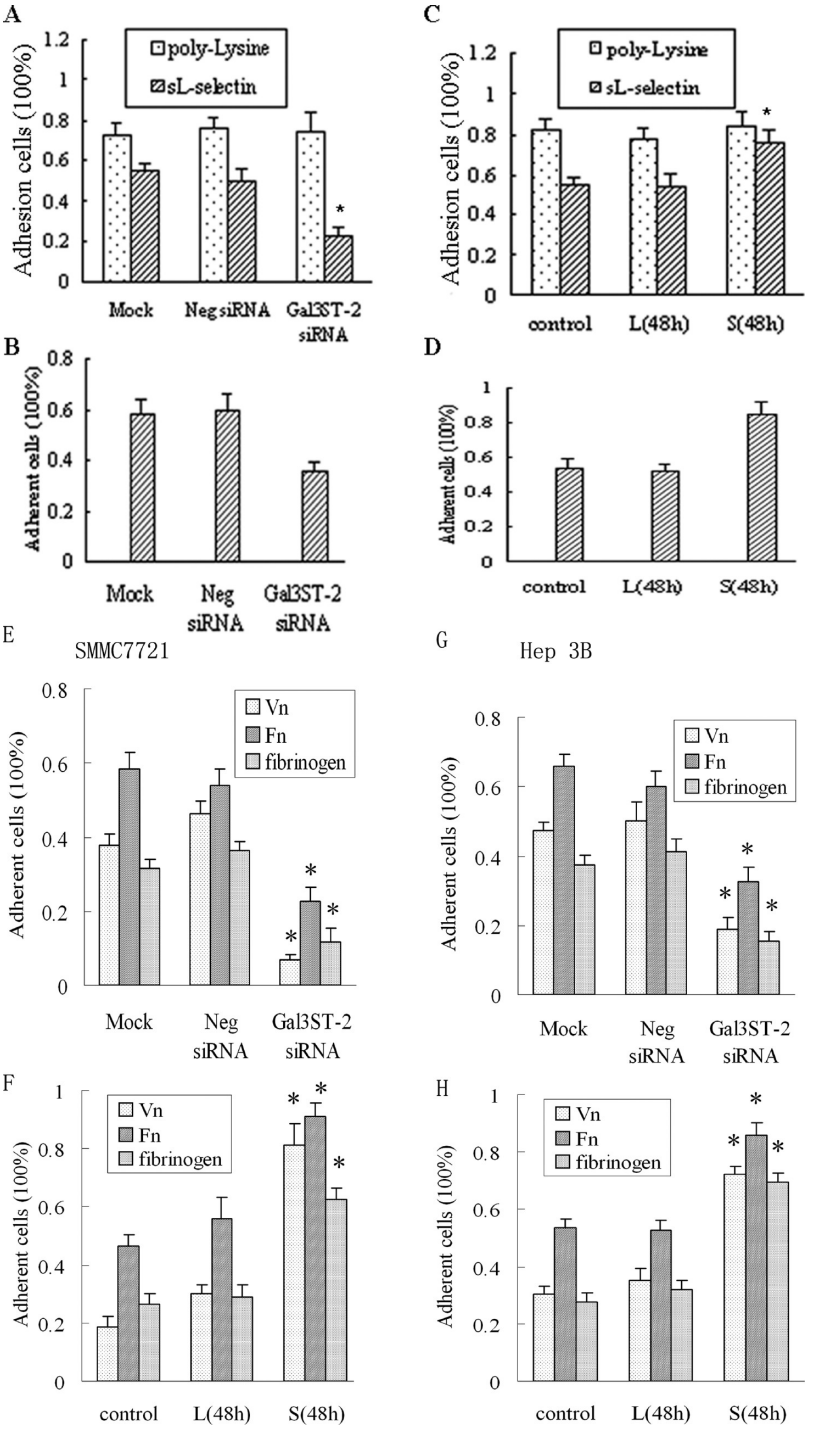

FIGURE 2: Adhesion assays in transfected and treated cells. (A) Adhesion of transfected cells to sL-selectin. Each well was coated with 100 μL of sL-selectin (5 μg/mL) at 4 °C for 16 h. After being blocked with 1% BSA for 1 h at room temperature, the transfected cell suspension was added to each well. Incubated at 37 °C for 1 h, attached cells were measured by the MTT assay. The adhesion of cells to polylysine was used as the positive control. (B) Adhesion to TNF-α-induced HUVECs in Gal3ST-2 siRNA-transfected cells. The HUVEC monolayer was stimulated with 10 ng/mL TNF-α for 4 h prior to the addition of transfected SMMC7721 cells in 200 μL of RPMI 1640 and 1% BSA. After being incubated for 1 h at 37 °C and washed with PBS, the attached cells were counted under a phase contrast microscope in five random fields in each well. The percentage of adherent cells in total was calculated to show the affinity of cells for HUVECs. (C and D) Adhesion to sL-selectin and TNF-α-induced HUVECs to cells treated with in Lewis-x trisaccharide (L) and Lewis-x trisaccharide 3′-sulfate sodium salt (S). (E) SMMC7721 cells transfected with Gal3ST-2 siRNA were examined for their adhesive capability. Forty-eight hours post-transfection, the numbers of cells adhering to vitronectin (Vn), fibronectin (Fn), and fibrinogen were represented by A_{490}. (F) Cell adhesion was tested after incubation for 48 h with 40 μM Lewis-x trisaccharide (L) or 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) at 37 °C. (G and H) Hep3B cells were transfected with Gal3ST-2 siRNA (top) or incubated with 40 μM Lewis-x trisaccharide (L) or 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) for 48 h at 37 °C and assayed for adhesion to vitronectin, fibronectin, and fibrinogen. Shown are the means ± SD from five separate experiments with equivalent results. Bars indicate the SD (*$P < 0.05$).

another kind of hepatocellular carcinoma cell, the adhesive results were also similar (Figure 2G,H)

Regulation of Integrin Subunit αV. Integrins are skeleton proteins that are important in cell morphology, adhesion, migration, and the cancer metastasis process. Vitronectin and fibronectin are associated with integrin αVβ3 as the ligand. Our previous studies also showed that the level of integrin subunit αV expression increased as the CST was overexpressed and sulfated

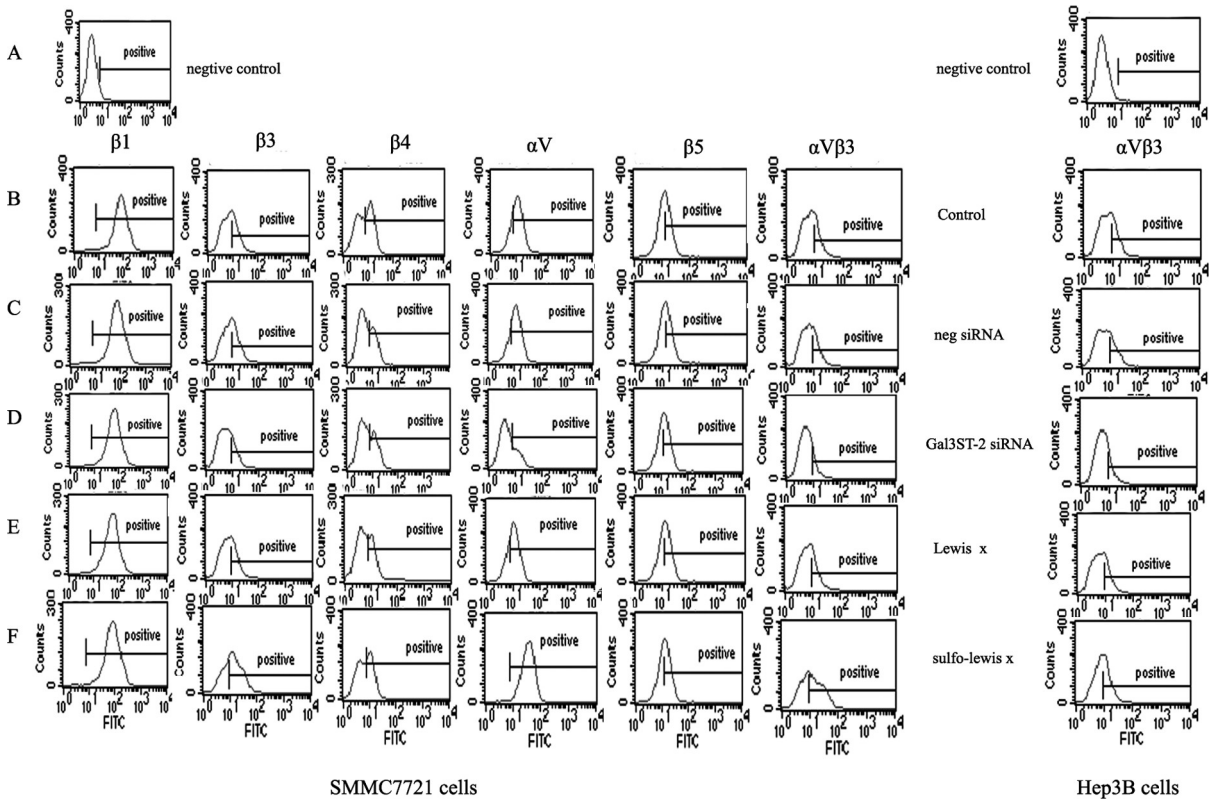

FIGURE 3: FACS analysis of cell surface integrin expression. SMMC7721 and Hep3B cells were transfected with siRNA vectors or treated with Lewis-x trisaccharide 3′-sulfate sodium salt as in adhesion assays; 48 h later, cells were harvested, stained with FITC-labeled integrin specific antibodies as indicated in the figure, and analyzed by FACS. (A) Negative control in which mouse IgG was used as the unrelated primary antibody. (B) Untransfected SMMC7721 cells. (C) Negative siRNA-transfected cells. (D) Gal3ST-2 siRNA-transfected cells. (E) Lewis-x trisaccharide-treated cells. (F) Lewis-x trisaccharide 3′-sulfate sodium salt-treated cells.

products were produced (*24*), which belonged to Gal3ST families and promoted metastasis. To further explore the regulation effect of Gal3ST-2 and its product sulfo-Le^x on integrin, we used flow cytometry, RT-PCR, and Western blotting to investigate integrin expression. After SMMC-7721 cells were detached and incubated with integrin antibodies, cellular surface immunofluorescence was analyzed with a flow cytometer. The data indicated that integrin subunit αV was significantly downregulated in the cells with Gal3ST-2 siRNA (Figure 3) and up regulated in cells treated with Lewis-x trisaccharide 3′-sulfate sodium salt (Figure 3) compared with their controls. The β subunits seemed not to be affected by such treatments, except subunit β3 had a change similar to that with the αV subunit but much less significantly. The level of adhesion of integrin αVβ3 was reduced slightly from 32 to 15% in the cells with Gal3ST-2 siRNA and elevated from 31 to 57% with positive staining after treatment with Lewis-x trisaccharide 3′-sulfate sodium salt (Figure 3). A similar regulation of integrin αVβ3 could be seen in Hep3B cells (Figure 3). Also as shown in Figure 4A, the level of integrin subunit αV expression was markedly decreased in either mRNA or protein level, while the total level of integrin subunit β3 expression was hardly affected. In SMMC7721 cells, the αV protein level decreased from 155.7% (negative siRNA) to 78.6% (48 h post-transfection with Gal3ST-2 siRNA) of actin expression.

Exogenous Lewis-x trisaccharide 3′-sulfate sodium salt was then used to treat cells in comparison with the control Lewis-x trisaccharide. In the SMMC7721 cells treated with exogenous Lewis-x trisaccharide 3′-sulfate, a significant upregulation of integrin αV expression was observed (Figure 4B) as compared with that in the control cells, while there were no or slight changes in the levels of total subunit β3. Lewis-x trisaccharide treatment for 48 h did not influence the level of integrin β3 protein (Figure 4B) but interestingly resulted in elevation of the level of expression of integrin αV to 222.2% of that in the controls by densitometry analysis in the cells.

To test if Lewis-x trisaccharide 3′-sulfate sodium salt indeed promoted αV expression, Gal3ST-2 siRNA constructs were transfected in SMMC7721 cells for 48 h and Gal3ST-2 gene expression was silenced. The transfected cells were then incubated with Lewis-x trisaccharide 3′-sulfate sodium salt or Lewis-x trisaccharide for an additional 48 h. Then the effect of Lewis-x trisaccharide 3′-sulfate sodium salt on the expression of integrin αV was evaluated. As seen in Figure 4C, both mRNA and protein levels of integrin subunit αV increased in cells treated with Lewis-x trisaccharide 3′-sulfate sodium salt, in comparison to the levels in control and Lewis-x trisaccharide-treated cells.

Furthermore, a significant sulfation of integrin αV was observed (Figure 4D) in the cells treated with Lewis-x trisaccharide 3′-sulfate, while the sulfated αV subunit almost disappeared in Gal3ST-2 siRNA-transfected cells during immunoprecipitation analysis (Figure 4E).

Stimulation of AKT and ERK Pathways. The proliferation of the cells with Gal3ST-2 siRNA was measured by the MTT assay in 96-well microplates. By 48 h after transfection, the Gal3ST-2 siRNA-transfected SMMC7721 cells showed a significant decrease in proliferation rate relative to that of the negative control (Figure 5A). By 48 h after exogenous Lewis-x trisaccharide 3′-sulfate treatment, the survival rate was upregulated 38.1% compared with those of the controls. The proliferation rate in the cells treated with Lewis-x trisaccharide was not significantly different from that of the controls (Figure 5C).

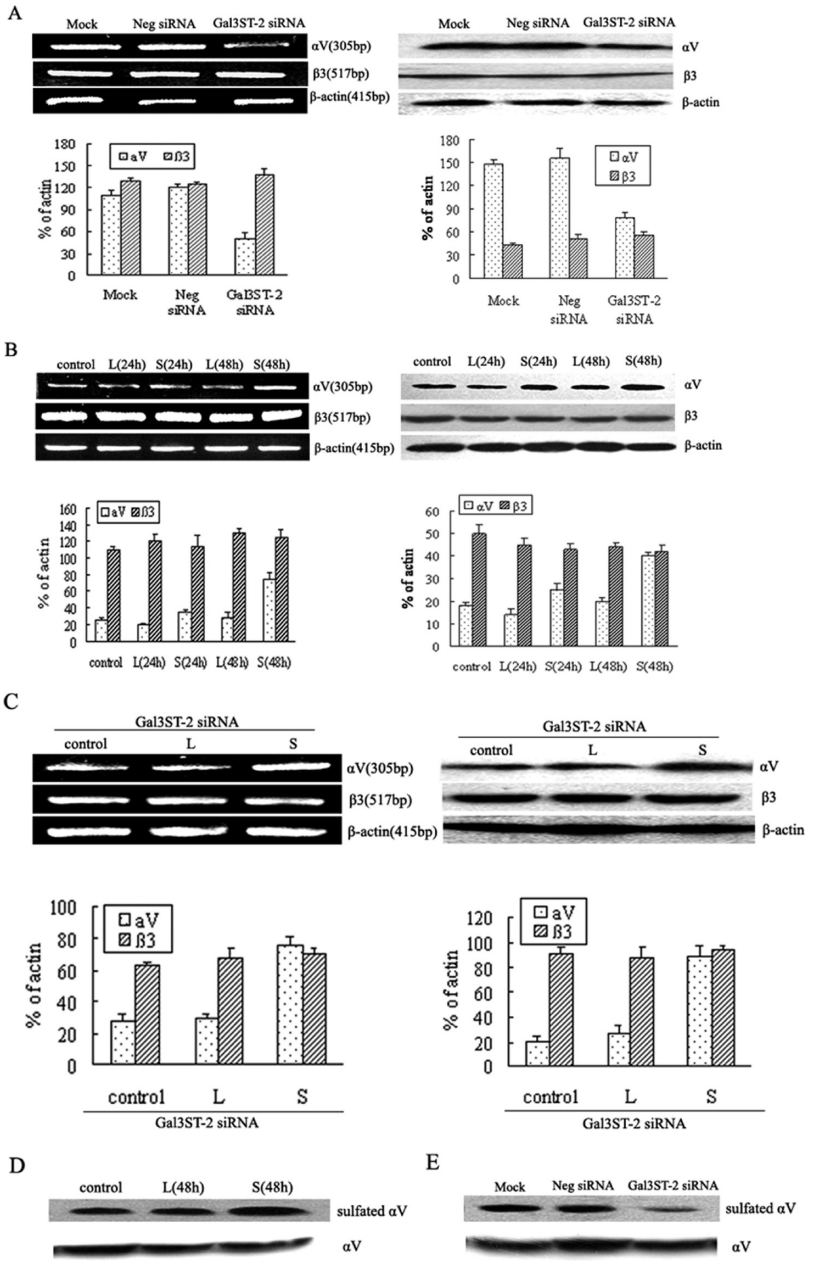

FIGURE 4: Expression analysis of integrin subunits αV and β3 in SMMC7721 cells. (A) The mRNA and protein levels of integrin subunits αV and β3 were detected by RT-PCR and Western blot in the transfected SMMC7721 cells. (B) The expression levels of integrin subunits αV and β3 were detected by RT-PCR and Western blot in SMMC7721 cells treated with Lewis-x trisaccharide 3′-sulfate sodium salt. (C) Cells were transfected for 48 h and treated without (control) or with 40 μM Lewis-x trisaccharide (L) or with 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) for an additional 48 h. Integrin αV/β3 mRNA and protein levels were assayed. (D and E) The 3′-sulfated subunit αV expression levels were measured by immunoprecipitation in transfected or Lewis-x trisaccharide 3′-sulfate sodium salt-treated SMMC7721 cells. The supernatants with equal amounts of lysed proteins were mixed with 100 μL of streptavidin-agarose and 5 μL of Bio-MALII followed by rotation at 4 °C overnight, and then 50 μL of an elution solution (100 mM lactose) was added and applied to SDS−PAGE. The blotted member reacted with the anti-αV antibody. The sample lacking Bio-MALII was regarded as the negative control.

The integrin-linked kinase (ILK), AKT, and ERK are intracellular protein kinases that couple integrins and growth factors to downstream signaling pathways involved in the suppression of apoptosis and in promoting cell cycle progression. Thus, we investigated ILK and total and phosphorylated forms of AKT and ERK (a principal MAPK) under the various test conditions. As shown in Figure 5B, SMMC7721 cells exhibited little ILK and phosphorylation of AKT and ERK after Gal3ST-2 siRNA transfection, as compared with cells transfected with negative control siRNA. The levels of ILK and phosphorylated forms of AKT and ERK expressed by Lewis-x trisaccharide 3′-sulfate-treated cells were higher than those expressed by the control cells. However, SMMC7721 cells exposed to Lewis-x trisaccharide had no significant change in ILK or phosphorylated AKT and ERK over the same time period versus the controls (Figure 5D). Total AKT and ERK protein levels were loaded as a control and remained unchanged regardless of treatment conditions.

Effect of Anti-Apoptosis. In cancer development, the resistance of cells to apoptosis is one of the most crucial steps. To ascertain whether apoptosis was occurring in the treated SMMC7721 cells, we used Hoechst 33258 to stain the cells at room temperature for 10 min to evaluate the apoptosis rate. Forty-eight hours after transfection, only 6.83% of the mock cells and 7.23% of the negative control SMMC7721 cells were apoptotic. By contrast, at

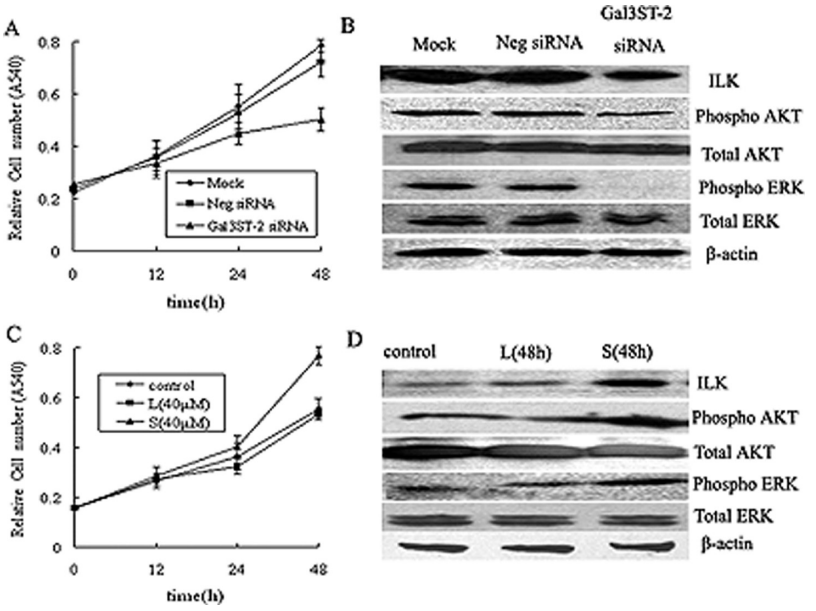

FIGURE 5: Proliferation assay and phosphorylation of AKT and ERK in SMMC7721 cells. (A) The growth curve was analyzed in SMMC7721 cells transfected with vector, negative control vector (Neg siRNA), and Gal3ST-2 siRNA vector (Gal3ST-2 siRNA). As described in Materials and Methods, the numbers of viable cells were assessed by the MTT assay. Shown are the means ± SD from three separate experiments. (B) SMMC7721 cells transfected with control vector (Neg siRNA) or Gal3ST-2 siRNA vector (Gal3ST-2 siRNA) were analyzed by Western blotting with the anti-ILK antibody, anti-phospho-AKT antibody, anti-AKT antibody, anti-phospho-ERK antibody, and anti-ERK1/2 antibody, 48 h after transfection. Equal loading of proteins in each lane was confirmed by probing the membranes with anti-β-actin antibody. (C and D) Cells were treated with 40 μM Lewis-x trisaccharide (L) or 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) for 48 h, and the numbers of viable cells and the levels of expression of proteins were assessed.

this time point, 22.36% of the Gal3ST-2 siRNA-treated cells were apoptotic. However, when the transfected cells were reposed to Lewis-x trisaccharide 3′-sulfate for 48 h, the apoptosis rate decreased slightly (Figure 6A). To examine the possible mechanism of apoptosis in such cells, we measured the expression ratio of Bcl-2 over Bax in SMMC7721 cells by immunoblotting described above. After the samples had been transiently transfected with Gal3ST-2 siRNA plasmids, the amount of prosurvival Bcl-2 protein was markedly decreased in SMMC7721 cells, as was the Bcl-2:Bax ratio (Figure 6B). Conversely, Bcl-2 levels were found to be significantly higher in cells treated with exogenous Lewis-x trisaccharide 3′-sulfate (Figure 6C).

DISCUSSION

Sulfate groups can be located at the 3′-hydroxyl position of galactose in various glycoconjugates, but little is known about their functions in biological processes such as cell adhesion, inflammation, and cancer metastasis, although carbohydrate sulfation has long been shown to be important for the formation of ligands that bind adhesion molecules belonging to the selectin family (*25*). The lack of efficient tools such as highly specific antibodies (*26*) greatly limited and retarded the progress of research. Sulfate groups serve as the terminal residue of the saccharide chain like fucosyl groups (*27*), which directly affects the functions of the molecules modified. Recently, our study showed that serum 3′-sulfated Lewis a is closely associated with the metastasis incidence in patients with gastric and colorectal cancers (*22*, *28*). Serum 3′-sulfated Lewis a is a promising reference for a patient's prognosis during the follow-up because of its relation to tumor metastasis. As sulfated trisaccharides, sulfated Lewis-x and Lewis a are widely distributed in tumor cells. Sulfated Lewis-x can also be the predominant determinant of tumor mucin, as in the LS174T-HM7 human colon carcinoma mucin (*7*). In in vitro assays, sulfo-Lewis antigens were shown to be more likely to bind to E-selectin than sialyl-Lewis antigens could (*29*). Thus, sulfo-Lewis antigens are more powerful in binding to E- and L-selectin, which are adhesion molecules important in the tumor metastasis process (*30*). Sulfated Lewis-x, the product of Gal3ST-2, might thus be involved in tumor metastasis.

Our previous study revealed that overexpression of Gal3ST-2 which produces the sulfated galactose mioties plays an important role in the process of tumor metastasis (*20*). In this study, we demonstrated, for the first time, that the functional role of Gal3ST-2 and its product, sulfated Lewis-x, in cell adhesion and growth might be associated with integrin sulfation and integrin-related signaling. Sulfated Lewis-x-repressed cells via Gal3ST-2 siRNA showed a striking morphological change from polygon to shuttle shape and weakened adhesive ability so they can be trypsinized more easily than control cells. Adhesion to HUVECs and sL-selectin was significantly attenuated in Gal3ST-2 siRNA-transfected cells. This suggested that the cellular sulfated Lewis-x was significantly reduced in the transfected cells. Moreover, the level of adhesion to vitronectin, fibronectin, and fibrinogen, which are ligands mainly for integrin $\alpha V\beta 3$, was also reduced significantly in Gal3ST-2 siRNA-transfected cells. RT-PCR and Western blotting analysis showed that the level of integrin subunit αV expression was markedly decreased and sulfated subunit αV almost disappeared. The expression of integrin subunit αV was upregulated when exogenous sulfated Lewis-x was added to the cells. Surface immunofluorescence analysis by FACS confirmed that the level of integrin $\alpha V\beta 3$ was reduced in Gal3ST-2 siRNA transfectants and greatly elevated after exogenous Lewis-x trisaccharide 3′-sulfate treatment, which were similar to the regulation effect of 3′-sulfated lactosylceramide catalyzed by CST (*31*). These results indicate that Gal3ST-2 was responsible for 3′-sulfated Lewis-x

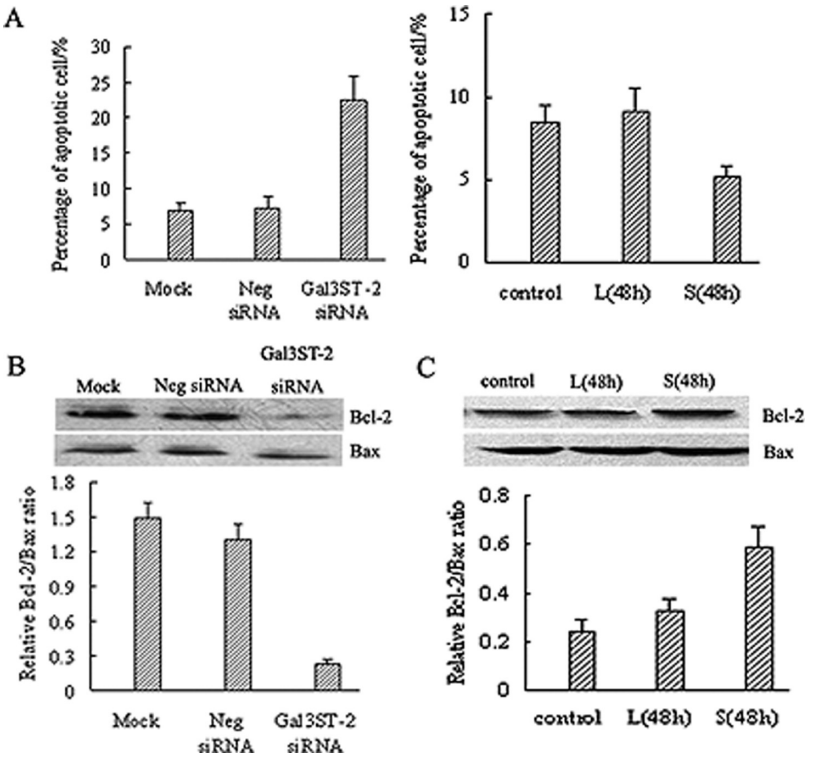

FIGURE 6: Apoptosis in Gal3ST-2-interfered or Lewis-x trisaccharide 3′-sulfate sodium salt-treated SMMC7721 cells. (A) The left panel shows that SMMC7721 cells were transfected for 48 h with negative control vector (Neg siRNA) or Gal3ST-2 siRNA vector (Gal3ST-2 siRNA) separately. The right panel shows that cells were exposed to 40 μM Lewis-x trisaccharide (L) or 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) for 48 h. Apoptotic cells were analyzed by cytofluorometric detection of apoptotic phenotypes. Data points are from three experiments with replicate cultures. The results are presented as means ± SD. (B) Western blot analysis of Bcl-2 and Bax expression in SMMC7721 cells, transfected with the Gal3ST-2 siRNA plasmid, or negative control siRNA as indicated. The chart shows the quantification of the Bcl-2:Bax expression ratio by densitometric analysis. (C) Cells were incubated without (control) or with 40 μM Lewis-x trisaccharide (L) or with 40 μM Lewis-x trisaccharide 3′-sulfate sodium salt (S) for 48 h. Cells were then processed and analyzed by immunoblotting vs Bcl-2 and Bax antibodies. The chart shows the quantification of the Bcl-2:Bax expression ratio by densitometric analysis.

synthesis and involved in the regulation of integrin subunit αV. Although the detailed mechanism needs to be studied further, the regulation seems to occur at the level of transcription because the integrin subunit αV mRNA was regulated simultaneously, which might be related to integrin subunit αV sulfation-related signaling. The cells treated with Lewis-x trisaccharide 3′-sulfate sodium salt, an exogenous 3′-sulfo-Le^x, promoted the expression of integrin αV and sulfation and enhanced adhesion to HUVECs and sL-selectin. However, Lewis-x trisaccharide did not have the same effect. This suggested that exogenous sulfated Lewis-x glycan could incorporate into cellular glycochains at a high concentration and triggered a similar response in the cells. On the other hand, the expression of integrin subunit αV was regulated by either exogenous 3′-sulfo-Lewis-x or cellular 3′-sulfo-Lewis-x via Gal3ST-2 catalysis. 3′-Sulfo-Lewis-x epitopes might bind to its ligands such as selectin, which, in turn, activated downstream effectors of signaling. The sulfation of integrin αV itself might also trigger signaling related to the integrin pathway, including ILK and ERK activation. However, this cannot exclude the possibility that the sulfation would affect the stability of integrin subunit αV.

The integrin family of cellular adhesion molecules is a major class of receptors through which cells interact with extracellular matrix components (ECM), and evidence has implicated the integrins as being closely involved in the pathology of many diseases (*32*−*34*). Integrins have been shown to participate in intracellular signal transduction pathways that may contribute to tumor cell growth and survival, although many of their functions have yet to be characterized. The membrane distribution of some integrins is restricted to subcellular structures known as focal adhesions, which contain structural and signaling molecules including actin, focal adhesion kinase (Fak), Srk, protein kinase C, and paxillin. Integrin activation can generate multiple signals that regulate cell behavior through the modulation of lipid metabolism. ILK is an intracellular protein kinase that couples integrins and growth factors to downstream signaling pathways involved in the suppression of apoptosis and in promoting cell cycle progression. ILK regulates the cell cycle by stimulating the phosphorylation of PKB/AKT on serine 473 in a PI-3K-dependent manner. Inhibition of ILK activity in tumor cells results in the inhibition of PKB/AKT serine 473 phosphorylation and activation, inhibition of cell cycle progression, and stimulation of apoptosis (*35*). In a thyroid cell line obtained by immortalizing human fetal thyroid cells, integrin clustering by binding to fibronectin (FN) generates two signaling pathways: Fak/Ras/Raf-1/Mek/ERK that mediates FN-induced proliferation and the PI-3K pathway involved in cell survival (*36*). PI-3K inhibition may induce severe attenuation of integrin-dependent ERK activation (*37*), and the lipid products of PI-3K provide a protective signal acting through AKT (*38*). In this paper, we observed the total and phosphorylated forms of AKT and ERK under the various test conditions. Our results demonstrated that knockdown of 3′-sulfo-Lewis-x via Gal3ST-2 siRNA reduced the level of ILK and phosphorylated AKT and ERK compared with those of the controls. SMMC7721 cells exposed to exogenous Lewis-x trisaccharide 3′-sulfate showed an increase in the level of ILK and phosphorylated ERK and AKT versus the controls.

In a hepatoma cell culture model, it was shown that the antiapoptotic effect of integrins in response to a variety of chemotherapeutic agents was mediated by ERK and p38 MAPK (*39*). Apoptosis can result not only from the loss of integrin-mediated survival signals but also from induction of pro-apoptosis signaling, as some unligated integrins themselves can induce apoptosis (*40*). Induction of apoptosis in fludarabine-, etoposide-, or bleomycin-treated human leukemia cells was prevented by integrin-mediated adhesion to ECM on the basis of upregulation of Bcl-2-like proteins in parallel with downregulation of pro-apoptotic proteins such as Bax or Bim (*41*−*43*). In addition, many reports also implicated the PI-3K/AKT pathway as having a major influence on cellular apoptotic commitment, and PI-3K activation is a known inhibitor of apoptosis in hematopoietic cells (*44*). Consistent with these results, we found that the apoptotic cell had a much higher level of expression in Gal3ST-2 siRNA-transfected SMMC7721 cells in conjunction with less Bcl-2 expression. Conversely, Bcl-2 levels as well as the Bcl-2:Bax ratio were found to be significantly higher in cells to which Lewis-x trisaccharide 3′-sulfate sodium salt had been added.

In summary, our findings suggested that 3′-sulfo-Lewis-x, the product of Gal3ST-2, modulated both the expression and sulfation of integrin subunit αV and regulated integrin-associated adhesion behavior in SMMC7721 cells. The 3′-sulfated modification on the Le^x structure thus played important roles in cell adhesion as well as in cell growth regulation.

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