Regulation of integrin αV subunit expression by sulfatide in hepatocellular carcinoma cells

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Abstract  Integrin is important in migration and metastasis of tumor cells. Changes of integrin expression and distribution will cause an alteration of cellular adhesion and migration behaviors. In this study, we investigated sulfatide regulation of the integrin αV subunit expression in hepatoma cells and observed that either exogenous or endogenous sulfatide elicited a robust upregulation of integrin αV subunit mRNA and protein expression in hepatoma cells. This regulatory effect occurred with a corresponding phosphorylation (T739) of the transcription factor Sp1. Based on the electrophoretic mobility shift assay, sulfatide enhanced the integrin αV promoter activity and strengthened the Sp1 complex super-shift. The results of chromatin immunoprecipitation analysis also indicated that sulfatide enhanced Sp1 binding to the integrin αV promoter in vivo. Silence of Sp1 diminished the stimulation of integrin αV expression by sulfatide. In the early stage of sulfatide stimulation, phosphorylation of Erk as well as c-Src was noted, and inhibition of Erk activation with either U0126 or PD98059 significantly suppressed Sp1 phosphorylation and integrin αV expression. We demonstrated that sulfatide regulated integrin αV expression and cell adhesion, which was associated with Erk activation.—Wu, W., Y. W. Dong, P. C. Shi, M. Yu, D. Fu, C. Y. Zhang, Q. Q. Chai, Q. L. Zhao, M. Peng, L. H. Wu, and X. Z. Wu. Regulation of integrin αV subunit expression by sulfatide in hepatocellular carcinoma cells. J. Lipid Res. 2013. 54: 936–952.

Supplementary key words cell adhesion • cerebroside • sulfation • signaling

Integrins have been implicated as very important adhesion molecules that are involved in multiple physiological processes, such as cell adhesion, proliferation, and survival (1–3). Each integrin generally consists of a noncovalently linked α- and β-subunit, with each subunit having a large extracellular domain, a single membrane-spanning domain, and a short, noncatalytic cytoplasmic tail. Integrins seem to be the major receptors by which cells attach to components of the extracellular matrix (ECM), such as vitronectin, etc. (4), and are involved in the metastasis signaling of hepatocellular carcinoma (HCC) (5).

The integrin αV subunit associates with one of five integrin β subunits, β1, β3, β5, or β8, to form five distinct αVβ heterodimers (6). The integrin αVβ heterodimers on the cell surface interact with cell adhesive proteins, such as collagen, fibrinogen, fibronectin, and vitronectin. These interactions play an important role in cell adhesion or migration, especially in tumor metastasis. Integrins increase in invasive tumors and distant metastases, characterize the metastatic phenotype, and play a key role in tumor metastasis (7, 8). Many studies have documented marked differences in the surface expression and distribution of integrins between malignant tumors and preneoplastic tissues. For example, the integrin αVβ3 complex is strongly expressed in the invasive front cells of malignant melanoma and angiogenic blood vessels, but it is weakly expressed on preneoplastic melanomas and quiescent blood vessels (9). Also, it has been demonstrated that αVβ3 integrin is specifically required to sustain neovascularization induced in vivo by fibroblast growth factor-2 (10). Integrin αVβ3 physically associates with phosphorylated and activated insulin-like growth factor receptor, and it may be involved in the HCC cell migration and progression (11). Furthermore, inducing the expression of the integrin αV (7) or β3 (12) subunit in melanoma cells increases their metastatic potential.

Abbreviations:  ChIP, chromatin immunoprecipitation; CST, cerebroside sulfotransferase; cyclo-ManN-pro, cyclo-ManN propanoyl perac; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; Gal-Cer, galactocerebroside; HCC, hepatocellular carcinoma; HUVEC, human umbilical vein endothelial cell; Lacto-Cer, lactocerebroside; ManN-pro, ManN propanoyl perac; SM3, lactosyl sulfatide, sulfated lactocerebroside, Sulfo-Lacto-Cer; SM4, galactosyl sulfatide, sulfated galactocerebroside.
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A change of the expression and distribution of integrins on the cell surface can modulate the strength of cell adhesion and migration. The regulation of cell adhesion and migration involves coordinated events of tumor metastasis.

Many primary and metastatic cancer cells display altered integrin expression levels and/or activation status, leading to abnormal cell adhesion, growth, and survival, which are pathological hallmarks of cancer. Although the influence of integrin αV and β3 on metastasis has been documented in several studies (13, 14), there is little information about cerebroside sulfation signaling, and the molecular regulation mechanisms underlying integrin expression have not been elucidated. Sulfatide, the product of galactose-3-O-sulfotransferase 1 (Gal3ST-1; also called cerebrosido sulfotransferase, CST), is highly expressed in high metastasis potential HCC cells (MHCC97H) compared with low metastasis potential HCC cells (MHCC97L) (15). After retinoic acid treatment and inhibition of cell migration, the cellular sulfatide production is decreased (15), but the precursor cerebroside is not affected, suggesting the inhibition of CST activity. Hep3B cells overexpressing CST that produces sulfatide significantly promotes the metastasis behaviors in nude mice. Apart from CST (Gal3ST-1), Gal3ST-2 is also observed to be involved in tumor metastasis (16). Both genes encode galactose-3-O-sulfotransferases, which catalyze and transfer sulfate to the 3’ hydroxyl group of the galactosyl residue in the glycol chain. This sulfation makes the substrate molecule negatively charged, changes its affinity with binding molecules, and involves HCC metastasis or progression (17). The HCC Hep3B cells transfected by CST expressed an elevated level of integrin αV and intensively adhered to vitronectin, the ligand of integrin αVβ3 (15, 18). However, the mechanism by which the CST is involved in regulation of integrin αV and cell adhesion is not fully understood. The relationship between the promotion mechanism of cancer cells and elevated expression of sulfatide remains unknown (19). A recent study showed that sulfatide can serve as a laminin-binding glycolipid and can anchor laminin-1 and laminin-2 to the Schwann cell surface, form a laminin-associated complex, and enable basement membrane assembly to initiate c-Src activation (9). Sulfatide was also identified as an interacting partner of P-selectin and promoted a P-selectin-mediated metastasis process in colon cancer cells (20). Sulfatide and P-selectin interactions led to subsequent platelet aggregation (21) and played an important role in the formation of cancer embolus. Our previous study (15, 18) revealed that hepatoma cells expressed sulfatide after CST transfection. The enzyme CST in HCC can only catalyze the production of sulfatide, which acts as the endogenous sulfated cerebroside. We thus hypothesize that the enzyme product sulfatide is responsible for the regulation of the integrin αV subunit and involves the metastasis process. To test this, we investigated, in this study, the regulatory effect of both exogenous and endogenous sulfatide, the product of CST, and demonstrated that sulfatide, but not cerebroside, played an important role in the regulation of the expression of the integrin αV subunit, which can form the molecule αVβ3 in hepatocellular carcinoma cells.

**Materials and Methods**

**Materials**

Lactocerebroside (Lacto-Cer), galactocerebroside (Gal-Cer), and sulfatide from bovine brain were obtained from Sigma-Aldrich (St. Louis, MO). ManNpropanoyl perac (ManN-pro) and cyclo-ManN propanoyl perac (cyclo-ManN-pro) were kindly provided by Professor Werner Ruettner (Institut für Biochemie und Molekularbiologie, Campus Benjamin Franklin, Germany) (22, 23). Peptides GRGDSP and GRGESP with 99% purity were provided by China Peptides Co. (Shanghai, China). Polyclonal rabbit, monoclonal mouse anti-human integrin αV antibodies, and polyclonal rabbit anti-human Sp1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse O4 antibody (anti-galactosyl sulfatide and lactosyl sulfatide) was obtained from Chemicon (Temecula, CA). Monoclonal mouse anti-lactosyl sulfatide antibody was obtained from Seikagaku (Tokyo, Japan). FITC-anti-human integrin αV was obtained from BioLegend (San Diego, CA). Antibodies against Erk, p38, Rac, mTOR, JNK, and phospho-Raf (S338) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Phospho-Raf (Y341) was from Beijing Biosynthesis Biotechnology (Bios, Beijing, China). Akt antibody was from Bio-world (Atlanta, GA). Anti-phosphoserine antibody was from Millipore (Billerica, MA). Fibrinogen was from Sigma-Aldrich, collagen type I was from Roche (Indianapolis, IN), and fibronectin and vitronectin were from Calbiochem (San Diego, CA).

**Cell culture**

Hepatoma cells (SMMC-7721, BEL-7404), human umbilical vein endothelial cells (HUVEC), HeLa cells, and HER-293T cells were obtained from the Institute of Cell and Biochemistry Research of the Chinese Academy of Science. CST-overexpressing cells (CST-1, CST-8), CST-knockdown cells (Chp2, Chp5), and their corresponding Mock cells were established by our laboratory previously (18). CST overexpressed HCC cells mainly produced lactosyl sulfatide. The cells were maintained in RPMI 1640 medium supplemented with 10% newborn bovine serum (PAA, Austria) at 37°C under a 5% CO₂ atmosphere. For the treatment, cells were cultured in RPMI 1640 medium containing 2 μM sulfatide, lactocerebroside, or galactocerebroside added from stock solution in DMSO. An equal amount of DMSO (0.1% v/v) was added to control group.

**Plasmid construction**

The short hairpin sequences, including 5’-AGGAGUUG-GUGGCAAAUAUU-A3’ and 5’-UAAUAGGCAUCACUCCAGG-3’, which specifically interfered and targeted Sp1 mRNA, were designed according to the protocol from Ambion (24). The synthesized 35 bp forward and reverse oligonucleotides containing the siRNA sequence were annealed and ligated to the pSilencer 4.1 vector. The pDNA3.0-Sp1 expression plasmid was kindly provided by Dr. Jian-Hai Jiang (Fudan University, P.R. China). A human CST cDNA expression plasmid was previously constructed (15, 18). The integrin αV promoter fragments were inserted from -1295 to +207 bp, -795 to +207 bp, -309 to +207 bp, and -16 to +207 bp were obtained by PCR from the genomic DNA of SMMC-7721 cells. The following primers were used: integrin αV/Kpn I 1–1295: 5’-CCCCGTAC-GGTTCCACACATGCACTAAA-3’, integrin αV/Kpn I 1–795: 5’-AAAAAGTACCGAAAGGGCATTCGCTGCC-3’, integrin αV/Kpn I 1–309: 5’-AAAAAGTACGCGTCCCTTCCAGGTCTCC-3’, integrin αV/Kpn I 1-16: 5’-AAAAAGTACGTGGCGGGGGGAGGT-3’, integrin αV/Xho I +207: 5’CGCGTGCGAGGAAATACTACCGAGCCGAA-3’. The PCR products were inserted into the Xho I/Kpn I sites of the pGL3-basic vector (Promega, Madison, MI) and designated as pGL3-integrin αV. All of the constructs were verified by sequencing.
RT-PCR and real-time PCR

Total RNA was extracted from cells with the Trizol reagent according to the manufacturer’s instructions and was used as the template for cDNA synthesis. Reverse transcription was then carried out by M-MLV. The following primer sets were used for RT-PCR and real-time PCR: integrin αv subunit (sense 5′-GA-CAGTCTCGGAAGATGA-3′, anti-sense 5′-CTGGGTTGTTTGTTCC-3′); Sp1 (sense 5′-TCACAAGGCGTGACCTTCG-3′, anti-sense 5′-GGTGTCACCTTCTGGA-3′); EGR2 (sense 5′-CAGCAGCTGGCTGAA-3′, anti-sense 5′-CATCGCTCCTGGCAAACT-3′); EGR1 (sense 5′-CAGCGAGTCTGCTAACC-3′, anti-sense 5′-CATCGCTCCTGGAACATG-3′); EGR1 (sense 5′-CAGCGAGTCTGCTAACC-3′, anti-sense 5′-CATCGCTCCTGGAACATG-3′); CST (sense 5′-CAAGTCAGAGCGGGAGAACC-3′, anti-sense 5′-TCGTCAGAGCGGGAGAACC-3′); AP2 (sense 5′-GGTGTCCACCTGGGTAACG-3′, anti-sense 5′-ACTTGGGACAGGGACACG-3′); EGR1 (sense 5′-CAGCGAGTCTGCTAACC-3′, anti-sense 5′-CATCGCTCCTGGAACATG-3′); Sp1, and histone 4. After washing, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin. The absorbance was measured at 450 nm after incubation with the alkaline phosphatase substrate p-nitrophenyl phosphate (pNPP).

Immunofluorescence

The immunofluorescence method was based on the previous report (26). The cells of CST-1 and CST-8 were fixed with 2% paraformaldehyde and not permeabilized, but exogenous sulfatide-treated cells were permeablized. After blocking and staining with a mouse anti-sulfatide antibody for 2 h and subsequently with FITC-conjugated secondary antibody for 1 h, the coverslips containing the cells were placed on glass slides with Vectashield, and the cells were viewed under a fluorescence microscope.

Cell adhesion to HUVEC

HUVECs in 150 μl of medium were seeded onto 96-well plates at 1 × 10^4 cells per well and incubated overnight at 37°C to confluence. Next, the HUVEC monolayer was stimulated with 10 ng/ml TNF-α for 4 h prior to the addition of the variously treated SMMC-7721 (1 × 10^4/ml) cells in 200 μl RPMI 1640 medium with 1% newborn bovine serum. 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. The numbers of adherent cells in all groups were analyzed and compared to show the cell adhesion to HUVEC as described earlier (16). Data were reported as mean ± SD for at least three wells, and the experiment was repeated three times independently.

Flow cytometry analysis

Based on our previous report (25), subconfluent SMMC-7721 cells were detached with 0.02% EDTA, and the single-cell suspensions were washed and maintained in suspension for 1 h in RPMI 1640 medium with 10% newborn bovine serum. Cells, which were not permeabilized, were then incubated with 2 μg of FITC-labeled primary anti-human CD51 antibody per million cells for 2 h at 4°C with gentle shaking. After washing, the cells were resuspended in 1% paraformaldehyde, and cell surface immunofluorescence was analyzed by a flow cytometer (Becton Dickinson, Mountain View, CA).

Luciferase assay

SMMC-7721 cells were seeded into 96-well plates and the next day transiently transfected with 0.2 μg of specific expression vectors. The cells were incubated for 24 h at 37°C, washed once with PBS, and then lysed in 20 μl of lysis buffer (Promega, Madison, WI) to measure the luciferase reporter gene expression by the luciferase reporter assay system (Promega). The intensity of luminescence was measured by a luminometer (Lumat LB 9507, Berthold, Germany). The data of relative luciferase activities were normalized to the control. The results are presented as the mean of the experiments performed in triplicate. Experiments were repeated at least three times.

Western blotting

Western blot analysis was carried out essentially as described previously (17). Briefly, 30 μg of protein was resolved on 10% SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was then incubated with a series of antibodies against GAPDH, integrin αv, Sp1, and histone 4. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody, and
protein bands were visualized using an enhanced chemiluminescence detection kit.

**Nuclear extracts**

Nuclear extracts were prepared with the nuclear extraction kit (Chemicon, Cat. No. 2900). The concentration of protein was determined by the Lowry method.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) was performed with a modified method according to a previous protocol (27). The double-stranded probes were labeled with fluorescence by a 5′ oligolabeling kit (RPN 5755, Amersham Pharmacia Biotech) according to the manufacturer’s instruction. EMSA reaction mixtures were incubated in 1× binding buffer [1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5)], 4% glycerol, and nuclear extracts at room temperature for 20 min with or without unlabeled competitors, and then with fluorescence-labeled oligonucleotides for 30 min at room temperature. For the super-shift assays, anti-Sp1 antibody was added to the EMSA reaction mixture prior to the addition of the labeled oligonucleotide probes for 30 min at room temperature. For the competitive binding assay, the nonlabeled probe was added to the binding reaction at 100-fold excess over the labeled probe. The samples were electrophoresed on a native 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 80 V for 1 h at 4°C and then at 120 V for 60 min. Finally, the binding reactions were visualized and analyzed by a multifunction imaging scanner (Typhoon Tyio).

**Chromatin immunoprecipitation assays**

Chromatin isolated from SMMC-7721 cells was used for the chromatin immunoprecipitation (ChIP) assays according to the manufacturer’s instructions (EZ-ChIP, Millipore, Temecula, CA). Briefly, SMMC-7721 cells, treated as indicated, were washed and chemically cross-linked using 1% formaldehyde in PBS for 10 min at room temperature. After quenching by the addition of 2 ml of 10× glycine solution (2.5 mol/l), cells were washed twice with ice-cold PBS and collected in 2 ml of PBS containing protease inhibitor cocktail II and centrifuged at 700 g at 4°C for 5 min. Next, the cell pellets were resuspended in 1 ml of SDS lysis buffer containing 1× protease inhibitor cocktail II and sonicated on wet ice by a Bioruptor sonicator (Ningbo, China) to disrupt genomic DNA to an average size of 200–750 bp. After preclearing with protein A-agarose for 1 h at 4°C, the chromatin was incubated at 4°C overnight with a specific antibody against Sp1, with anti-RNA polymerase II as the positive control and anti-IgG as the negative control, followed by incubation with protein A-agarose for 1 h at 4°C for 20 min with or without unlabeled competitors, and then with fluorescence-labeled oligonucleotides for 30 min at room temperature. The double-stranded probes were labeled with fluorescence by a 5′ oligolabeling kit (RPN 5755, Amersham Pharmacia Biotech) according to the manufacturer’s instruction. EMSA reaction mixture prior to the addition of the labeled oligonucleotide probes for 30 min at room temperature. For the competitive binding assay, the nonlabeled probe was added to the binding reaction at 100-fold excess over the labeled probe. The samples were electrophoresed on a native 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 80 V for 1 h at 4°C and then at 120 V for 60 min. Finally, the binding reactions were visualized and analyzed by a multifunction imaging scanner (Typhoon Tyio).

**RESULTS**

**Exogenous sulfatide stimulates integrin αV subunit expression**

SMMC-7721 cells were treated with exogenous sulfatide, Gal-Cer, or Lacto-Cer for 12, 24, and 36 h (Fig. 1A), and then the mRNA level of the integrin αV subunit in the cells was analyzed by RT-PCR. As shown in Fig. 1A, treatment with sulfatide stimulated the mRNA expression of the integrin αV subunit gene in a time-dependent manner, and the upregulation of the integrin αV subunit was the most obvious at 24–36 h after the treatment (Fig. 1A). After 48 h, the upregulation effect attenuated (data not shown). The stimulation by sulfatide was also observed in a dose-dependent manner, and a concentration of 2 μM was necessary to stimulate the expression of the integrin αV subunit (Fig. 1B). Furthermore, the protein expression level of the integrin αV subunit was promoted by sulfatide but not by galactocerebroside (Gal-Cer) or lactocerebroside (Lacto-Cer) (Fig. 1C). Lacto-Cer was then used as the control for sulfatide in the following experiments since both Lacto-Cer and Gal-Cer did not affect integrin αV expression significantly. To determine the distribution on the cell membrane, flow cytometry analysis of nonpermeabilized cells with integrin αV-specific antibody revealed that upregulation of the surface integrin αV subunit was observed after sulfatide treatment (Fig. 1D, E). The positive rate was elevated from 37.8 ± 0.8% to 54.5 ± 0.95%. No significant increase of the integrin αV subunit expression was observed on the cells induced by exogenous Lacto-Cer. Other subunits, such as β3, β5, and α5, did not change.
significantly after the treatment (Fig. 1F) and after sulfatide overexpression as in our previous reports (15, 16). After sulfatide treatment of the cells, high positive staining with O4 antibody, which recognizes sulfatide, was seen in the treated cells (Fig. 1G) but not in the control (LactoCer treatment), confirming that glycosphingolipids could incorporate into cells.

Sulfatide is the sulfation product of cerebroside. The addition of a sulfate moiety gives the sulfatide molecule a negative charge. To test whether the sulfatide or just the negative charge was the cause of upregulation of the integrin αV subunit gene, SMMC-7721 and BEL-7404 cells were incubated with 2 μM ManN-pro and cyclo-ManN-pro, which were reported to incorporate into a cellular sugar chain with a sialyl group (22, 23, 28). RT-PCR (Fig. 1H) and real-time PCR (Fig. 1I, J) showed that in both the SMMC-7721 and the BEL-7404 cells, only sulfatide enhanced the expression of the integrin αV subunit, while the negatively charged ManN-pro and cyclo-ManN-pro did not. The increased αV subunit was mainly joined with β3 subunit to form αVβ3 as observed in our previous studies (15–17). Current results suggest that exogenous sulfatide upregulated the expression of the integrin αV subunit.

Endogenous sulfatide promotes the expression of integrin αV subunit

To further confirm the regulation of the integrin αV subunit expression by sulfatide, we established stable transfection cell lines with a CST plasmid in SMMC-7721 cells to produce endogenous sulfatide. We obtained CST-1, CST-8, and the corresponding Mock cells, which overexpress CST and sulfatide (18). A cell ELISA assay (Fig. 2A) and immunofluorescence staining (Fig. 2B) demonstrated that there was overexpression of CST and its product sulfatide. Next, we established CST-knockdown cells by stable transfection with specific siRNA plasmids in SMMC-7721 cells and established cell lines Chp2 and Chp5 that reduce CST expression. The RT-PCR results (Fig. 2C) confirmed successful CST interference in the cells. The mRNA and protein levels of the integrin αV subunit gene were significantly increased in SMMC-7721 cells that were transfected with CST plasmid but not in the Mock cells (Fig. 2D). In addition, the expression of the integrin αV subunit was decreased significantly in CST siRNA cells, especially in the Chp2 cells (Fig. 2E). Therefore, either exogenous or endogenous sulfatide could stimulate the expression of the integrin αV subunit gene.

Fig. 2. Regulation of the integrin αV subunit gene expression by endogenous sulfatide. (A and B) In CST transfectants CST-1, CST-8, and Mocks, sulfatide was analyzed by a cell ELISA assay (A) and immunofluorescence detection with O4 antibody under a confocal microscope. The cells were not permeabilized (B). (C) CST knockdown was achieved in the CST RNAi transfectants Chp2 and Chp5 and confirmed by RT-PCR. Scr, scrambled. (D) Expression of the integrin αV in CST-transfected SMMC-7721 cells (CST-1 and CST-8) was analyzed by RT-PCR and Western blotting. Mock3 and Mock4 were transfectants of the control vector. (E) The expression of integrin αV in si-CST SMMC-7721 cells by CST RNAi (Chp2 and Chp5) was analyzed by RT-PCR and Western blotting. Scr1 and Scr2 were transfected with the control vector. All figures were the representative study and at least three additional independent experiments were repeated. *P < 0.05.
**Rationale for the Study**

High expression of the integrin αV subunit can influence tumor progression through regulation of cell migration and adhesion (29). The results above showed that sulfatide could stimulate the expression of the integrin αV subunit gene. We then examined the influence of sulfatide on cell adhesion. As shown in Fig. 3A, the adhesion to HUVECs, stimulated by TNF-α, was significantly increased in cells treated with sulfatide compared with the control, Lacto-Cer, ManN-pro, and cyclo-ManN-pro groups (Fig. 3A). The adhesion of the treated SMMC-7721 cells to vitronectin, collagen type I, fibrinogen, and fibronectin is shown in Fig. 3B–E. With the exogenous sulfatide, the cells were more adhesive to vitronectin, collagen type I, fibrinogen, and fibronectin than to the control. The adhesion rate increased to 22.7 ± 4.04% from 1.763 ± 0.234% for vitronectin, and to 63.75 ± 1.43% from 6.05 ± 1.472% for collagen type I. The adhesion to vitronectin was significantly inhibited by adding the antibody against integrin αV (64.4%) (Fig. 3B). Adhesion to collagen was diminished by integrin αV antibody, whereas adhesion to fibrinogen was not (Fig. 3C, D). Adhesion to fibronectin was also significantly inhibited by the antibody (Fig. 3E). GRGDSP peptide suppressed the adhesion to vitronectin and fibronectin, as well as to collagen. The heparin seemed mainly to reduce the adhesion to vitronectin and fibronectin in these cells. The adhesion of ManN-pro and cyclo-ManN-pro groups was similar to the Lacto-Cer group (data not shown). Thus, sulfatide stimulated the expression of the integrin αV subunit and enhanced the adhesion of SMMC-7721 cells to ECM proteins that were the ligands of the integrin αV subunit.

**Sp1 is involved in the integrin αV subunit gene regulation induced by sulfatide**

According to bioinformatic promoter analysis and previous reports (29, 30), transcription factors, including Sp1, Ets1, Ap2, Egr1, Egr2, and Sp3, may be important for the expression regulation of the integrin αV subunit gene. To test and screen these transcription factors that might be involved, we measured all these transcription factors.

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**Fig. 3.** Sulfatide enhanced cell adhesion. SMMC-7721 cells were treated with 2 μM sulfatide, Lacto-Cer, ManN-pro, or cyclo-ManN-pro for 24 h. Then the cells were used for adhesion assay. (A) The adhesion of the treated cells to TNF-α induced HUVECs. The monolayer HUVECs were stimulated with 10 ng/ml TNF-α for 4 h prior to the adhesion assay. After incubation and washing, the attached cells were counted under a phase contrast microscope in five random fields in each well, and the cell number was averaged among the test groups and compared with the affinity of the cells to HUVECs. (B–E) The treated SMMC-7721 cells were examined for their adhesive capability to vitronectin (B), collagen type I (C), fibrinogen (D), and fibronectin (E). For the inhibition, the cells were preincubated with 30 mg/ml GRGESP, GRGDSP, 60 μg/ml antibody against integrin αV, and 1 mg/ml heparin, respectively, for 2 h at 4°C. The numbers of cells adhering were represented by A570, and the adhesive rate was calculated. Shown are the means ± SD from five independent experiments with equivalent results. Bars indicate the SD. *P < 0.05, **P < 0.01.
Among them, Sp1 expression was elevated (Fig. 4A, B), and its phosphorylation on threonine 739 (T739) was significantly enhanced after sulfatide treatment (Fig. 4C, D). However, the tyrosine phosphorylation was not obvious on Sp1 (data not shown) by the detection with phosphotyrosine antibody after Sp1 immunoprecipitation. The serine phosphorylation on Sp1 was slightly enhanced in the blot with anti-phospho-serine antibody after precipitation with Sp1 antibody (Fig. 4C, D). To investigate the role of Sp1 in the sulfatide-induced regulation of the integrin αV subunit further, we constructed an Sp1 interference plasmid and confirmed its activity by RT-PCR (Fig. 4E). We transfected Sp1 siRNA plasmid (si-Sp1) into SMMC-7721 cells after treatment with exogenous sulfatide (Fig. 4F) and into CST transfectants (Fig. 4G). Results showed that Sp1 siRNA could disturb the expression of the integrin αV subunit gene that is induced by both exogenous and endogenous sulfatide. The Sp1 plasmid (Sp1) was transfected into si-CST transfectants (Chp2 and Chp5), in which CST was knocked down by siRNA. Sp1 upregulated the expression of the integrin αV subunit gene, and the expression of Sp1 directly correlated with both the mRNA and the protein expression levels of the integrin αV subunit gene (Fig. 4F–H). Additional Sp1 overexpression increased the integrin αV subunit expression on the surface of SMMC-7721 cells (Fig. 4I, J). These observations are consistent with the results above, indicating that sulfatide regulated the Sp1 transcription factor and enhanced the gene expression of the integrin αV subunit.

To investigate the interaction partner of Sp1 after its phosphorylation, Sp1 protein complex was precipitated by Sp1-specific antibody for the detection of Western blot. In the complex immunoprecipitated by Sp1 antibody, Stat3 was observed (Fig. 4K, L). Furthermore, in the sulfatide group, much more Stat3 was noted than in the control. Interestingly, Stat3 was highly phosphorylated after sulfatide stimulation in both SMMC-7721 and BEL-7404 cells (Fig. 4K, M). Therefore, Sp1 was complexed with Stat3 after phosphorylation induced by sulfatide.

**Analysis of the human integrin αV subunit gene promoter**

An analysis of the human integrin αV subunit gene promoter (−1295/+207) (GenBank accession no. 23999) based on the Transfac website predicted Sp1 binding sites, which had four Sp1 binding enrichment regions. The first site includes one Sp1 binding consensus site, the second includes four Sp1 binding consensus sites, the third includes three Sp1 binding consensus sites, and the fourth includes one Sp1 binding consensus site (Fig. 5). To define the integrin αV subunit gene promoter, we cloned the Sp1 transcription factor binding sites in the promoter. These promoter fragments (Fig. 6) were inserted into the promoterless pGL3-basic vector. Fig. 6A shows the integrin αV subunit gene promoter fragments, including different Sp1 binding sites. The basal and proximal regulatory regions of the integrin αV subunit promoter were characterized by constructing a series of luciferase reporter plasmids and transfected into HeLa and HEK293T cells. Deletion of the sequence between −1295 and −794 increased the transcriptional activity (Fig. 6B). To further define the roles of the Sp1 binding sites of the integrin αV subunit gene promoter in response to sulfatide regulation, HeLa and HEK293T cells were transfected with pGL3 (−795/+207) along with treatments of 2 μM sulfatide and controls (Fig. 6C). Sulfatide significantly increased the activity of the integrin αV subunit gene promoter. To test whether sulfatide increased the activity of the Sp1 transcription factor regulation of the integrin αV subunit gene promoter, HeLa and HEK293T cells were cotransfected with pGL3 (−795/+207) and pcDNA3.0-Sp1 or si-Sp1, along with treatments of 2 μM sulfatide and Lacto-Cer. A significant increase in the reporter activity by the treatment with sulfatide (Fig. 6D, E) supported the Sp1 transcription factor being involved in transcriptional regulation of the integrin αV subunit gene and Sp1 regulation activity being enhanced by sulfatide.

**Sulfatide enhances binding of Sp1 to the integrin αV subunit gene promoter**

EMSA was performed to test whether sulfatide enhanced the binding of the Sp1 transcriptional factor to the integrin αV subunit gene promoter. As shown in Figs. 5 and 6, there are four binding sites of Sp1 in the integrin αV subunit gene promoter, but the fourth Sp1 binding site did not affect the activity of the integrin αV subunit gene promoter. Therefore, we designed three pairs of probes for the

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*Fig. 4.* Observation of Sp1 in the sulfatide-induced expression of integrin αV. (A) The expression level of Sp1 was analyzed with real-time PCR in both SMMC-7721 and BEL-7404 cells with various treatments. (B) After treatment for 24 h, the expression of Sp1 mRNA and protein in the cells was analyzed by RT-PCR and Western blotting. (C) After SMMC-7721 cells were treated with 2 μM sulfatide and Lacto-Cer for 24 h, Sp1 protein expression and phosphorylation were analyzed by immunoblotting. Ser-P, phospho-serine antibody; Sp1-p, antibody against phospho-Sp1 on threonine 739. (D) Densitometry analysis of Sp1 phosphorylation is summarized (Sp1-p, left; Ser-p, right). (E) Sp1 mRNA analysis by RT-PCR. The cells were transfected with Sp1 RNAi plasmid and negative siRNA plasmid, and the Sp1 mRNA was analyzed by RT-PCR. Neg siRNA, negative control siRNA. (F) Influence of Sp1 silence by RNAi (si-Sp1) on the exogenous sulfatide-induced integrin αV expression in SMMC-7721 cells. Scr was the transfection with the control vector containing a scramble sequence. (G) Influence of Sp1 silence by si-Sp1 transfection on the endogenous sulfatide-induced αV expression in CST-1 and CST-8 cells. (H) Sp1 overexpression influence on sulfatide regulation in Si-CST-transfected cells (Chp2 and Chp5). Sp1 was overexpressed by Sp1 transfection (Sp1), which enhanced the endogenous sulfatide-induced αV expression. (I) Flow cytometry analysis of the cell surface integrin αV expression in cells transfected with Sp1 and a vehicle and in cells without transfection (control). (J) The positive rates of the cell surface integrin αV staining were averaged from the experiments of flow cytometry. (K) Stat3 protein was observed in the complex immunoprecipitated by Sp1 antibody (upper panel). Phosphorylation of Stat3 on tyrosine 705 was measured by Western blotting (bottom panel). (L and M) Graphs summarize the densitometry analysis of Stat3/Sp1 (L) and Stat3-p Y705/Stat3 (M). All figures are from a representative study and at least three additional experiments yielded similar results. *P < 0.05, **P < 0.01. IP, immunoprecipitation; IB, immunoblotting.
Fig. 5. Nucleotide sequence of the human integrin αV promoter. The major transcriptional start site as indicated is at position +1. The Sp1 transcriptional factor binding sites are boxed, and the promoter fragment primers are shaded.

first three binding sites, which encode the Sp1 consensus sequences and the corresponding mutant probes (Fig. 7A). According to the luciferase analysis (Fig. 6B), the second Sp1 binding site had the strongest promoter activity. The nuclear extracts from the untreated SMMC-7721 cells were incubated with the fluorescence-labeled DNA probes for EMSA. The super-shift band of the protein-DNA complex was detected in the presence of the Sp1 antibody (Fig. 7B, lane 5), and the probe designed for the second Sp1 binding site had the highest binding capability among the three pairs of probes (Fig. 7C). This band was eliminated in the presence of unlabeled double-stranded oligonucleotide competitor (Fig. 7B, lane 3), but it was not affected by mutated oligonucleotides (Fig. 7B, lane 4). Histone 4 was the internal loading control of the nuclear extracts of the variously treated cells (Fig. 7D). The level of the super-shift bands was enhanced in cells transfected with pcDNA3.0-Sp1 (Fig. 7E) and also in the cells treated with sulfatide (Fig. 7F). This finding suggested the impact of sulfatide on Sp1 binding to the integrin αV subunit gene promoter.

Sulfatide regulates Sp1 binding to the integrin αV subunit promoter in vivo

The status of the Sp1 on the human integrin αV subunit gene promoter was determined using ChIP. The presence of the integrin αV subunit gene promoter in the chromatin immunoprecipitates was analyzed by...
Fig. 6. Characterization of the human integrin αV promoter. (A) The promoter fragments were designed according to the Sp1 binding sites in the integrin αV promoter. (B) 5′ deletion analysis of the integrin αV promoter was performed in HEK293T and HeLa cells. The activity of luciferase was statistically analyzed between the construct and the vector pGL3-basic. (C) The luciferase activity was assayed in HEK293T and HeLa cells transfected with the human integrin αV promoter plasmids pGL3(-795/+207) or treated with 2 μM sulfatide for 24 h. The comparison was made between sulfatide and Lac-Cer groups. (D) HEK293T and HeLa cells were cotransfected with the human integrin αV promoter plasmids pGL3(-795/+207) and si-Sp1(pSilence-Sp1) or with a control vector. After transfection for 12 h, HEK293T and HeLa cells were treated with sulfatide and Lacto-Cer for an additional 12 h. The luciferase activities were assayed and expressed as the percentage of the test groups over the control siRNA as the mean ± SD of three separate experiments. *P < 0.05.
Fig. 7. EMSA analysis of the integrin αV subunit gene promoter. (A) Three EMSA probes of the Sp1 transcriptional factor binding sites. SB, Sp1 binding site; SM, mutated Sp1 binding site. (B) EMSA was performed for the (−541/−519) binding site using the nuclear extract from SMMC-7721 cells without treatment. SB2, Sp1 binding site 2; SM2, mutated Sp1 binding site 2. (C) EMSA for the (−619/−597), (−541/−519), and (−176/−154) binding sites using the nuclear extract from SMMC-7721 cells. (D) Immunoblotting with histone 4 as the loading control was performed to adjust the amount of nuclear extracts from the various groups. (E) EMSA for the (−541/−519) binding site. The nuclear extract was from SMMC-7721 cells transfected with pcDNA3.0 and pcDNA3.0-Sp1. The group without adding nuclear extract was the negative control for the complex, and α-IgG was the negative control for Sp1 antibody. (F) EMSA for the (−541/−519) binding site. The nuclear extract was from SMMC-7721 cells with various treatments including sulfatide, Lacto-Cer, and the vehicle. The group without adding nuclear extract was the negative control of the complex, and α-IgG was the negative control for the super-shift.

Semiquantitative PCR using specific primer pairs that spanned the integrin αV subunit gene promoter region from −632 to −452. To test whether Sp1 directly binds the regulatory regions of the integrin αV subunit gene promoter, we performed ChIP assays with untreated SMMC-7721 cells. As shown in Fig. 8A, chromatin immunoprecipitation with an antibody against Sp1 indicated that Sp1 could directly bind the regulatory regions of the integrin αV subunit gene promoter. The anti-RNA Polymerase II was used as a positive control, which could bind to the integrin αV subunit gene promoter, and normal mouse IgG was used as a negative control. We next probed whether sulfatide or Sp1 overexpression affected the recruitment of Sp1 to the integrin αV subunit gene promoter.
As shown in Fig. 8B, C, chromatin immunoprecipitation with the specific antibody against Sp1 indicated that treatments with sulfatide or pcDNA3.0-Sp1 transfection induced a significant increase in Sp1 binding to the integrin αV subunit gene promoter compared with Lacto-Cer or pcDNA3.0. Input DNA from each sample was used as the control. These data suggested that sulfatide enhanced the Sp1 occupancy of the integrin αV subunit gene promoter.

Sulfatide regulation is associated with Erk signaling

Sp1 transcription factor can change its regulation activity by phosphorylation. To further study the reason that sulfatide enhanced Sp1 phosphorylation and integrin αV subunit gene promoter, the gene expression profiles stimulated by sulfatide were investigated. Among the genes regulated, the mitogen-activated protein kinase (MAPK) signaling pathway triggered in these cells was noted for its enrichment (Fig. 8D). After 24 h of treatment with sulfatide, the extracellular signal-regulated kinases (Erk)1/2 pathway activation was found in hepatocellular carcinoma cells (BEL-7404), and the levels of active phosphorylated Erk1/2 increased significantly (Fig. 8E, F), whereas Akt phosphorylation was not affected. In addition, the cells treated with Lacto-Cer or Gal-Cer had a comparable level of the phosphorylated Erk1/2 compared with the control. Apart from BEL-7404, a similar result could also be seen in SMMC-7721 cells (Fig. 8E). Further detection showed that Erk1/2 was phosphorylated 2 h after sulfatide treatment, suggesting that sulfatide stimulates Erk1/2 activation. As Erk1/2 can be activated by Raf and Src signaling, we further measured the Src activation in the treated cells and observed that phosphorylation of Src (on tyrosine 416) was significantly higher in sulfatide group than that in Lacto-Cer (Fig. 8E, F). c-Raf, especially on tyrosine 341, was found highly phosphorylated in the sulfatide group (Fig. 8G, H). The phosphorylation on the serine 338 of c-Raf was also enhanced (Fig. 8I). Although not as strong as the Erk1/2 phosphorylation, a significant activation of p38 was also observed in the sulfatide group (Fig. 8G, J). Additionally, JNK phosphorylation was increased in the sulfatide group (Fig. 8G, K), whereas RAC was less phosphorylated in the sulfatide group (Fig. 8G, L). We thus reasoned that the activation of Raf/MEK/Erk pathway might be responsible for regulation. To test this, an inhibitor of MEK1 was used in the next experiment. In the results, the active phosphorylation of Erk1/2 was inhibited by the pretreatment with 50 μM PD98059, an inhibitor of MEK1/2, even under sulfatide stimulation. Interestingly, the phosphorylation of Sp1 induced by sulfatide was also suppressed (Fig. 9A). U0126, another inhibitor with different structure, also showed a significant inhibition of Erk1/2, Sp1 phosphorylation (Fig. 9B), and integrin αV expression, consistent with the effect of PD98059. This finding suggested that Erk pathway was associated with the Sp1 phosphorylation in sulfatide-induced regulation of integrin αV subunit expression.

DISCUSSION

Integrins are very important in both adhesion and metastasis of tumor cells. Integrin αV expression is required for the acquisition of a cancer metastatic stem or progenitor cell phenotype (31). Transcriptional silencing of αV and β3 integrin subunits suppresses cancer metastasis (32). In hepatocellular carcinoma, the expression of integrins such as α5β1 and αVβ3 is greatly altered, which is directly involved in the angiogenesis and metastasis of HCC (5). Hepatocellular carcinoma expresses integrin αⅤβ3, which leads to extravasation of HCC cells in the liver through a process of metastasis possibly mediated by vitronectin (33). However, less is known about the regulation mechanism of integrin αVβ3 expression in HCC, especially by sulfated cerebroside (sulfatide). Sulfatide is the product of CST. In HCC cells of both Hep3B and SMMC-7721, CST could be overexpressed by transfection to produce sulfatide (15, 18). The enzyme CST overexpression was found to be involved in metastasis of HCC in nude mice and adhesion to vitronectin (15, 18), which were ascribed to the production of sulfatide. We then postulated that sulfatide leads to the expression of the integrin αV subunit that is involved in the metastasis of HCC. However, the pathway and mechanism of sulfatide regulation of integrin αV subunit expression have not been known. A recent report indicated that sulfatide-laminin binding can initiate basement membrane assembly and signaling (9). Sulfatide expressed on cell surface exerts biological functions through mediating interactions with various proteins, such as laminin, hepatocyte growth factor, and disabled-2 (34). Furthermore, the expression and distribution of sulfatide are often altered during development and oncogenic transformation (35). The elucidation of the biological significance of sulfatide in the regulation of integrin is one of the most important issues.

In this study, we observed that either exogenous or endogenous sulfatide could increase the expression of the integrin αV subunit, whereas cerebrosides did not. Furthermore, this effect was rendered at the transcriptional level in the regulation of the integrin αV subunit expression. The transcription expression of the integrin αV subunit gene is commonly controlled by transcriptional factors, among which Sp1 has binding sites in the integrin αV subunit gene promoter, and phosphorylation on threonine 739 was enhanced after sulfatide stimulation. Sp1 mediates its target genes and can change its activity by phosphorylation on threonine 799 was enhanced after sulfatide stimulation. Sp1 mediates its target genes and can change its activity by phosphorylation on threonine 739 (36). Regarding the integrin αV subunit, Sp1 promoted its expression by enhanced binding activity to the promoter because Sp1 protein was phosphorylated and its expression was elevated. Our data confirm that sulfatide stimulated the phosphorylation and expression of Sp1, which subsequently binds to and enhances the integrin αV subunit gene promoter. The issue that sulfatide stimulated Sp1 expression will be addressed in another report. More importantly, the molecule sulfatide could send a regulatory signal that stimulates Sp1 phosphorylation and promotes integrin αV subunit expression. However, the signaling pathway is currently unknown. Through large-scale screening via gene expression analysis, effectors associated with MAPK pathway
Sulfatide regulation of integrin αV

**Figure A**
- Input RNA Pol II
- a-IgG
- a-Sp1

**Figure B**
- Sulfactide-Cer
- Lacto-Cer
- Control

**Figure C**
- Sp1
- Mock
- Input
- IgG
- Sp1

**Figure D**
- Sulfactide
- Lacto-Cer

**Figure E**
- SMMC-7721
- BEL-7404
- control
- Gal-Cer
- control
- Lacto-Cer
- Sulfactide

**Figure F**
- Erk1/2-p (T202/Y204)
- Erk1/2
- Src-p (Y416)
- Src
- Akt-p (S397)
- Akt

**Figure G**
- SMMC-7721
- BEL-7404
- control
- Gal-Cer
- control
- Lacto-Cer
- Sulfactide

**Figure H**
- Raf-p (Y341)
- Raf-p (S338)
- Raf
- p38-p (T180/Y182)
- p38
- JNK-p (T183)
- JNK
- Rac-p (S71)
- Rac
- mTOR-p (S2481)
- mTOR

**Figure I**
- Raf-p (S338)

**Figure J**
- p38-p (T180/Y182)

**Figure K**
- JNK-p (T183)

**Figure L**
- RAC-P

**Figure M**
- Control
- Lacto-Cer
- Sulfactide

**Figure N**
- Relative protein level (fold)

**Figure O**
- Relative protein level (fold)
were noted for the most enrichment. Therefore, we investigated the MAPK-associated signal pathway triggered by sulfatide. Compared with control, Gal-Cer, and Lacto-Cer, sulfatide significantly stimulated and enhanced Erk1/2 phosphorylation. Erk1/2 is an important signaling molecule that can further activate Sp1 transcriptional factor via phosphorylation on T739 (37, 38). Pretreatment with both PD98059 and U0126, inhibitors of MEK1/2, significantly diminished and prevented the Erk1/2 and Sp1 phosphorylation that sulfatide stimulated. This finding demonstrated that Erk1/2 signaling activation is important in Sp1 phosphorylation and integrin αv subunit expression regulation by sulfatide. Activated p38 can also phosphorylate Sp1 on threonine 739 (36). Thus, activation of p38 contributes to the phosphorylation and activation of Sp1 as well, although the phosphorylation of p38 (T180/Y182) was not as robust as Erk1/2.

In current study, we noted that exogenous sulfatide could incorporate into the cells treated, which was consistent with the previous observation (39). After incorporation, the glycosphingolipids can cluster in the membrane microdomain where they interact with caveolae (40) and are internalized. Then, how is sulfatide recognized by the upstream components of the MAPK pathway? In this study, we noted the phosphorylation and activation of c-Src and c-Raf, the upstream regulators of Erk1/2, in the cells stimulated with sulfatide within a short period (2 h), but not in Gal-Cer- or Lacto-Cer-treated cells. Sulfatide can bind to laminin after intercalation into plasma membrane and initiate basement membrane assembly, which enables activation of c-Src by tyrosine phosphorylation (9, 12). Treatment with arylsulfatase abrogates the activation of c-Src (9), suggesting that the molecule without sulfate group is unable to activate c-Src. Activated c-Src can phosphorylate c-Raf on the site of tyrosine 341, which is the upstream kinase of MEK1/2, and it can further activate Erk1/2 cascade. On the other hand, we noted that c-Raf was phosphorylated on the site of serine 338, which is activated by p21-activated protein kinase (PAK) (41), a downstream effector of RAC1 (42, 43) that can be activated by Src signaling (44). RAC, a small G protein, can also be activated by integrin-related focal adhesion kinase (FAK). Activated RAC1 binds to PAK protein kinase. Activated PAK in turn phosphorylates and activates c-Raf and MEK1. However, the phosphorylation on serine 71 of RAC1 was inhibitory for its GTP binding (45). In the present study, RAC1 phosphorylation on serine 71 was attenuated in sulfatide group, although the Akt phosphorylation was not significantly affected. This may synergize the Src signaling to activate Erk1/2. Binding its ligands, sulfatide forms a complex with laminin and acts as a scaffold for cell signaling to activate c-Src subsequent c-Raf/MEK/Erk, which in return phosphorylates Sp1. Phosphorylated Sp1 recruits Stat3, binds to, and enhances the promoter of integrin αv subunit gene. This constitutes at least one major part of the integrin αv subunit gene regulation pathways that are caused by sulfatide in HCC cells. This study demonstrates that sulfatide is apt and able to regulate integrin αv subunit expression in the hepatocellular carcinoma cells. The increased αv subunit mainly forms the molecule αvβ3 (15–17) and involves the process of metastasis of HCC. HCC cells with increased integrin αv expression showed significantly higher adhesion to vitronectin, which could significantly be inhibited by specific integrin αv antibody. Vitronectin binds to a cysteine loop region of β3 subunit, and this interaction is required for the positive effects of αvβ3 ligand occupancy on IGF-I signaling (46).

Interestingly, cerebroside failed to regulate both Sp1 and the integrin αv subunit, which suggested that the sulfation of cerebroside was important in triggering the regulatory signal. However, according to Zou et al., the nonglycosylated C6-cerebroside significantly increased GLTP expression by altering the binding affinity of the Sp1 and Sp3 coeffectors to the GLTP promoter (47). This hints that ceramide is also able to send signals to regulate the expression and binding promoter activity of Sp1, although the fatty acid chain in the molecule is short. Glycosylation of ceramide might hinder the signaling of the molecule. The sulfation of cerebroside may relieve such hindrance and trigger the regulatory signaling for Erk activation, Sp1 phosphorylation, and integrin αv expression; Moreover, we previously observed that sulfation of a type I or type II sugar chain could promote the expression of the integrin αv subunit in hepatoma cells (16, 17), suggesting that the sulfo group in the sulfatide molecule may directly trigger the regulatory signal for integrin αv subunit expression.

In conclusion, the present study demonstrated that sulfatide increased the integrin αv subunit expression level and adhesion to vitronectin in human hepatocellular carcinoma cells, which was associated with Erk signaling and Sp1 regulation.

![Fig. 8. Sulfatide enhancing Sp1 binding to the promoter of integrin αv.](image-url)
Fig. 9. Inactivation of Erk prevented Sp1 phosphorylation induced by sulfatide. (A) Prior to the treatment with sulfatide, BEL-7404 cells were preinhibited with 25 and 50 μM PD 98059, respectively, and assayed by Western blotting for the measurement of Erk1/2 (T202/Y204) and Sp1 (T739) phosphorylation. (B) The effect of MEK1/2 inhibitor on the phosphorylation of the Erk1/2 (T202/Y204) and Sp1 (T739) was assessed by Western blotting in both SMMC-7721 and BEL-7404 cells pretreated with 10 μM U0126 before sulfatide treatment (top panel). The graphs at the bottom summarize the densitometric analysis of Erk-p (left) and Sp1-p (right). All figures are from a representative study, and at least three additional experiments yielded similar results. *P < 0.05, **P < 0.01.

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