Abstract. It has been reported previously that the expression of glucose transporter member 3 (GLUT3) is increased in malignant glioma cells compared with normal glial cells. However, the regulating mechanism that causes this phenomenon remains unknown. The present study investigated the regulating role of transcription factor specific protein 1 (Sp1) in GLUT3 expression in a human glioma cell line. In the present study, Sp1 was identified to directly bind to the GLUT3 5'-untranslated region in human glioma U251 cells. Small interfering RNA - and the Sp1-inhibitor mithramycin A -mediated Sp1 knockdown experiments revealed that Sp1 depletion decreased glucose uptake and inhibited cell growth and invasion of U251 cells by downregulating GLUT3 expression. Therefore Sp1 is an important positive regulator for the expression of GLUT3 in human glioma cells, and may explain the overexpression of GLUT3 in U251 cells. These results suggest that Sp1 may have a role in glioma treatment.

Introduction

Malignant glioma is one of the most common types of malignant primary brain tumor, and has high morbidity and mortality. Even with optimal treatment, which consists of surgery, chemotherapy and radiotherapy, the median survival time of patients with malignant glioma is extremely short; only 12-15 months for patients with glioblastomas and 2-5 years for patients with anaplastic gliomas (1). It is important to understand the genetic and epigenetic factors that cause the activation of oncogenes and inactivation of tumor suppressor genes in cancer cells. An increasing number of studies indicate that accelerated glycolysis is one of the most important and vital biochemical characteristics of cancer cells (2,3). Glucose is the major energy substrate of most eukaryotic cells, and tumors, particularly solid cancer cells, often possess a growth advantage compared with non-tumorous cells due to increased anaerobic glycolysis (4,5).

Glucose transporter (GLUT) proteins allow the energy independent transport of glucose across the hydrophobic cell membrane and against a concentration gradient, and this is the first rate-limiting step for sugar metabolism in cells (6). GLUTs are regarded as important regulators of glucose metabolism in transformed cells. Among the 14 members of GLUTs, GLUT member 3 (GLUT3), which has a high affinity for glucose, is reported to be upregulated in samples obtained from patients with brain tumors (7,8). Furthermore, the level of GLUT3 expression in gliomas has a significant association with pathological grading of tumors (7,9). It has been reported previously that GLUT3 is highly expressed in glioblastoma, and promotes the growth of brain tumor initiating cells (3). Therefore, it is probable that GLUT3 may become a therapeutic target in cancer treatment. However, the mechanism by which GLUT3 is upregulated remains unknown.

Transcription factor Sp1 (Sp1) was the first transcription factor to be isolated, and it regulates the expression of numerous genes involved in cell proliferation, apoptosis and differentiation (10). Previous studies have demonstrated that an increase in Sp1 transcriptional activity is associated with tumorigenesis (11-13). In addition, Sp1 has been suggested as a therapeutic target for cancer (14), and the Sp1 inhibitor mithramycin A and analogues have been used as a novel strategy...

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for the treatment of various types of cancer (15-17). In muscle cells, insulin-like growth factor-1 controls GLUT3 expression via Sp1 (17). In human glioma, it has been reported that Sp1 is upregulated, which promotes matrix metalloproteinase (MMP)-2-mediated cell invasion and predicts poor clinical outcomes of patients (18). However, the clinical significance and biological role of the association between Sp1 and GLUT3 expression in glioma requires further investigation. The aim of the current study was to identify whether the expression of GLUT3 is regulated by Sp1, and to investigate the role of Sp1 in regulating glucose transport and the biological function of glioma cells.

Materials and methods

Cell culture and materials. Human embryonic kidney (HEK)-293T and human glioma U251 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HEK-293T and U251 cells were maintained in Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin (Gibco®; Thermo Fisher Scientific, Inc.). The cells were free from mycoplasma contamination. All cell lines were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Mitomycin A, a selective inhibitor of Sp1-mediated transcriptional activation, was assayed according to previous report (15). The cells were treated with mitomycin A (300 nM; Sigma-Aldrich, St. Louis, MO, USA) and were then harvested and lysed for either RNA or protein extraction.

Plasmids constructs and luciferase assay. To determine the possible effect of the Sp1 binding site on the solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3) gene, which encodes GLUT3 and GLUT3 5′-untranslated region (5′-UTR) containing putative binding sites for Sp1 (-306 to -297 nt and -312 to -303 nt) was amplified from normal human genomic DNA using the PCR and the following primers: Forward, 5′-TACGGTACCACAACCCAGGGTGGAGAGAGAG-3′ and reverse, 5′-TAGCTCGAGACCTGAAAGGGCG-3′. The binding sites were cloned into the KpnI and XhoI sites of the firefly luciferase reporter gene vector pGL3-Basic (Promega Corporation, Madison, WI, USA), which was named as pGL3-GLUT3-WT. GLUT3 mutant 5′-UTR was generated using site-directed mutagenesis (Forward, 5′-GGGCCGGGG-3′ and reverse, 5′-GTTTTTTGG) and was recombined into pGL3-Basic, which was named as pGL3-GLUT3-MUT. Silencing of Sp1 expression was achieved using small interfering (si) RNA duplex sequences according to a previous study by et al (19). Short hairpin (sh) Sp1.1, 5′-CCTTGCATCCTGTCAAACAGCGTTTCTGCA-3′ and shSp1.2, 5′-AGCACGCTCGATGTTGGAGCAATGTCAC-3′. The siRNA sequence was synthesized by Genetimes Technology, Inc. (Shanghai, China) and named as siRNA-S1. All constructs were confirmed by DNA sequencing.

All GLUT3-associated promoter-luciferase constructs were co-transfected into U251 cells using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.). For the reporter assay, 100 ng pGL3-GLUT3-WT or pGL3-GLUT3-MUT were co-transfected together with 50 ng p-renilla luciferase reporter vector-TK with or without siRNA-S1. The U251 cells were cultured in 24-well plates under serum-free conditions. The cells were harvested 48 h following transfection, and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega Corporation) according to the manufacturer’s protocol. Firefly luciferase activity was normalized to renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay. To detect any in vivo binding of Sp1 to the SLC2A3 gene, a ChIP assay was performed using a ChIP Assay kit (EMD Millipore, Billerica, MA, USA). Briefly, U251 cells (1x10⁶ cells) were cross-linked with 1% formaldehyde (Sigma-Aldrich) at 37°C for 10 min. The cross-linked chromatin was subsequently extracted, diluted with lysis buffer and sheared by sonication. Following centrifugation (Eppendorf 5415R; 7,500 x g, 10 min), supernatants were diluted 10-fold with ChIP dilution buffer and an aliquot of the diluted supernatant (1%) was saved as a positive control for polymerase chain reaction (PCR). Subsequent to pre-clearing the lysate with protein A and protein G-agarose beads (1:2 ratio; EMD Millipore), the chromatin was divided into equal samples for immunoprecipitation with either anti-Sp1 (mouse monoclonal IgG₁; 1:5000; sc-17824) or anti-immunoglobulin (Ig)G (negative control; mouse monoclonal IgG₂; sc-53740) antibodies (purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Immunoprecipitated DNA was recovered using the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) and 1:100 of the precipitated DNA was used for PCR. Prior to adding the antibody, 1:100 of the solution was used as an internal control for the quantitative accuracy of the DNA. Purified DNA was subjected to PCR with primers specific for a region in the SLC2A3 promoter (-381 to -200 nt), which spanned two putative Sp1-binding sites. The primer sequences used for the PCR reactions were as follows: Forward, 5′-AAACCCAGGGGTGGAGAGAG-3′ and reverse, 5′-AGCTGAAAGGGCGAC-3′. Quantitative (q) PCR was performed using SYBR green. The following cycling conditions were used: 95°C for 1 min; and amplification for 40 cycles of 95°C for 30 sec and 58°C for 45 sec. The comparative threshold cycle method was used to calculate the relative alterations in the SLC2A3 gene. The data were normalized using the negative control, an untranscribed region upstream of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and was represented as fold-change of the negative control.

Virus production and infection. The role of GLUT3 in glioma cell proliferation was examined using RNA interference-mediated gene silencing. The siRNA sequence that targeted the GLUT3 gene plasmid (pLV-SLC2A3-shRNA) were synthesized by Genetimes Technology, Inc. as follows: 5′-CGGTGCAGA TAGATCTGGAACACTGATTTTCGAGATTCGAGCCGTGTTT-3′. HEK-293T cells were transfected using the calcium-phosphate precipitation method, co-transflecting the lentiviral plasmid of interest in conjunction with proteolipid protein (PLP)-1, vesicular stomatitis virus (VSV-G) and pLP2. Viral supernatants were harvested 48 h post-transfection, filtered and used directly for infection of U251 cells. Puromycin selection was performed to select cells with stable pLV genomic integration (with control or GLUT3 shRNA). Fluorescence-activated cell
sorting was used to select cells with stable constructs, based on green fluorescent protein expression.

\[^{1}H\] -2-deoxyglucose (\[^{1}H\] -2-DG) uptake experiment. The glucose uptake experiment was measured by modifying the method described previously (20). Briefly, U251 cells were plated in 24-well plates at a density of 2x10^5 cells/well 48 h prior to the uptake experiment. The cells were washed with phosphate-buffered saline (PBS) and uptake was initiated by the addition of 0.5 ml incubation buffer containing \[^{1}H\] -2-DG (DuPont USA, Wilmington, DE, USA) at a final concentration of 600 nM (0.1 µCi/500 µl media in well) for 20 min at 37°C. Uptake was halted by washing the cells with ice-cold 0.1 M PBS. The cells were dissolved in 200 µl of lysis buffer [10 mM Tris-HCl (pH 8.0) and 0.2% sodium dodecyl sulfate (SDS)] and the incorporated radioactivity was measured by liquid scintillation spectrometry. Non-specific uptake was determined in the presence of cytochalasin B (10 µmol/l; control), which was subtracted from all values.

RNA purification and qPCR amplification. Total RNA was extracted from the U251 cells using TRIzol® Reagent (Invitrogen™; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Nucleic acid concentrations were determined using Quant-iT™ RiboGreen® Assay Kit (Invitrogen); Molecular Probes®; Thermo Fisher Scientific, Inc.). The RNA was stored at -80°C until required.

SLC2A3 expression level was detected by one-step qPCR using TaqMan probe specific for SLC2A3 mRNA. SLC2A3 primers and probe were as follows: Forward 5'-CTC TTCGTCAAACCCTTTGG-3'; reverse 5'-TCAACCGACTTA GCTATTCTAC-3' and TaqMan probe, 5'-FAM-AGC CACGGTGACAGCCAA-BAHQ1-3'. One-step qPCR was performed on ABI 7500 Read-Time PCR system (Applied Biosystems®; Thermo Fisher Scientific, Inc.) using 25 µl QuantiTect Probe RT-PCR kit (Qiagen, Inc.) on 1-2 µg of total RNA. PCR conditions were as follows: 50°C for 15 min; 95°C for 15 min; 45 cycles at 94°C for 30 sec; annealing at 55°C for 45 sec. A no-template control was included in each assay. β-actin was used as an endogenous control and vehicle control was used as a calibrator. Each sample was run three times. The comparative threshold cycle method was used to calculate the relative changes in SLC2A3 gene expression. The relative changes of gene expression were calculated using the 2^{-ΔΔCq} method, where ΔCq = Cq (detected gene) - Cq (β-actin), and Cq represents threshold cycle number.

Scratch assay. The scratch motility assay was used to measure two-dimensional movement of the cells. Aliquots of 1x10^6 cells were plated in individual wells of 6-well tissue culture plates. Following 48 h, a line of adherent cells was scraped from the bottom of each well using a sterile 200 µl pipette tip to generate a wound, and medium containing serum and blasticidin (Thermo Fisher Scientific, Inc.) was added and cells were incubated at 37°C. The cells were allowed to proliferate and migrate into the wound for 36 h. The extent of migration of cells into the region from which cells had been scraped was determined using photographs. The experiment was repeated three times with multiple scratches each time.

Statistical analysis. Data are presented as the mean ± standard error. Statistical significance was determined by Student's t test using SPSS version 17.0 was used (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Spl induces GLUT3 through transcriptional activation in glioma cells. To test the hypothesis that Spl is critical for the production of GLUT3, which is encoded by the SLC2A3 gene, putative Spl binding site sequences of the GLUT3 promoter were transfected into a luciferase reporter plasmid. Compared with the empty luciferase vector, pGL3-GLUT3-WT reporters, which comprised nucleotides (nt) -381 to -200, generated a high level of luciferase activity [32.8-fold compared with pGL3-Basic (empty vector); P<0.001] in U251 cells. However, this increase was greatly attenuated in Spl-depleted cells (11.2-fold compared with pGL3-GLUT3-WT; P<0.001) using Spl siRNA (Fig. 1A). Using reporters containing mutated binding sites, the present study identified that the Spl-binding site -306 to -297 nt was required for activation of the GLUT3 promoter. The pGL3-GLUT3-MUT reporters containing a mutated Spl binding site was unable to respond to Spl depletion, indicating that the putative Spl binding site is critical in Spl activation of GLUT3 transcription (Fig 1A).

Subsequently, the Spl putative binding site within the promoter of SLC2A3, composed of a GC-box (GGGGGC GGGGG), was analyzed using ChIP. The assay revealed that a 182 bp DNA fragment corresponding to the putative Spl binding site of the SLC2A3 gene (-381 to -200 nt) was amplified from U251 cells when chromatin was immunoprecipitated with a Spl antibody (Fig. 1B).

Spl activate GLUT3 expression in glioma cells. The present study transduced U251 cells with lentiviruses, which delivered shRNAs targeting GLUT3, to generate cells with stable GLUT3 knockdown. The expression level of GLUT3 in U251 cells were clearly reduced following an increase in the GLUT3 shRNA treatment concentration (Fig. 2A). By contrast, there was no reduction in GLUT3 expression observed with untransfected cells when the cells were treated with the negative control shRNA. Furthermore, Spl depletion decreased GLUT3 mRNA expression with an increasing concentration of siRNA-Spl. Mithramycin A may also lead to a decrease in GLUT3 mRNA. Therefore, the present study analyzed if GLUT3 expression was affected by self-targeted shRNA or an Spl-regulated mechanism, either transfection with siRNA-Spl or treatment with mithramycin A (Fig. 2B).

GLUT3 promotes glucose uptake. The present study used stable lentivirus-transfected U251 cells to assay the contribution of GLUT3 to the import of glucose. Silencing of GLUT3 expression led to a decrease of ~50% in the efficiency of radioactive 2-DG incorporation, revealing that GLUT3 is important in glucose uptake in glioma cells (Fig. 2C). Furthermore, the present study demonstrated that Spl expression silencing and mithramycin A also led to a reduction in the efficiency of radioactive 2-DG incorporation in the cells (Fig. 2C). The present data indicates that Spl participates in the GLUT3-dependent glucose uptake by regulating GLUT3 expression.

Reduced GLUT3 expression attenuated the invasive potential of glioma cells in vitro by Spl regulation. Subsequently, the effect of Spl depletion and silencing of GLUT3 expression on the biological characteristics of glioma cells was evaluated. The proliferation rates of U251 cells treated with shRNA-GLUT3 (10 pmol), siRNA-Spl (20 pmol) or mithramycin A (300 nM) were detected using an MTT assay. The treated cells demonstrated significant growth inhibition compared with the untreated cells (P<0.05; Fig. 3A).

The present study next examined the role of Spl in GLUT3-mediated glioma invasion using Transwell assay. Spl depletion using Spl RNAi or mithramycin A clearly inhibited glioma invasion and the role of shRNA-GLUT3. Representative photomicrographs and quantification of tumor cell invasion through matrigel are shown in Fig. 3B and C.

In the scratch assay, the present study observed that shRNA-GLUT3 transected and Spl depleted cell migrated more slowly compared with control cells (Fig. 3D). The values at 0 and 12 h demonstrated a significant difference (Fig. 3E). The present data confirmed that siRNA-Spl inhibits glioma cell migration in the in vitro assay.

Discussion

The present study identified that in glioma cells Spl is a transcription factor for GLUT3, and GLUT3 expression is...
Figure 2. (A) Western blot analysis for the expression of GLUT3 in control and human glioma U251 cells treated with siRNA-SP1 (10 and 20 pmol), shRNA-GLUT3 (10 and 20 pmol) and SP1-inhibitor (300 nM). Notably, GLUT3 expression is decreased following treatment with siRNA-SP1, shRNA-GLUT3 and SP1-inhibitor. (B) GLUT3 relative mRNA level. GLUT3 mRNA expression was detected using quantitative polymerase chain reaction in control and U251 cells treated with GLUT3 shRNA (5, 10 and 20 pmol), SP1 siRNA (10 and 20 pmol) and SP1-inhibitor (300 nM). (C) Glucose uptake in U251 cells. Uptake of 2-DG in control and U251 cells treated with GLUT3 shRNA (5, 10 and 20 pmol), SP1 siRNA (10 and 20 pmol) and SP1-inhibitor (300 nM). Data are the means of three independent experiments performed in triplicate. Error bars indicate the standard deviation from the mean; *P<0.05; **P<0.01 vs. control. GLUT3, glucose transporter 3; control, untreated U251 cells; siRNA, small interfering RNA; SP1, specific protein 1; shRNA, short hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SP1-inhibitor, mithramycin A; mRNA, messenger RNA; 2-DG, [3H]-2-deoxyglucose; pm, picomole.

Figure 3. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used as an in vitro cell proliferation assay following GLUT3 knockdown or SP1 depletion in human glioma U251 cells. The control cells were transfected with non-targeting siRNA or were untreated. (B) Transwell assay. U251 cells transfected with (a) control, (b) shRNA-GLUT3 and (c) siRNA-SP1 and (d) treated with mithramycin A (300 nM) were plated into Transwell plates. Tumor cells invaded through matrigel were photographed. (C) Quantification of the Transwell assay. (D) Scratch assay. U251 cells treated with shRNA-GLUT3, siRNA-SP1 mithramycin A migrated more slowly compared with control cells. (E) Quantification of the scratch assay. Data represent the mean of three experiments. Error bars indicate the standard deviation from the mean; *P<0.05; **P<0.01 vs. control. GLUT3, glucose transporter 3; SP1, specific protein 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; control, untreated cells.
regulated by the Sp1 interaction with the GLUT3 promoter. The present data also suggest that Sp1 knockdown or the use of an Sp1 inhibitor decreases glucose uptake and inhibits the biological function of glioma cells and the GLUT3 blockade. Therefore, demonstrating the contribution of Sp1 to tumor proliferation through the regulation of GLUT3-mediated glucose metabolism.

It is well known that a variety of cancer cells exhibit increased levels of glucose uptake and glycolysis as a fundamental metabolic alteration, which is often associated with elevated levels of GLUT family members, including GLUT1 and GLUT3 (9,21). In normal brain cells, GLUT3 gene expression is confined to neurons; however, in highly malignant glial cells of the human brain, GLUT3 may be the predominant glucose transporter (7). Furthermore, GLUT3 may contribute to the maintenance of human brain tumors, and the expression of GLUT3 may be closely associated with various pathological types of tumor (9). However, the regulation of GLUT3 expression and its biological significance in glial tumorigenesis requires clarification. The present study demonstrated that knockdown of GLUT3 expression decreased the proliferation, invasion and migration of human glioma cells. This indicates that GLUT3 is important in glioma cell survival, growth and invasion. Furthermore, knockdown of GLUT3 expression decreased glucose uptake of the cells, suggesting that GLUT3 may be critical in sugar metabolism.

It has been reported that Sp1 is an important transcription factor, which enhances or represses the activity of gene promoters involved in cell differentiation, cell cycle progression and oncogenesis (10). Sp1 is upregulated in human glioma and predicts a poor clinical outcome (18). Sp1 mediates transcriptional regulation of numerous genes, including MMP-2 and purinergic receptor P2X7 in brain cells (18,19). In addition, the Sp1 inhibitor mithramycin A has therapeutic effects in numerous diseases, including brain cancer and neurodegeneration (14,15,22,23). Mithramycin A competitively binds to GC-rich promoter regions; thereby replacing the transcription factor Sp1 (24,25). Through this mechanism, mithramycin A inhibits the transcription of Sp1-regulated genes, including v-myc avian myelocytomatosis viral oncogene homolog and SRC proto-oncogene, non-receptor tyrosine kinase (24,26). However, few studies demonstrate the role of Sp1 in the regulation of glioma cell metabolism through GLUT3 expression.

The present data identify that Sp1 regulates the expression of GLUT3 directly, and mithramycin A inhibits GLUT3 expression by binding to GGGGGCGGGGG regions of the GLUT3 promoter in glioma cells. The present results were different from a previous study in mice, in which Sp1 bound to the mouse Glut3 gene that mediated the suppression of Glut3 gene expression in murine neuroblasts and trophoblasts. Furthermore, in the present study glucose uptake was decreased in Sp1 depleted cells or Sp1 inhibitor treated cells. This suggests that Sp1 may also have a role in glioma cell metabolism through GLUT3 regulation. Additionally, Sp1 depletion or inhibition decreases glioma cells proliferation, invasion and immigration. This indicates that Sp1 is important in glioma cells survival, growth and invasion, which has been revealed by certain studies in other types of tumors (14,16,17,26). Indeed, mithramycin A is approved by the Food and Drug Administration for the treatment of leukemia and testicular cancer. In the future, it is possible that Sp1 may become a crucial therapeutic target.

In summary, the present study suggests that GLUT3 is extremely important in human glioma, and that its knockdown may contribute to decreased biological activity and glucose uptake in glioma cells. In addition, the present study identified that the role of GLUT3 in glioma may be regulated by the Sp1 transcription factor directly through binding to Sp1-sites in the GLUT3 promoter. These results may provide a novel insight into glioma drug treatment.

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