NEOPLASIA
www.neoplasia.com

Volume 10 Number 12 December 2008 pp. 1335–1342

Insulin-like Growth Factor Binding Protein 7 Mediates Glioma Cell Growth and Migration[1]

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Abstract

Insulin-like growth factor binding protein 7 (IGFBP-7) is the only member of the IGFBP superfamily that binds strongly to insulin, suggesting that IGFBP-7 may have different functions from other IGFBPs. Unlike other IGFBPs, the expression and functions of IGFBP-7 in glioma tumors have not been reported. Using cDNA microarray analysis, we found that expression of IGFBP-7 correlated with the grade of glioma tumors and the overall patient survival. This finding was further validated by real-time reverse transcription–polymerase chain reaction and Western blot analysis. We used RNAi to examine the role of IGFBP-7 in glioma cells, inhibiting IGFBP-7 expression by short interfering RNA transfection. Cell proliferation was suppressed after IGFBP-7 expression was inhibited for 5 days, and glioma cell growth was stimulated consistently by the addition of recombinant IGFBP-7 protein. Moreover, glioma cell migration was attenuated by IGFBP-7 depletion but enhanced by IGFBP-7 overexpression and addition. Overexpression of AKT1 in IGFBP-7–overxpressed cells attenuated the IGFBP-7–promoted migration and further enhanced inhibition of IGFBP-7 depletion on the migration. Phosphorylation of AKT and Erk1/2 was also inversely regulated by IGFBP-7 expression. These two factors together suggest that IGFBP-7 can regulate glioma cell migration through the AKT-ERK pathway, thereby playing an important role in glioma growth and migration.

Neoplasia (2008) 10, 1335–1342

Introduction

Insulin-like growth factor (IGF) is a potent mitogen involved in normal growth and development. In the central nervous system, IGFs promote the proliferation of oligodendrocytes and myelin synthesis [1] and are thought to play a critical role in the proliferation of brain tumors [1]. The growth-promoting and metabolic activities of IGFs are modulated by insulin-like growth factor binding proteins (IGFBPs) and their receptors [2,3]. The biological actions of IGFs may be regulated by IGFBPs either negatively or positively, depending on the tissue type and the physiological and/or pathological status [4].

Insulin-like growth factor binding proteins are soluble secreted proteins that bind IGF-I and IGF-II with affinity that is equal to or greater than those of IGF receptors. The IGFBP family consists of two groups: 1) high-affinity IGFBPs (i.e., IGFBPs 1-6) and 2) low-affinity IGFBPs (i.e., IGFBP-related proteins 1-4) [5,6]. The low-affinity IGFBPs are classified as IGFBP family members by virtue of their structure homology with the high-affinity IGFBPs. Their low affinity for IGF in addition to the conserved structure homology to the IGFBP family leads to the proposal that these IGFBPs may have unique biological properties independent of their capacity to bind IGF [7]. Insulin-like growth factor binding protein 7—also known as IGFBP-related protein 1 (IGFBP-rP1), mac25/angiomodulin, tumor adhesion factor, and prostacyclin-stimulating factor—is one of the low-affinity IGFBPs [8]. It is distinct from other low-affinity IGFBP-rPs in that it can bind strongly to insulin [9]. The expression of IGFBP-7 is related to tissue type and tumor pathology. *IGFBP-7* has been known as a tumor-suppressor gene in prostate and breast cancers. Detectable *IGFBP-7* mRNA levels are lost significantly in metastatic prostate tissue [10]. Both *in vitro* and *in vivo* studies have shown that overexpression of IGFBP-7 in human prostate cancer M12 suppresses cell proliferation, colony formation in soft agar, and tumor formation in male nude mice, suggesting that IGFBP-7 might have a suppressive effect on prostate cancer development [11,12]. However, using immunohistochemistry to stain prostate

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[1]This work was supported in part by the National Institutes of Health grants CA095809 (T.M.) and CA-109196 (C.B.).
Received 16 June 2008; Revised 3 September 2008; Accepted 5 September 2008

DOI 10.1593/neo.08694

cancer and benign prostate tissues, Degeorges et al. [5] showed that IGFBP-7 was expressed in invasive prostate neoplasms but not typically in normal secretory or benign prostatic hyperplasia epithelium. Furthermore, low IGFBP-7 was also found to be associated with poorly differentiated breast cancer tumors and higher-stage disease when immunohistochemical staining and microarray were used to identify IGFBP-7 expression in the tumor cells [14]. Expression of IGFBP-7 has been found to be up-regulated in human colorectal cancer [15] and glioma cell lines [16]. So far, there is limited understanding of the biological and mechanistic functions of IGFBP-7 in different cancers, especially gliomas.

In this paper, we report the association of IGFBP-7 expression levels with glioma grade and outcome. We investigated the roles of IGFBP-7 in glioma cell growth and migration, as well as the molecular mechanisms involved.

Materials and Methods

Materials

Polyclonal anti–IGFBP-rP1 antibody and recombinant IGFBP-rP1 protein were purchased from R&D Systems, Inc. (Minneapolis, MN). silMPORTER short interfering RNA (siRNA)/plasmid DNA transfection reagent was purchased from Upstate, Inc. (Charlottesville, VA). Transwell Permeable Supports were purchased from Corning Life Science (Acton, MA). A Matrigel Invasion Chamber was obtained from BD Discovery Labware (Bedford, MA). SeaPlaque Agarose was purchased from Cambrex (Rockland, MN), and CytoMatrix Cell Adhesion strips were purchased from Chemicon International (Temecula, CA).

Glioma Cells

The glioma cell lines, A172, U87, U251, LN18, LNZ308, and LN443, purchased from American Type Culture Collection (Manassas, VA), were grown on tissue culture dishes in medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, and 0.05 mg/ml streptomycin.

Tumor Samples

Glioma tumor samples from 192 patients were obtained from the Glioma Biorepository at Henry Ford Hospital, Detroit, MI. All tumor samples were obtained with written consent in accordance with institutional guidelines. Tumor histologies included 81 glioblastomas multiforme (GBMs), 49 oligodendrogliomas, 28 astrocytomas, 11 mixed gliomas, and 21 nontumor brain tissues obtained from epilepsy surgery. All tissues had been freshly frozen and stored at −80°C.

Microarray Construction

We used an array, constructed at the Neuro-Oncology Branch of the National Cancer Institute/National Institute of Neurological Disorders and Stroke, containing the 28,896 Research Genetics cDNAs (15,935 of them have been sequence-verified) and an additional 5664 Cancer Genome Anatomy Project (CGAP) sequence-verified cDNAs obtained through data mining of the CGAP databases. The clones we selected were those related to genes that had been described previously as potentially important for cancer or glioma development, such as genes involved in invasion, angiogenesis, and transformation. We also selected a series of clones that have been shown previously to be over- or underexpressed highly in the glioma SAGE database. Finally, we incorporated a number of cDNA clones that were obtained by screening approximately 20 glioma cDNA libraries for highly represented and/or unusual transcripts. The arrays were printed using an OmniGrid Microarrayer (GeneMachines, San Carlos, CA) on poly-L-lysine–coated glass slides.

RNA Preparation and Microarray Experiments

Total RNA was isolated using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The RNA was further purified using RNeasy Mini kit (Qiagen, Inc., Valencia, CA). Total RNA was then linearly amplified one round as described previously [1]. Amplified RNA (1 μg) was reverse-transcribed and labeled directly with Cy3-dUTP and Cy5-dUTP using a cDNA labeling kit according to manufacturer's instructions (Amersham, Inc., Piscataway, NJ). Tissue sample RNA was labeled with Cy5, and Universal Human Reference RNA (Stratagene, La Jolla, CA) was labeled with Cy3. Labeled cDNA were hybridized to the 34,561-feature glass cDNA microarrays overnight at 42°C with 25% formamide. The arrays were scanned on a GenePix 4000A microarray scanner (Axon Instruments, Foster City, CA) to generate TIFF images. GenePix pro 3.0 microarray analysis software (Axon Instruments) was used to measure fluorescence signals. All of the array data were deposited in the National Cancer Institute Center for Information Technology microarray database (http://nciarray.nci.nih.gov/).

Short Interfering RNA Transfection

Short interfering RNA duplexes were synthesized and purified by Dharmacon (Lafayette, CO). We used a pool of four IGFBP-7 siRNA duplexes that were also obtained from Dharmacon. Transfection of siRNA was done using 100-nM IGFBP-7 or scrambled control siRNA and silMPORTER kit (Upstate, Inc.) according to the manufacturer's instructions. Protein levels of IGFBP-7 were determined using Western blot analysis.

Proliferation Analysis

The cell lines, LN18 and LN443, expressing very low or medium levels of IGFBP-7 protein and mRNA, were grown in DMEM/10% FBS medium containing 1 μg/ml of IGFBP-7 recombinant protein for 6 days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied to analyze cell proliferation at days 0, 1, 3, and 6 of the treatment. Briefly, 1×10^3 cells were planted into 96-well plates and grown overnight in DMEM supplemented with 10% FBS and cultured at 37°C with 5% CO_2. At day 0, one 96-well plate was used for the MTT assay. Meanwhile, the medium of the other 96-well plates was changed with DMEM supplemented with 10% FBS containing 1 μg/ml of IGFBP-7 recombinant protein. Fresh medium was added every day up to day 6 of the treatment. Cell proliferation was analyzed with MTT at days 1, 3, and 6.

Transwell Migration Assay

Corning Costar Transwell plates (8 μM) were pretreated according to the manufacturer's instructions. The cells that had been transfected with IGFBP-7 siRNA or control siRNA for 3 days were trypsinized, collected in DMEM supplemented with 10% FBS, and centrifuged at 1200 rpm for 5 minutes at 4°C. Cell pellets were suspended in DMEM supplemented with 10% FBS medium for counting. Cell concentration was adjusted to 4×10^5 cells/ml, and 250 μl

of cell suspension was plated into each membrane chamber of a 24-well Transwell plate, with 400 μl of medium. Plates were incubated at 37°C under 5% CO_2 for 2 days. Migrated cells were stained and counted as reported previously [18].

Scratch Assay

Parental or transfected cells (1×10^6 cells per well) were seeded into a 12-well plate and cultured overnight under 5% CO_2 at 37°C. When the cells were 80% confluent, a straight scratch was gently made through the central axis of the plate using a micropipette tip. The plates were rinsed with Hanks solution, and serum-free DMEM was added. After a 24-hour incubation, the distance of cells migrating into the acellular area created by scratching was measured in randomly selected visual fields ($n = 9$) at a magnification of ×20 using an inverted microscope. The measurement was repeated three times for each group of cells. The percentage of migration was determined by dividing the migrated distance by the scratched distance.

Cell Growth Analysis

A172 cells (1×10^5) in 500 μl of medium were plated onto 12-well plates a day before transfection. Insulin-like growth factor binding protein 7 and control siRNA were transfected using silMPORTER kit from Upstate, Inc. Cell number was counted on days 3 and 5 of transfection. On the day the assay was performed, 50 μl of 10 mM EDTA was added to each well and incubated at 37°C for 2 minutes. Cells were suspended with 1 × PBS (450 μl), and cell suspension was collected in a 5-ml tube. The remaining cells in the well were washed with 1 × PBS (500 μl) and collected in the same tube. The cell suspension was kept on ice and then counted through a microscope with Trypan blue staining.

Western Blot Analysis

Total cell lysates were prepared in a RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors (Roche Diagnostics, Basel, Switzerland). Electrophoresis was performed on either 10% or 12% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred onto PVDF Plus membrane (MSI, Inc., Minnetonka, MN) using the Bio-Rad Mini Protean II transfer system.

RNA Extraction

Total RNA was isolated from nontumor brain tissues (i.e., from epilepsy surgeries and postmortem from patients with causes of death other than brain disease) and brain tumor samples (i.e., glioblastomas, astrocytomas, and oligodendrogliomas) using an RNeasy lipid tissue mini RNA-isolation kit (Qiagen) following the manufacturer's instructions. The quality of the RNA samples was determined by measuring their absorbance at 260 nm as well as by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under UV.

Reverse Transcriptional Reaction

Reverse transcription of RNA was done in a final volume of 20 μl containing 13 reverse transcription–polymerase chain reaction (RT-PCR) buffer (500 mM each deoxynucleotide triphosphate, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3), 10 units of RNasin RNase inhibitor (Promega Corp., Madison, WI), 10 mM DTT, 50 units of Superscript II RNase H2 reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1.5 mM random hexamers (Pharmacia, Uppsala, Sweden), and 1 μg of total RNA. The samples were incubated at 20°C for 10 minutes and at 42°C for 30 minutes, and the reverse transcriptase was inactivated by heating at 99°C for 5 minutes and cooling at 5°C for 5 minutes.

Real-time PCR Amplification

All PCRs were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers for the *IGFBP-7* gene were chosen with the assistance of the computer program, Vector NT. We conducted BLASTN searches against refSeq_rna to confirm the total gene specificity of the nucleotide sequences chosen for the primers and the absence of DNA polymorphisms. To avoid amplification of contaminating genomic DNA, the two primers were placed in two different exons. For each PCR run, 8 μl of 30-fold diluted cDNA was mixed with 2 μl of primer mixture (10 μM per primer) and 10 μl of Platinum SYBR Green qPCR SuperMix UDG with ROX (No. 11744; Invitrogen, Carlsbad, CA) on ice. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 4 minutes, 45 cycles at 95°C for 30 seconds, 60°C for 30 minutes, and 70°C for 1 minute and finished with incubation at 72°C for 7 minutes.

Statistical Analysis

The results are presented as the mean ± SD. Data were analyzed using analysis of variance and Student's *t* test to determine the level of significance between the different groups.

Results

Expression of IGFBP-7 in Glioma Tumors

The expression of IGFBP-7 has been found in the literature to be down-regulated in prostate and breast cancer cells [11,12,14]. In contrast, our microarray analysis of glioma patients' cDNA showed that IGFBP-7 was highly up-regulated in GBM compared to nontumor brain tissue (Figure 1*A*). To verify the microarray results, we randomly picked samples from our tumor bank for quantitative real-time RT-PCR analysis to verify the range of expression of IGFBP-7. Its expression by quantitative real-time RT-PCR in tumors confirmed the microarray results (Figure 1*C*). A $P < .05$ supported our hypothesis that up-regulation of IGFBP-7 expression is statistically significant in GBM samples. We also randomly picked GBM specimens for Western blot analysis not necessarily matched to those included in microarray analysis. Western blot analysis further demonstrated IGFBP-7 protein expression in GBM specimens. As shown in Figure 2*A*, IGFBP-7 protein was strongly expressed in GBM specimens but not in nontumor brain tissues. Therefore, the results of RT-PCR and Western blot analysis increased the number of tumors examined to support the microarray results. Owing to the lack of a robust antibody for IGFBP-7 immunohistochemistry staining in human brain specimens, we analyzed IGFBP-7 expression in primary tumor culture. As shown in Figure 2*C*, IGFBP-7 was expressed in the primary cultured cells. In addition, we also analyzed the IGFBP-7 expression in GBM xenografts in nude rats that were implanted with primary cultured GBM cells using Western blot analysis as well as RT-PCR of *IGFBP-7* mRNA with human-specific primers. Both

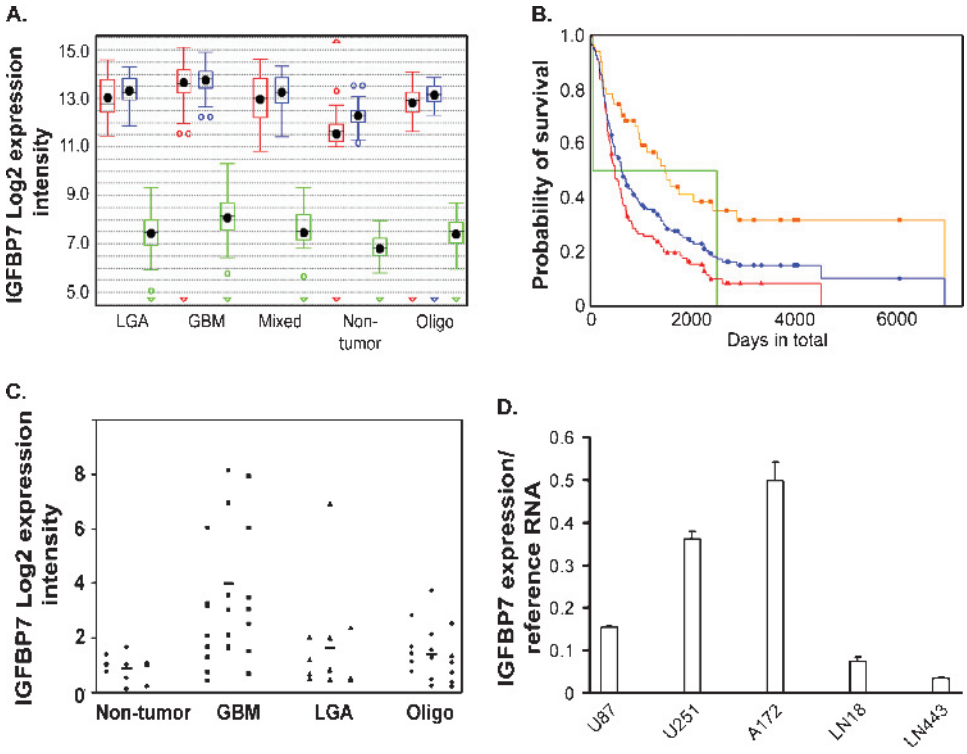

Figure 1. Insulin-like growth factor binding protein 7 is up-regulated in gliomas, and its expression correlates with overall patient survival. (A) Microarray analysis of *IGFBP-7* mRNA level in different grades of glioma tissues. Tumor specimens included 89 glioblastomas (GBMs), 49 oligodendrogliomas (Oligo), 28 astrocytomas (Astro), and 21 nontumor brain tissues obtained from epilepsy surgery (nontumor). The *t* test analysis shows that $P < .005$ for GBM *versus* nontumor, oligodendroglioma *versus* nontumor, and astrocytoma *versus* nontumor. Different color bars show the analysis using different probes. (B) Probability of GBM patient survival and IGFBP-7 expression level. The yellow line indicates the survival of GBM patients with intermediate levels of *IGFBP-7* mRNA (i.e., IGFBP-7 expression in the tumors falls within the two-fold change compared to the nontumor samples) in specimens; the red line indicates the survival of GBM patients with high levels of *IGFBP-7* mRNA (i.e., the threshold for IGFBP-7 up-regulation of two-fold or higher) in specimens; and the blue line indicates the overall GBM patient survival rate. The number of patients with up-regulated IGFBP-7 expression in the group is 74, whereas the number of patients with intermediate levels of IGFBP-7 is 13 and two tumors showed down-regulation of IGFBP-7 expression (i.e., two-fold or less, shown in green line). The *t* test analysis showed that the *P* value between the intermediate and up-regulated levels is $< .01$. (C) Real-time RT-PCR data validated that IGFBP-7 expression level correlates to tumor grades. The samples used in this analysis did not have to be the same as those used for the microarray assay and were randomly selected from our tumor bank. The *t* test analysis shows that $P < .05$ for GBM *versus* nontumor and for oligodendroglioma *versus* nontumor. (D) *IGFBP-7* mRNA expression in different glioma cell lines. Seven glioma cell lines were analyzed by real-time PCR. The *IGFBP-7* expression level in these cells is presented compared with the housekeeping gene *S12*.

analyses showed IGFBP-7 expression in human glioma cells but not rat brains (data not shown). These data together suggested that IGFBP-7 was overexpressed in GBM specimens.

Moreover, statistical analysis of patient survival showed that the expression of IGFBP-7 correlated inversely with overall GBM patient survival rates (Figure 1*B*), that is, the GBM patients with high levels of IGFBP-7 (i.e., the threshold for IGFBP-7 up-regulation was two-fold or higher) had shorter survival than those with intermediate levels (i.e., IGFBP-7 expression in the tumors falls within the two-fold change compared to the nontumor samples). In addition, using real-time RT-PCR and Western blot analysis, we found that IGFBP-7 was expressed in different glioma cell lines (Figures 1*D* and 2*B*).

Influence of IGFBP-7 on Glioma Cell Growth

According to the Western blot analysis of the glioma cell lines, we chose A172 cells for our experiments owing to their relatively high level of IGFBP-7 expression. We used RNA interference to diminish the expression of IGFBP-7 in A172 cells. The Western blot assay shown in Figure 2*A* suggests that more than 95% of IGFBP-7 protein expression in these cell lines was inhibited by siRNA transfection. Cell growth analysis showed that cell growth was significantly suppressed by *IGFBP-7* gene RNA interference after IGFBP-7 siRNA had been transfected for 5 days (Figure 3*A*).

Because IGFBP-7 is a secreted protein, we treated cells with IGFBP-7 recombinant protein to examine whether IGFBP-7 can stimulate cell proliferation. LN18 and LN443 showed lower levels of IGFBP-7 expression when analyzed by real-time RT-PCR and Western blot assay (Figures 1*D* and 2*B*). The addition of IGFBP-7 recombinant protein in cell culture medium of these cells stimulated the cell proliferation significantly (Figure 3*B*). Results from the addition and depletion of IGFBP-7 suggest that IGFBP-7 plays an important role in the regulation of glioma cell growth and proliferation. In addition, the apoptosis assay (Annexin-V staining) showed that depletion of IGFBP-7 expression in glioma cells did not induce cell apoptosis (data not shown). Taken together, these data suggest that IGFBP-7 regulates glioma cell proliferation and growth but not cell survival.

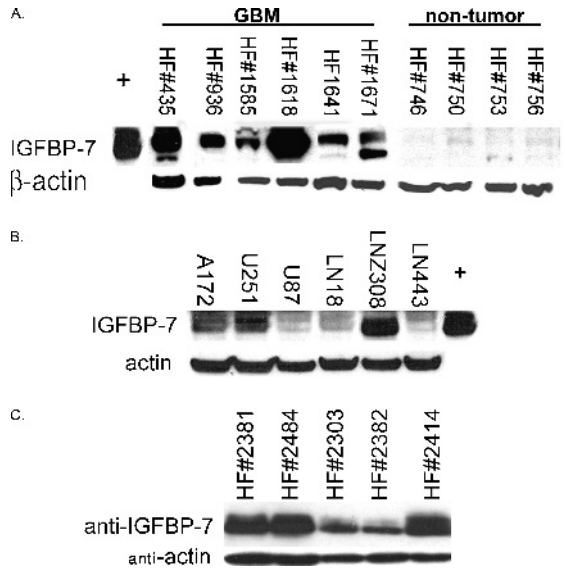

Figure 2. Insulin-like growth factor binding protein 7 expression in GBM specimens and glioma cell lines. (A) Expression of IGFBP-7 protein in GBM and nontumor specimens was analyzed by Western blot. Protein extracts (30 μg) from GBM specimens and nontumor specimens were separated by 12% SDS-PAGE and were transferred to PVDF membrane. Immunoblot analysis was performed with anti–IGFBP-7 antibody and antiactin antibody in the same membrane. (B) Expression of IGFBP-7 protein in glioma cell lines was studied by Western blot analysis. Total cell lysates (30 μg) were separated by 12% SDS-PAGE. (C) Expression of IGFBP-7 protein in primary cultured GBM tumor cells (passages were <5) was analyzed by Western blot assay.

IGFBP-7 Intervenes in the Migration of Glioma Cells Through Regulating AKT and Erk1/2 Activation

Cell migration assays were conducted on uncoated transwell plates (i.e., they were not coated with extracellular matrix proteins) and uncoated six-well plates using scratch assay. After IGFBP-7 was knocked down, glioma cell migration decreased. After 48 hours of plating the transfected cells into the Transwell plates, we counted the migrated cells. The results were presented as the mean migrated cell number ± standard error. We used Student's *t* test to determine whether migration differs significantly between IGFBP-7–depleted cells and control cells. As shown in Figure 4*A*, compared to control transfection, migrated A172 cells of IGFBP-7–depleted cells were decreased approximately 44%. Figure 4*B* shows the same migration analyzed with scratch assay. The data confirmed that the two assays are consistent.

To further confirm the impact of IGFBP-7 on glioma cell migration, IGFBP-7 was added into the growth medium or overexpressed in glioma cells. Shown in Figure 4, *C* and *D*, the addition or overexpression of IGFBP-7 promoted the migration of LN18 and LN443 cells that have low levels of endogenous expression of this protein. Taken together, the data suggested that IGFBP-7 can mediate glioma cell migration.

Insulin-like growth factor binding proteins can compete with IGF-R to bind to IGFs to prolong the half-life of IGFs. Previous published data suggested that IGF-I or IGF-II can regulate cell growth, motility, and other biological processes through regulating the activation of PI3K/AKT and MEK/ERK pathways. To verify whether IGFBP-7 can mediate the activation of AKT and ERK1/2, we analyzed the phosphorylation of ERK1/2 and AKT after IGFBP-7 was added into the cell medium up to 6 hours. Western blot analysis

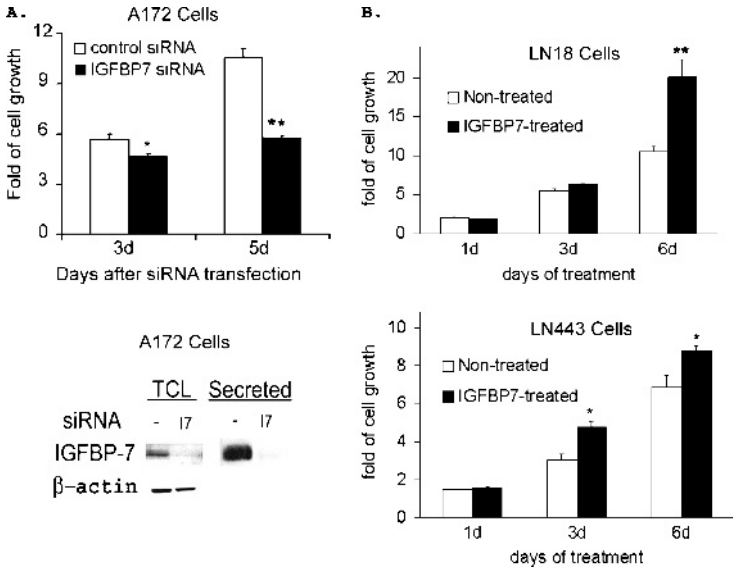

Figure 3. Insulin-like growth factor binding protein 7 mediates glioma cell growth. (A) A172 cell growth after IGFBP-7 was silenced for 3 and 5 days. Open columns show the cell growth of control siRNA transfected cells. Black columns represent the cell growth of IGFBP-7–depleted cells. Significance is as follows: $*P < .05$, $**P < .01$. Western blot analysis confirmed the depletion of IGFBP-7 in IGFBP-7–silenced A172 cells. To silence IGFBP-7 expression in glioma cells, 100 nM IGFBP-7 or control siRNA was transfected into A172 cells using siIMPORTER kit. Total cell lysates were collected in RIPA buffer after 72 hours of transfection. Serum-free medium was also collected. Total cell lysates (30 μg) or serum-free cell culture medium was separated by 12% SDS-PAGE and analyzed for IGFBP-7 expression. (B) LN18 and LN443 cells grew in the DMEM containing 1 μg/ml IGFBP-7 for 6 days. Fresh medium was added every day to keep the concentration of IGFBP-7 consistent. Cell growth was analyzed using Trypan Blue staining and counting.

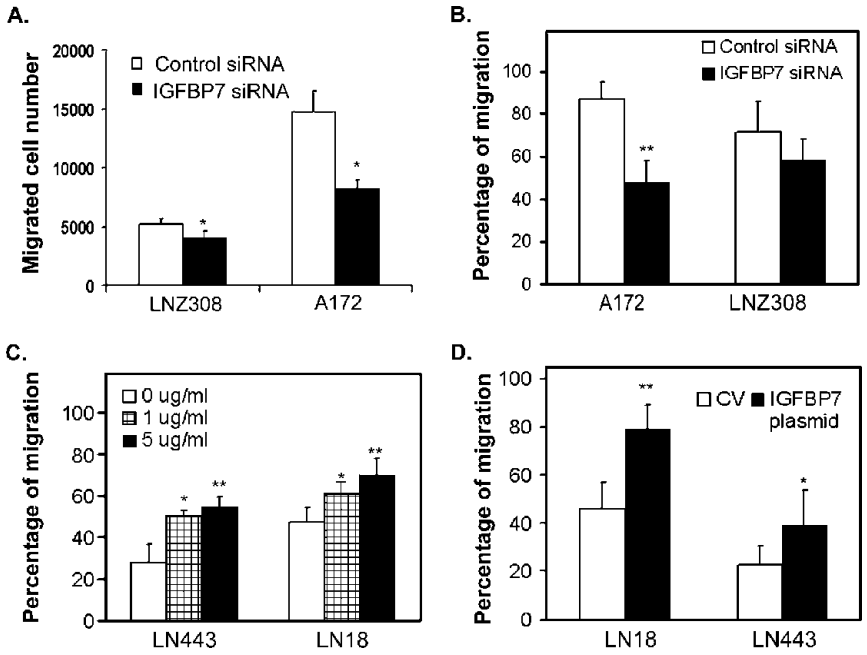

Figure 4. Insulin-like growth factor binding protein 7 regulates glioma cell migration. (A) Migration assay of IGFBP-7–depleted A172 and LNZ308 glioma cells was conducted on Corning Costar Transwell plates (8 μM). Cells (1×10^5) that were transfected with IGFBP-7 or control siRNA for 3 days were plated into each membrane chamber of a 24-well Transwell plate with 400 μl of medium. Plates were incubated at 37°C under 5% CO_2 for 2 days before migrating cells were counted. The data represent the average of three independent experiments with triplicate samples in each experiment. The significance is $P < .01$. (B) Migration of IGFBP-7–depleted A172 and LNZ308 glioma cells was analyzed using scratch assay. The distance of migrated cells from the scratched point was measured randomly. The percentage of migration was determined by dividing the migrated distance by the scratched distance. The cells that were transfected with IGFBP-7 or control siRNA for 3 days were plated into a 12-well plate to 80% confluence before the day of assay. The scratched cells were grown in serum-free medium for 24 hours before analyzing the migration. (C) The effect of IGFBP-7 on the migration of glioma cells that have low endogenous levels of this protein was analyzed by adding IGFBP-7 recombinant protein (0, 1, 5 μg/ml) into the growth medium of LN18 and LN443 cells. The cells were grown in serum-free and IGFBP-7–enriched medium after scratching. The migration was analyzed after 24 hours. (D) The IGFBP-7–mediated migration was analyzed with IGFBP-7–overexpressed LN18 or LN443 cells. Scratch assay was conducted as described in panel (B). Significance values compared to control cells are as follows: *$P < .05$, **$P < .01$.

(Figure 5) showed that the levels of ERK1/2 and AKT phosphorylation varied with the incubation time of glioma cells in IGFBP-7–added medium. To further verify the effect of AKT activation on IGFBP-7–regulated glioma cell migration, we overexpressed AKT in IGFBP-7–silenced A172 and IGFBP-7–overexpressed LN18. The migration assay (Figure 6) showed that overexpression of AKT further enhanced the decreased migration caused by IGFBP-7 silencing but overcame the IGFBP-7–promoted cell migration in IGFBP-7 overexpressed cells. These data together suggest that IGFBP-7 promoted glioma cell migration through regulating the AKT and ERK1/2 signal transductions.

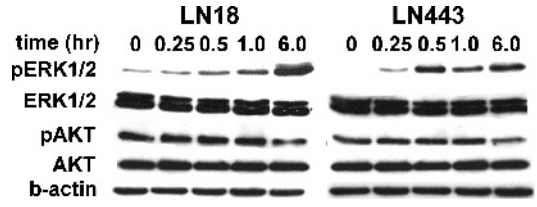

Figure 5. Activation of AKT and ERK1/2 in IGFBP-7–treated cells. The LN18 cells were treated with IGFBP-7 recombinant protein (1 μg/ml) at several time points as seen in this figure. The total cell lysates (30 μg) were collected for Western blot analysis using anti–phosphorylated AKT (Ser473), anti-AKT, anti–phosphorylated ERK1/2, and anti–ERK1/2 antibodies.

Discussion

Although RT-PCR [22] detected the expression of *IGFBP-7* mRNA in 87% of established glioma cell lines, the expression and function of IGFBP-7 in primary glioma tumor specimens have not yet been studied. The biological function and expression of IGFBP-7 seems to be diverse and cell type– or host-environment–specific. Subtractive hybridization assays have shown that IGFBP-7 was preferentially expressed in normal leptomeningeal cells compared with meningiomas [2]. *In situ* hybridization studies of IGFBP-7 expression in normal prostate tissue *versus* prostate tumors have shown that a marked decrease in IGFBP-7 expression was associated with increasing malignancy [3]. Furthermore, a prostatic carcinoma cell line stably transfected with IGFBP-7 cDNA showed poor tumorigenicity in both *in vitro* and *in vivo* assays compared with vector controls [4]. These observations suggest that IGFBP-7 has a potential tumor-suppressive function. Contradictory to these findings, Degeorges et al. [5] used immunohistochemical staining to analyze the location of IGFBP-7 in prostate cancer. They found that the most intense staining was observed in nerves, whereas smooth muscle cells in the prostate stained weakly. Lymphocytes were always negative. When normal

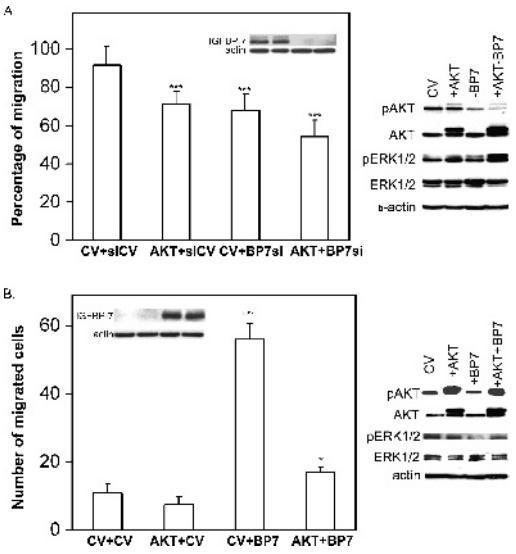

Figure 6. The effect of AKT on IGFBP-7–mediated migration. (A) Overexpression of AKT1 in IGFBP-7–depleted cells enhanced the inhibition of IGFBP-7 depletion on cell migration. AKT1 plasmid and IGFBP-7 siRNA were cotransfected into A172 cells using siIMPORTER siRNA and plasmid DNA Transfection Reagent (Upstate). After 48 hours of transfection, cells were plated in a 24-well plate, and migration was analyzed using scratch assay. Western blot analysis of IGFBP-7, phosphorylation of AKT and Erk1/2, and total AKT and Erk1/2 expression in total cell lysates is included. (B) Overexpression of AKT1 in IGFBP-7–overexpressed cells inhibited the promoted migration by IGFBP-7 overexpression. AKT1 and IGFBP-7 plasmids were cotransfected into LN18 cells using Lipofectamine 2000 reagent (Invitrogen). The migration assay was conducted on Corning Costar Transwell plates (8 μM). The expression of IGFBP-7 in cell lysates was analyzed by Western blot assay as shown in the figure. The effect of IGFBP-7 and AKT expression on the activation of AKT and Erk1/2 was analyzed by Western blot assay. The significance between the transfected cells and control cells are as follows: ***$P < .001$, **$P < .01$, and *$P < .05$.

prostatic secretory epithelium was present, staining was usually absent. The lining secretory epithelium stained positively in 0 (0%) of 12 cases of benign prostatic hyperplasia, 57 (90.5%) of 63 primary adenocarcinomas, and 7 (100%) of 7 prostate cancer metastases. Prostatic intraepithelial neoplasia showed a similar pattern of staining to that observed for the invasive tumors. Although prostate cell lines such as LNCap and C4-2 have no IGFBP-7 expression, the researchers found that tumor xenografts generated by direct intraosseous injection of LNCaP or C4-2 to bone marrow space resulted in tumors that stained positively for IGFBP-7, suggesting that the expression of IGFBP-7 can be induced in human prostate cancer cell lines *in vivo* on interaction with an appropriate host environment. In addition, Creighton et al. [6] also showed that IGFBP-7 was up-regulated in tumor xenografts generated by the implantation of human lung adenocarcinoma (A549) cells. In agreement with these results, we found that IGFBP-7 was highly overexpressed in glioma tissues. Our RT-PCR and Western blot assay data suggest that IGFBP-7 expression corresponds to tumor grade at both protein and mRNA levels. Together, these results suggest that IGFBP-7 plays different roles in different organs or host environments, a finding which is common in the IGFBP family. For instance, IGFBP-2 has also been ascribed properties of both suppressor and enhancer of cell proliferation. In small cell lung cancer cells, soluble IGFBP-2 inhibited IGF-dependent DNA synthesis [7]. In nontransformed IEC-6 cells, exogenous IGFBP-2 resulted in decreased proliferation [8], whereas expression of antisense IGFBP-2 resulted in growth stimulation [9]. In contrast, a positive correlation between IGFBP-2 level and cell proliferation has been observed in human colon carcinoma [10], in conjunction with a positive correlation between tumor grade and level of IGFBP-2 in colon [11], adrenal [12], prostate [13], and central nervous system tumors [14,15]. Similarly, IGFBP-5 has been associated with both the inhibition and stimulation of cell growth with variable cell–type specificity [16,17].

One of the biological attributes of glioma cell progress is the persistent formation of new vessels. A previous report of immunohistochemical staining of various tumor tissues indicated that IGFBP-7 seems to specifically accumulate in new blood vessels in various human cancer tissues including GBM specimens but not in those of normal tissues and capillary tube–like structures of cultured vascular endothelial cells [18]. A recently published paper showed similar findings, i.e., IGFBP-7 was exclusively associated with laminin-stained GBM vessels but was not observed in the vessels from nonmalignant brain [19]. These results indicate that IGFBP-7 may correlate with glioma cell progression. On the basis of the fact that IGFBP-7 is secreted protein, these observations and our data suggest that IGFBP-7 can promote glioma cell migration and growth along the newly formed tumor vessels.

Some research has suggested that IGFBP-7 has the ability to be involved in signal transduction in both IGF-dependent and IGF-independent pathways [20,21]. A previous study [22] showed that phosphorylated Erk and Akt were increased in the M12 and LNCaP transfected IGFBP-7 cells and that inhibition of PI-3 kinase results in a marked decrease in viability of the M12–IGFBP-7 cells compared to M12 controls, suggesting that IGFBP-7 could regulate prostate cancer cell through the PI3K/AKT pathway. Similarly, we showed the activation of AKT and ERK1/2 in IGFBP-7–treated glioma cells. Furthermore, we analyzed the impact of IGFBP-7/AKT on the glioma cell migration. Our data suggested that IGFBP-7 promoted glioma cell migration through down-regulating the signaling of AKT. Although previous studies indicated that AKT activation induces cancer cell invasion, recent studies that agree with our finding suggest that AKT1 can block the cell migration and invasion. Irie et al. [23] showed that down-regulation of AKT1 enhanced the migration of IGF1-receptor–overexpressing cells induced by EGF. In addition, Irie et al. [23] also found that enhanced ERK1/2 activation is necessary for the migration induced by AKT1 down-regulation and that down-regulation of AKT1 enhanced ERK1/2 activation. Our data showed that ERK1/2 activation increased with the addition of IGFBP-7 recombinant protein in medium up to 0.5 hours and then decreased lower than the basal level and then increased again after incubation for 8 hours. The activation of AKT was decreased whereas phosphorylation of ERK1/2 was enhanced. Together, it implies that IGFBP-7 may regulate glioma cell growth and tumorigenesis through signals that are regulated by the AKT/PKB pathway.

In conclusion, we demonstrate for the first time that expression of IGFBP-7 correlates with tumor (i.e., glioma) grade and overall GBM patient survival and that IGFBP-7 mediates glioma cell growth and migration through regulating AKT and ERK1/2 activation.

Acknowledgments

The authors thank Sandra Rempel for her invaluable discussion and comments. The authors acknowledge the support of the Glioma

Molecular Diagnostics Initiative, neuro-oncology branch NCI/NINDS for the microarray data.

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