Silencing of insulin-like growth factor binding protein-2 (IGFBP-2) in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24

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Running title: Role of CD24 in IGFBP-2-induced glioblastoma invasion.

SUMMARY

Glioblastoma multiforme (GBM) is a malignant brain tumor characterized by rapid growth and extensive invasiveness. Overexpression of insulin-like growth factor binding protein-2 (IGFBP-2) has been reported in GBM. However, it remains to be determined how IGFBP-2 is involved in the progression of GBM. We utilized short hairpin-RNA (shRNA) expression retroviral vectors to inactivate the IGFBP-2 gene permanently in two human GBM cell lines, U251 and YKG-1. The stable knockdown of IGFBP-2 resulted in decreased invasiveness, decreased saturation density of the cells in vitro, and decreased tumorigenicity in nude mice. Transcriptional profiling of both lines revealed several genes which were significantly down-regulated by inactivation of IGFBP-2. One such gene was CD24, which has been implicated in progression of various cancers. Indeed, CD24 was expressed in most GBM cases and the inactivation of CD24 in GBM cells suppressed cellular invasiveness, as was the case for IGFBP-2. Forced overexpression of CD24 led to increased invasiveness of both IGFBP-2-inactivated GBM cell lines and also A172, a human GBM cell line with low endogenous CD24. Further supporting the inter-relationship between IGFBP-2 and CD24, knockdown of IGFBP-2 suppressed the CD24 promoter activity. Moreover, both CD24 promoter activity and in vitro invasiveness were restored in knockdown cells by transfection with an IGFBP-2 expression plasmid. These results indicate that CD24 is modulated by IGFBP-2 and contributes to IGFBP-2-enhanced invasiveness of GBM cells.

INTRODUCTION

Gliomas are the most frequent type of brain tumor. Glioblastoma multiforme (GBM) is the most malignant form and is characterized by rapid growth, extensive invasiveness, and angiogenesis. Although extensive resection of the tumor may be achieved by surgery, recurrence is almost inevitable (1,2). Even with the latest updated combination of treatments, the prognosis of patients with GBM remains extremely poor and the 2-year survival rate is only 26% (1). Limitations to therapy include the infiltrative nature of GBM cells, their enhanced capacity for angiogenesis and their resistance to current modalities of irradiation, chemotherapy, and immunotherapy. Therefore, effective treatment of patients with GBM requires greater understanding of the biology of GBM cells and development of innovative clinical approaches.

Insulin-like growth factor binding proteins (IGFBPs) comprise a family of secreted proteins that modulates the bioavailability of insulin-like growth factors (IGFs) in the IGF-I/IGF-I receptor (IGF-IR) signaling axis (3). Six IGFBPs (designated IGFBP-1 ~ -6) have been identified. Although all IGFBPs have been shown to inhibit the IGF signaling axis, a number of IGFBPs also mediate IGF-independent actions, which could
be related to tumor progression (4). IGFBP-2 is a 36-kDa protein that is widely expressed in the fetus. After birth, its expression decreases significantly under normal conditions. Recently, elevated expression of IGFBP-2 has been reported in many tumors, such as prostate cancer (5), ovarian cancer (6), colon cancer (7), breast cancer (8), and glioma (9, 10). IGFBP-2 may play important roles in malignant progression. For example, in gliomas, expression of IGFBP-2 increases with tumor grade (10), and recent studies have suggested that IGFBP-2 enhances invasion by GBM cells by up-regulating invasion-enhancing proteins such as matrix metalloproteinase-2 (MMP-2) (11). However, it remains to be determined precisely how IGFBP-2 is involved in the growth and invasive behavior of tumor cells. Recent reports indicate that interaction between integrins and IGFBP-2 can have either positive or negative regulatory effects on the invasiveness of tumor cells (12, 13). Song et al. recently identified an IGFBP-2-binding protein, IIp45, an intracellular molecule which regulates IGFBP-2-induced invasiveness of GBM cells (14). IIp45 appears to antagonize the IGFBP-2-induced expression of transcriptional NF-κB (14).

CD24 is a glycoprotein that is anchored to the cell surface (15, 16). It has many potential sites for O-linked glycans, indicating that CD24 is a mucin-type protein. Although CD24 was initially identified as a B-cell specific marker, considerable attention has recently been focused on the roles of CD24 in tumor biology, in which CD24 appears to be involved in cell adhesion, invasion and metastasis (15, 16). Indeed, CD24 is expressed in a variety of tumors, such as ovarian cancer, breast cancer, lung cancer, prostate cancer, pancreatic cancer, and medulloblastoma, and a number of recent studies have indicated that high expression level of CD24 is significantly associated with a more aggressive course (17-24). Although little is known regarding the expression of CD24 in GBM, engineered overexpression of CD24 in C6 rat glioma cells resulted in enhanced invasiveness of the cells in vivo (25). However, there is little understanding of the mechanisms regulating CD24’s expression and its role in tumor progression.

This study examined the significance of IGFBP-2 in GBM in vitro and in vivo, and also elucidated the cellular pathway maintained by IGFBP-2 in human GBM cells. For this purpose, we established stable sublines of two different human GBM cell lines in which the IGFBP-2 gene was permanently inactivated by retroviral expression of short hairpin-RNA (shRNA). Using transcriptional profiling, we identified CD24 as an important candidate molecule potentially involved in IGFBP-2-induced invasion of GBM cells.

**EXPERIMENTAL PROCEDURES**

*Materials and cell culture - Specimens of four cases of GBM tissues were obtained at surgery. Informed consents were obtained from the patients and the protocol was approved by the Ethical Board of the Faculty of Medicine, University of Miyazaki. Nine human GBM cell lines (U251, YKG-1, T98G, A172, KS-1, U87, YH-13, U373, NYGM) and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5%CO₂. NYGM was established in our laboratory (26). U251, YKG-1, T98G, A172, and KS-1 were obtained from the RIKEN cell bank (Tsukuba, Japan). YH-13 was obtained from Health Science Research Resource Bank (Osaka, Japan). U87 and U373 were obtained from European Collection of Cell Culture via Dainippon Sumitomo Pharma (Osaka, Japan). Regarding p53 status, U251, YKG-1, T98G and U373 were mutant, while A172 and U87 have a wild-type p53 status. For the evaluation of in vitro cell growth, triplicated 60-mm dishes were seeded at 1 × 10⁵ cells/ml growth medium, and the number of viable cells was counted daily.

*Reverse-transcription polymerase-chain reaction (RT-PCR) and quantitative real-time RT-PCR - Total RNAs were extracted with Trizol™ reagent (Gibco BRL, Gaithersburg, MD), followed by DNase I (Roche Applied Science, Indianapolis, IN) treatment and phenol-chloroform-isoamylalcohol extraction. Total RNA of human whole brain was purchased from Clontech (Palo Alto, CA). For RT-PCR, 3 μg of total RNA was reverse-transcribed with a mixture of oligo dT and random primer using 200 units of SuperScript reverse transcriptase (Gibco BRL), and 1/30 of the resultant cDNA was processed for each PCR reaction with 0.1 μM of both reverse and forward primers and 2.5 U of HotStarTag DNA polymerase (Qiagen, Tokyo, Japan). The following primers were used: IGFBP-2, sense 5´-GGTATGAAGGAGCTGGCCGTGTTC-3´.
and antisense 5'-CGCTGCCGTTCAGAGACATCTTG-3'; IGF-I, sense 5'-TCAGAAGCAATGGGAAAAATCAGC-3' and antisense 5'-TCCTTAGACTACAGCTCCGGAAGC-3'; IGF-II, sense 5'-GCTTCCAGACACCAATGGGAATCC-3' and antisense 5'-TCATAATTGGAAGAACTTGCCCACG-3'; IGF-IR, sense 5'-ACTGTGGACTGGTCCCTGATCCTG-3' and antisense 5'-GGCACACAGACACCGGCATAGTAG-3'; CD24, sense 5'-GACATGGGCAGAGCAATGGTGGC-3' and antisense 5'-GAGTGAGACCACGAAGAGACTGGC-3'. The PCR products were analyzed by 2% agarose gel electrophoresis. For quantitative real-time RT-PCR for IGFBP-2 and CD24, PCR was performed in a LightCycler (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science) according to the manufacturer's instruction. The primer set for IGFBP-2 was purchased from Invitrogen (QuantiTect; Carlsbad, CA). For CD24, the above-described primers were used. For internal control, the level of β-actin mRNA measured using the following primers: sense 5'-ATTGCCGACAGGACATGGTGCC-3' and antisense 5'-GACATGGGCAGAGCAATGGTGGC-3'; and sense 5'-GAGTGAGACCACGAAGAGACTGGC-3' and antisense 5'-GGCACACAGACACCGGCATAGTAG-3'.

Detection of methylation - For methylation-specific PCR, 1 µg of genomic DNA was used for bisulfate modification using the EpiTect Bisulfite Kit (Qiagen). Bisulfite modified DNA was amplified with methylated IGFBP-2 5'-flanking region-specific primers 5'-ATAAGGTTCGCGGTTGCTC-3' (sense) and 5'-ATATCCCTCCGGTTGCTAGAC - 3' (anti-sense); unmethylated CD24 5'-flanking region-specific primers 5'-TGATAGGTGGTTGTTGTTTGTT -3' (sense) and 5'-ATATCCCCCTCATTCAATACACA-3' (anti-sense).

In study of the methyltransferase inhibitor, A172 and U87 cells were cultured in the presence of 10 µM 5-azacytidine (Sigma, St. Louis, MO) for 4 days and used for subsequent assays.

Construction of retroviral vectors and viral transduction into GBM cells - The knockdown vector was constructed by using a short hairpin-RNA (shRNA) expression retroviral vector pSINsi-hU6 (TAKARA Bio, Shiga, Japan). The most efficient target sequence for RNA interference was selected among six (IGFBP-2) or three (CD24) candidate sequences. The final selected target sequence for IGFBP-2 corresponded to coding region 905-925 (5'-ACTGTGACAAGCATGGCCTGT-3') and that for CD24 corresponded to coding region 446-466 (5'-GAGTCTTCATCGAATCTACTAA-3'). The following scrambled sequence of each target sequence was used as a control: IGFBP-2, 5'-ATCGCTAGGTCGGCGACATA T-3'; CD24, GGTACTGCTTAAA TCA TCTAC.

For retroviral expression of IGFBP-2 or CD24, human IGFBP-2 or CD24 cDNA was subcloned into pLNCX2 (Clontech), generating a retroviral expression vector pLNCX-IGFBP-2 or pLNCX-CD24. For infection of retroviral vectors, Amphotopack-293 packaging cells cultured in 60-mm dishes were incubated with 5 µg of recombinant retroviral vector and 10 µl of Transfectin (Bio-Rad, Hercules, CA) for 12 h. Subsequently, the cells were cultured in fresh DMEM, 10% FBS, for 48 h and the supernatant containing the retroviral particles was collected, filtered through a 0.45 µm filter, and used to infect target cells. Cultured GBM cells were tripinized, resuspended in the viral supernatant in the presence of polybrene (5 µg/mL), and incubated in a six-well plate at 3×10^5 cells/well for 12 h. Then the cells were incubated with a 1:1 mixture of fresh medium and viral supernatant with magnetofection reagent,
CombiMag (OZ Biosciences, Marseille, France), and placed on a magnetic plate for an additional 12 h. This process was repeated 3 times. The transfected cells were subcultured at an appropriate density in fresh DMEM containing 0.5 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The G418-resistant cell pools were readily established within 2 weeks.

**Re-expression of IGFBP-2 or CD24 in IGFBP-2-knockdown GBM cells** - For reversion of IGFBP-2 expression in IGFBP-2-knockdown cells, IGFBP-2 cDNA was subcloned into pcDNA3.1 (Invitrogen), generating the expression plasmid vector pcDNA-IGFBP-2. For the CD24 expression plasmid, CD24 cDNA was subcloned into pCIneo vector (Promega, Madison, WI), generating pCIneo-CD24. Transient or stable transfection was performed with TransFectin and CombiMag according to the manufacturer’s instruction.

**IGF treatment and immunoblot analysis** – Cultured cells at 60% confluency were maintained for 12 h in serum-free DMEM containing 0.1% bovine serum albumin (BSA), and then treated with recombinant human IGF-I or IGF-II (R & D systems, Minneapolis, MN) for 15 min, followed by washing in PBS. Then the cellular proteins were extracted on ice in cell lysis buffer (CelLytic™-M; Sigma) supplemented with protease inhibitor and phosphatase inhibitor cocktails (Sigma). After centrifugation (16,000 g × 10 min), equal amounts of proteins were electrophoresed by standard SDS-PAGE under reducing conditions, and transferred onto Immobilon (Millipore, Bedford, MA). After blocking with 5% phosphatase-free BSA in Tris buffered saline (TBS) with 0.05% Tween 20 (TBS-T), the membrane was incubated with primary antibody (see below) at 4°C overnight, followed by washing in TBS-T and incubation with a horseradish peroxidase-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark) or goat anti-mouse IgG (Bio-Rad) diluted in TBS-T with 1% BSA for 1 h at room temperature. The labeled proteins were visualized with a chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). The following primary antibodies were used: anti-human IGFBP-2 mouse monoclonal antibody (C-10; SantaCruz Biotechnology, Santa Cruz, CA), anti-β-actin mouse monoclonal antibody (AC-74; Sigma), anti-phospho-Erk1/2 rabbit monoclonal antibody (Thr185/Tyr187, clone AW39; Upstate Cell Signaling Solutions, Lake Placid, NY), anti-phospho-IGF-IR rabbit polyclonal antibody (Tyr1131), anti-IGF-IR β rabbit polyclonal antibody, anti-phospho-Akt mouse monoclonal antibody (Ser473), anti-Akt rabbit polyclonal antibody, anti-phospho-Erk1/2 rabbit monoclonal antibody (Thr185/Tyr187, clone AW39; Upstate Cell Signaling Solutions, Lake Placid, NY), anti-Erk1/2 rabbit polyclonal antibody, anti-caspase-3 and cleaved capase-3 (Asp175) rabbit polyclonal antibodies, and anti-heat shock protein 70 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA).

**Monolayer wounding assay and invasion assay** - For evaluation of in vitro motility of GBM cells, a monolayer wounding (scratch) assay was performed. Cells were allowed to form a monolayer on a culture dish surface, and a wound was made by scratching the monolayer with a pipette tip. After the scratched cells were removed, the cells were cultivated for a further 12 h. Photographs of the wound were taken at various time points after wounding.

Matrigel invasion assay was performed for the evaluation of invasive capability in vitro, by using Chemotaxicell containing an 8-µm pore size polycarbonate filter (Kurabo, Osaka, Japan) coated with 25 µg/well of Matrigel (Gibco BRL). As a chemoattractant, 1% FBS was added into the lower component. The transfected cells (1 or 5×10⁵ cells /100 µl DMEM, 0.1% BSA) were placed in the upper compartment and incubated for 48 or 72 hours. After incubation, the cells on the upper surface of the filter were wiped off with a cotton swab, then the cells on the lower surface were stained with hematoxylin. Invasion activity was quantified by counting the cells in 10 randomly selected fields (200-fold original magnification).

**Intracranial transplantation of GBM cells** - All animal work was carried out under protocols approved by the University of Miyazaki Animal Research Committee, in accordance with international guiding principles for biomedical research involving animals. For intracranial transplantation, GBM cells (1×10⁶ cells/10 µl DMEM) were stereotactically transplanted into the forebrain of six-week-old male nude mice (BALB/cAxC1-nu) as described previously (27).
Neurological defects and emaciation of the mice were carefully observed every day. Eight weeks after transplantation, the brain specimens were prepared after euthanasia. The brain tissues were fixed in 4% formaldehyde in PBS and sectioned coronally at the point of cellular implantation followed by embedding in paraffin. For immunostaining of IGFBP-2, rabbit polyclonal antibody to human IGFBP-2 (GroPep, Thebarton, Australia) was used according to the manufacturer’s instruction.

**Microarray analysis** - Five µg of total RNA extracted from IGFBP-2-knockdown GBM cells and control cells were reverse-transcribed with the T7-oligo(dT)24 primer followed by 2nd strand cDNA synthesis using a Super Script Choice System Kit (Invitrogen). The converted cDNA was repurified by phenol-chloroform-isooamyl alcohol and quality checked by electrophoresis. Biotinylated hybridization targets were prepared using the second strand cDNA with ENZO Bio Transcript Labeling Kit (Enzo Life Sciences, New York, NY), repurified, then hybridized to HG-U133A and HG-U133B Genechips (Affymetrix, Santa Clara, CA), and the expression data were analyzed with the Affimetrix Microarray Analysis Suit, version 5.0.

**Luciferase assay** - The 5’-flanking promoter region of the CD24 gene which showed the highest luciferase activity (28) was subcloned into a luciferase reporter vector, pGL3-Basic (Promega). IGFBP-2 knock-down cells were seeded at 1.0×10^4 cells/well in a 24-well plate in DMEM, 10% FBS. Transient transfection was performed with the reporter plasmid using TransFectin, and pRL-TK was co-transfected for inner control for normalizing transfection efficiency. After 48-h incubation, the cells were lysed and analyzed by a dual-luciferase reporter gene assay system (Promega).

**Statistical analysis** - Data were analyzed with the Statview 4.0 program (Brainpower, Calabasas, CA) and the results were expressed as the mean ± standard deviation (SD). Comparison between unpaired two groups was done with one-way ANOVA or Mann-Whitney U tests. Significance was set at P < 0.05.

**RESULTS**

**Expression of IGFBP-2 and related genes in human GBM.**

We first examined the expression of IGF-I, -II, their receptors, and IGFBP-2 in surgically resected human GBM tissues (4 cases) and 9 GBM cell lines, namely U251, YKG-1, T98G, A172, KS-1, U87, YH-13, U373 and NYGM. In addition, cDNAs derived from normal brain tissue and HeLa cells were also analyzed. As shown in Fig. 1a, all primary tissues derived from GBM as well as normal brain expressed IGFs and their receptors. Similarly, most of the cell lines expressed abundant mRNAs for IGF receptors (IGF-IR and IGF-IIIR). In contrast, IGF-1 mRNA was very low in cell lines. The expression of IGFBP-2 was observed in all brain tissues, but the level was apparently higher in GBM than in normal brain tissue. IGFBP-2 message was readily detected in 7 out of 9 human GBM cell lines (Fig. 1a) but expression was scarcely observed in HeLa cells.

**Establishment of GBM cells with silencing of IGFBP-2 gene.**

To examine the role of IGFBP-2 in GBM cell function, we first used shRNA directed against IGFBP-2 mRNA. Initially, we constructed six retroviral vectors each of which harbored distinct shRNA sequence for the IGFBP-2 gene. One of these sequences showed stable and significant silencing effects on IGFBP-2 genes of two different human GBM cell lines, U251 and YKG-1, after the infection of retroviral vector (Fig. 1b). The effect of stable shRNA expression was further confirmed by a quantitative real-time RT-PCR analysis for IGFBP-2 mRNA. As shown in Fig. 1c, we observed a 95% or 65% reduction in the IGFBP-2 mRNA level in U251 or YKG-1 cells, respectively. IGFBP-2 shRNA also reduced the level of IGFBP-2 protein (Fig. 1d). No decrease in the expression of IGFBP-2 was observed with a scrambled control shRNA. We therefore undertook further analyses of the roles of IGFBP-2 in GBM.

The basal phosphorylation level of IGF-IR was not altered by the silencing of IGFBP-2, as it was very low in both knockdown and control cells (Fig. 2). On the other hand, IGF-II-induced phosphorylation of IGF-IR was enhanced by the knockdown of IGFBP-2 (Fig. 2). This observation is consistent with the notion that IGFBP-2 normally suppresses the binding of IGF-II to IGF-IR (29). Hence, the results indicated that our attempt at silencing the IGFBP-2 gene did indeed disturb a function of the IGFBP-2 protein. In contrast, IGF-1-induced
phosphorylation of IGF-IR was not significantly altered by the knockdown of IGFBP-2 (Fig. 2). Consequently, although IGF treatment resulted in significantly enhanced phosphorylation (3 fold) of Akt, the knockdown of IGFBP-2 did not affect the IGF-induced phosphorylation of Akt. On the other hand, Erk phosphorylation was not significantly altered by the IGF treatment. In addition, consistent relationship between the expression level of IGFBP-2 and those of other IGFBPs (IGFBP-1, 3, 4, 5, 6) was not observed (Supplemental Fig. 1). These results suggested that phosphatidylinositol 3-kinase-Akt system may be a main downstream network of IGF-IR in these cell lines and IGFBP-2 was not regulating IGF pathway function significantly.

**Significant alteration of culture morphology follows silencing of IGFBP-2.**

Interestingly, potent long-term silencing of IGFBP-2 resulted in marked alterations of culture morphology. As shown in Fig. 3a, the IGFBP-2-knockdown GBM cells showed broad flattened lamellipodia compared with control cells in both U251 and YKG-1 cell lines. At confluency, the IGFBP-2-knockdown cells showed flattened and less overlapped morphology (Fig. 3b). This morphological alteration at the confluent culture resulted in significantly suppressed saturation density of the cells (Fig. 3c). The decreased cell numbers was not caused by the increased apoptosis (Supplemental Figure 2). The *in vitro* growth rate at the log phase of growth was also decreased by IGFBP-2 knockdown, particularly in YKG-1 (Fig. 3c). In high cell density, Erk phosphorylation was significantly suppressed in IGFBP-2-knockdown (Fig. 3c). Phosphorylation of Akt was not altered by IGFBP-2 knockdown in both cell lines.

**Decreased cellular invasiveness and tumorigenicity by stable knockdown of IGFBP-2.**

Next, we analyzed the effect of IGFBP-2 knockdown on *in vitro* invasiveness of GBM cells. Since cellular motility is a prerequisite for invasion, we carried out a cell monolayer scratch assay. In U251 cells, knockdown of IGFBP-2 resulted in decreased cell migration. Healing of the wounded monolayer was delayed and cellular morphology was altered (Fig. 4a). However, viability of the cells was not affected (data not shown). As the significant difference was observed 12 h after the plating, it is reasonable to postulate that the difference was caused by the decreased migratory activity of cells rather than decreased growth rate. Similar, though less prominent findings, were also observed in YKG-1 cells (Fig. 4a). We then performed an invasion assay using Matrigel, a solid gel of mouse basement membrane. Again, the knockdown of IGFBP-2 resulted in a significant reduction of the invasion through Matrigel in both GBM cell lines (Fig. 4b).

To further support the role of IGFBP-2 in the invasive growth of GBM, *in vivo* tumorigenesis was analyzed using an intracranial implantation model in nude mice. This was carried out with U251, since this line was quite susceptible to IGFBP-2 gene silencing by RNA interference. As shown in Fig. 5, while control U251 cells formed tumors in 11 out of 13 implanted mice (84.6%), the stable IGFBP-2 knockdown cells showed a tumorigenicity rate of only 7.7% (1/13). Consequently, knockdown of IGFBP-2 resulted in significantly improved survival (Fig. 5a). The *in vivo* expression of IGFBP-2 in control U251 cells was confirmed by an immunohistochemical analysis (Fig. 5b).

**Microarray analysis of gene expression modified by stable knockdown of IGFBP-2.**

In order to clarify possible downstream molecular targets of IGFBP-2 in GBM, we compared expression profiles of IGFBP-2-knockdown GBM cells with those from control cells. In both U251- and YKG-1-knockdown lines, 23 known genes (out of 31 probes) were significantly down-regulated (less than 0.5 fold relative to control cells) (Table 1). On the other hand, 25 known genes were significantly up-regulated (more than 2 fold) in both cell lines (Table 2). This microarray analysis also showed that IGFBP-2 shRNA reduced IGFBP-2 expression 0.05-fold and 0.29-fold in U251 and YKG-1, respectively, very similar to results obtained by real-time RT-PCR analysis (Fig. 6a). Among the 23 genes suppressed by IGFBP-2 knockdown, CD24 was remarkable: significant suppression was consistently observed with 5 different probes in both GBM cell lines (Table 1). Indeed, CD 24 has been implicated in tumorigenesis and metastatic progression of a variety of tumors (15, 16). Correlation of IGFBP-2 expression and CD24 expression was further confirmed by real-time RT-PCR (Fig. 6a). We also demonstrated expression of CD24 in human GBM tissues and cell lines (Fig 6b). All GBM tissues expressed notable levels of CD24. Six
out of nine GBM cell lines showed concomitant expression of IGFBP-2 and CD24. However, expression of CD24 was very low in both KS-1 and U87. In this regard, we found that the CD24 promoter region was hypermethylated in both cell lines (Fig 6c). In A172 and U87 cell lines, IGFBP-2 was hardly detectable, and methylation of IGFBP-2 promoter was observed. Although apparent methylation of CD24 promoter was not observed with this methylation-specific PCR in A172 cells, the treatment of the cells with DNA demethylation reagent, 5-azacytidine, enhanced the transcription of IGFBP-2 and CD24 by 2.1- and 1.4-fold, respectively, as judged by real-time RT-PCR.

Among the up-regulated genes in response to IGFBP-2 knockdown (Table 2), DOC1 (downregulated in ovarian cancer 1) and inhibin alpha chain may be involved in the decreased cellular growth of the knockdown GBM cells, as DOC1 is down-regulated in cancer cells and is expressed in senescent epithelial cell (30) and inhibin alpha subunit is a known tumor suppressor (31). The whole raw data of microarray analyses are available at CIBEX (Center for Information Biology Gene Expression Database; URL: http://cibex.nig.ac.jp). The accession numbers are CBX17 (for U251) and CBX18 (for YKG-1).

**Role of IGFBP-2 on the expression of CD24.**

The results of microarray analysis suggested that CD24 might be one of the downstream targets of IGFBP-2. To gather further support for this hypothesis, we performed dual luciferase assays using the promoter region of the CD24 gene (28). Indeed, the promoter activity of CD24 was suppressed by the knockdown of IGFBP-2 in both U251 and YKG-1, and the suppression was more evident in U251 which showed greater silencing of the IGFBP-2 gene by RNA interference (Fig. 7a). Importantly, re-expression of IGFBP-2 by transient transfection with IGFBP-2-expression plasmid (pcDNA-IGFBP2) in knockdown U251 cells resulted in gain of CD24 promoter activity, along with return of the invasive capability of the cells in vitro (Fig.7b). Similar results were also observed in YKG-1 cells (data not shown). These results indicated that IGFBP-2 positively regulated the expression of CD24 in U251 and YKG-1 cells.

In order to confirm the link between IGFBP-2 and CD24, we examined the effect of forced IGFBP-2 expression on two GBM cell lines (A172 and U87) lacking endogenous IGFBP-2 expression. Transient transfection of pcDNA-IGFBP-2 in A172 cells resulted in transient re-expression of IGFBP-2 by 48 to 72 h, followed by increased CD24 mRNA level at 96 h after the transfection (data not shown). Stable overexpression of IGFBP-2 in A172 cells also induced significantly enhanced expression of CD24 in this cell line (Fig. 8c). In U87, forced expression of IGFBP-2 induced CD24 expression in the presence of 5-azacytidine (supplemental Fig. 3), in accordance with the observation that CD24 promoter region of this cell line was hypermethylated (Fig. 6c).

**Role of CD24 in invasive capability of GBM cells.**

To further test the role of CD24 in IGFBP-2-induced invasion of GBM cells, we analyzed the effect of forced overexpression of CD24 on IGFBP-2-knockdown GBM cells. As described above, and also as shown in Fig. 5, the silencing of IGFBP-2 by shRNA resulted in a significant reduction of CD24 expression in GBM cells, accompanying reduced invasiveness. However, the engineered re-expression of CD24 in GBM cells by transfection of plasmid vector (pClneo-CD24) restored, at least partly, the reduced invasiveness caused by knockdown of IGFBP-2 may be mediated, at least partly, by the down-regulation of CD24.

Finally, we attempted to knockdown CD24 with shRNA. As expected, the knockdown of CD24 significantly suppressed the invasion of U251 and YKG-1 (Fig. 8b) as was observed in the case of IGFBP-2. Furthermore, engineered overexpression of CD24 enhanced the invasion of A172, which otherwise showed a very low basal level of expression for IGFBP-2 and CD24 as well as low invasive capability (Fig. 8b).

**DISCUSSION**

In the present study, we analyzed the roles of IGFBP-2 in human GBM cells by means of gene silencing techniques. Initially, we constructed six retroviral vectors each of which harbored a distinct shRNA sequence for the IGFBP-2 gene. One of these sequences showed stable and significant silencing effects on two different human GBM cell lines, U251 and YKG-1, both of which expressed endogenous IGFBP-2 abundantly. That outcome permitted us to perform further analyses of the biological roles of IGFBP-2 in GBM cells in vitro and also in

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vivo. The silencing of IGFBP-2 significantly reduced cellular migration and invasive capabilities of GBM cells. This observation appears to be in accordance with a previous study by Wang et al., which showed that overexpression of IGFBP-2 led to increased invasion of GBM (11). Our study also showed that IGFBP-2 knockdown led to a significant alteration of cellular morphology and reduced saturation density of cells in vitro. Furthermore, in U251-IGFBP-2-knockdown cells (in which IGFBP-2 expression was 95% reduced) in vivo tumorigenic potential was significantly attenuated. Collectively, these results suggested that IGFBP-2 might be critically involved in pathways regulating the invasive growth of GBM cells.

To identify the molecules related to IGFBP-2-induced signaling, recent studies have utilized real-time RT-PCR analyses of patients’ samples or gene expression profiling of GBM cells undergoing forced expression of IGFBP-2 (11, 32). The data indicated that expression of IGFBP-2 was correlated with expression of invasive growth-associated genes in GBM such as MMP-2, VEGF, fibronectin, thrombospondin, integrin α5 and α6 (11, 32). In the present study, gene silencing was combined with gene expression profiling. The experiments identified CD24 as another gene which appears to be regulated by IGFBP-2 in certain GBM cells. We offer the following evidence that CD24 is key to IGFBP-2-mediated invasion by GBM cells. First, CD24 expression was significantly down-regulated in two GBM cell lines (U251 and YKG-1) in which IGFBP-2 was knocked down. The decrease in expression was proportional to the degree to which IGFBP-2 was silenced. Second, forced expression of IGFBP-2 stimulated promoter activity of CD24. Third, reversion of CD24 expression by transfection of a CD24 expression plasmid in IGFBP-2-knockdown GBM cells resulted in the regain of invasive capability. Fourth, silencing of CD24 in GBM cell lines resulted in decreased invasiveness. Finally, all four GBM tissues expressed both IGFBP-2 and CD24 mRNAs, and six out of nine human GBM cell lines also expressed both genes.

In four GBM cell lines (A172, KS-1, U87 and U373), CD24 was scarcely expressed, and in A172 and U87, expression of IGFBP-2 was also lacking. It should be noted that A172 has poor invasive capacity compared with U251 and YKG-1 as judged by Matrigel assay. We previously showed that the tumorigenicity of KS-1 is also very low (33). Regarding the silencing of the CD24 gene in KS-1, we observed hypermethylation of the promoter region of CD24. On the other hand, in A172 cells, promoter methylation appears to be present in the IGFBP-2 gene. U87 showed hypermethylation in both genes. In A172, we observed induction of CD24 after re-expression of IGFBP-2, and the forced expression of CD24 in A172 cells resulted in significantly enhanced invasiveness. Interestingly, A172 and U87 have a wild-type p53 status, while U251 and YKG-1 are p53 mutants (34). Since another GBM cell line with high IGFBP-2 and CD24 expression, T98G, is also p53 mutant (34), the p53 status of a cell may have implications for a CD24-mediated IGFBP-2 invasiveness pathway.

CD24 is a heavily glycosylated, cell-surface, glycosylphosphatidylinositol anchored protein possibly having a role in B-cell maturation (35). In addition, CD24 may also be involved in the cell-cell interactions as a ligand for P-selectin (36). Importantly, CD24 is expressed in a variety of tumors including lung, breast, ovary, prostate, pancreas, and colorectal cancers. It is recognized as a prognostic marker and may be involved in tumorigenesis, invasion and metastasis of cancer cells (15-23). In brain tumors, high expression of CD24 has been reported in medulloblastoma, the most common malignant brain tumor of childhood (24). Moreover, engineered expression of CD24 in a C6 rat glioma cell line stimulated the invasiveness in vivo (25). All these lines of evidence suggest that CD24 is involved in progression and aggressiveness of tumors, though the mechanism underlying the CD24-induced invasiveness is poorly understood. This study indicates that human GBM cells also express CD24 and it is involved in the invasive phenotype of GBM cells as the silencing of the CD24 gene significantly reduced the invasiveness of the cells.

An important question then is how IGFBP-2 regulates CD24 expression. At present, the signaling pathway linking IGFBP-2 and CD24 remains to be clarified. It is generally accepted that IGFBP-2 negatively modulates IGF-induced signaling by binding IGFs, particularly IGF-II, and thereby inhibits IGF-mediated proliferation. In fact, we observed that the silencing of IGFBP-2 resulted in enhanced phosphorylation of IGF-IR following addition of IGF-II. However, in spite of the theoretically negative effect of IGFBP-2 on the IGF/IGF-R signaling...
axis implicated in tumor progression (3), this study clearly shows that knockdown of IGFBP-2 suppresses invasiveness and proliferation of GBM cells. Together with the previous reports that showed IGFBP-2-induced tumorigenic potential and mitogenesis in cancer cells, including GBM cells (11, 13, 37-42), IGFBP-2 proteins must have multifaceted functions that may be mediated by different signaling pathways. In this regard, recent reports suggest that IGFBP-2’s RGD region may interact with integrins and modulate outside-in signaling in GBM cells (12, 13). In support of that hypothesis, we observed that the silencing of IGFBP-2 induced considerable alteration of culture morphology. Notably, CD24 may also modulate integrin function as shown in leukocytes adhesion studies (43, 44), and thus, may also contribute to the IGFBP-2-mediated modulation of the interaction between GBM cells and the extracellular matrix.

As a potential regulatory molecule involved in IGFBP-2-induced signaling, Song et al. have identified an IGFBP-2 binding protein, Ilp45, which serves as a negative regulator of IGFBP-2-induced invasion by GBM cells (14). Ilp45 interacts with IGFBP-2 through the RGD region of IGFBP-2 and the binding eventually inhibits the expression of transcription factor NF-κB (14). This in turn suggests that IGFBP-2-induced signals might activate CD24 expression via NF-κB, as the promoter region of CD24 contains a possible NF-κB binding site as judged by TRANSFAC 7.0 public database. In this regard, we observed a modest decrease in the expression of NF-κB subunits in IGFBP-2 knockdown cells by DNA microarray analysis (data not shown), and this possibility is currently under investigation in our laboratory. As other possible regulators of CD24 expression, Ras-related small GTPases, RalA/B, were recently identified in urinary bladder carcinoma cells (45). Although, we did not observe apparent alterations of RalA/B expression levels in IGFBP-2 knockdown GBM cells (data not shown), further studies for a possible link between IGFBP-2- and RalA/B functions will be required.

In summary, silencing of the IGFBP-2 gene suppressed the invasive growth of GBM cells both in vitro and in vivo, indicating that IGFBP-2 is important in maintaining the malignant phenotype of GBM cells. CD24 may be one of the downstream molecules regulated by IGFBP-2-induced signaling, and may have a critical role in IGFBP-2-induced invasion by certain GBM cells. As such, IGFBP-2, CD24, and the signaling pathway(s) linking IGFBP-2 and CD24, may be potential targets to develop novel therapeutic strategies for the control of invasive growth of GBM cell. On the other hand, the cultured GBM cell lines in vitro may not demonstrate the molecular mechanisms that function in GBM in vivo in respect to the IGFBP-2-induced modulation of IGF/IGF-R signaling, as none of GBM cell lines used in this study expressed notable amounts of endogenous IGF-I. Therefore, further studies to test the relationship between IGFBP-2 and CD24 will be required by using clinical GBM specimens.

REFERENCES
10. Elmlinger, M. W., Deininger, M. H., Schuett, B. S., Meyermann, R., Duffner, F., Grote, E. H., and
10


**FOOTNOTES**

This work was supported in part by Grant-in-Aid for Scientific Research (B) No.17390116 and 21st Century COE program (Life Science) from the Ministry of Education, Science, Sports and Culture, Japan, and a grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare (15-13).

We are grateful to Ms. Tobayashi and Ms. Nomura for their skillful technical assistance.

The abbreviations used are: GBM: glioblastoma multiforme; IGFBP-2: insulin-like growth factor binding protein-2; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; IGF-IR: insulin-like growth factor-I receptor; shRNA: short hairpin-RNA; DMEM: Dulbecco’s modified Eagle’s medium; RT-PCR: reverse-transcription polymerase chain reaction; FBS: fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; BSA: bovine serum albumin; TBS: Tris buffered saline
FIGURE LEGENDS

Fig. 1. (A) Expression of IGFBP-2 and related molecules in human GBM. Results of RT-PCR (32 cycles of amplification) is shown and GAPDH mRNA was used as an internal control (30 cycles). All GBM cases and GBM cell lines except for A172 and U87 expressed IGFBP-2. (B) Effect of stable retroviral expression of IGFBP-2 shRNA on IGFBP-2 mRNA levels of U251 and YKG-1 cells. Representative results of RT-PCR with different amplification cycles are shown. control, cells expressing scrambled shRNA; IGFBP-2 KD, IGFBP-2-knockdown cells. (C) Quantitative real-time RT-PCR analysis for the expression of IGFBP-2 in knockdown cells (KD) and control cells (cont.). (D) Effect of IGFBP-2 shRNA on the level of IGFBP-2 protein. Significant reduction of IGFBP-2 protein level was achieved by infection of IGFBP-2-shRNA retroviral vector. cont., control cells; KD, IGFBP-2-knockdown cells.

Fig. 2. Modulation of IGF/IGF-IR signaling caused by knockdown of IGFBP-2. The IGFBP-2-knockdown cells (IGFBP-2 KD) and control cells were treated with indicated concentration of IGF-I or IGF-II in serum-free condition, and extents of phosphorylation of IGF-IR (p-IGF-IR), Akt (p-Akt) and Erk1/2 (p-Erk1/2) were analyzed by immunoblot and a representative result of U251 was shown. The band intensity was measured and the ratio of phosphorylated protein to the corresponding total protein was calculated.

Fig. 3. Effects of stable IGFBP-2 knockdown on culture characteristics of GBM cells. (A and B) Cellular morphology of IGFBP-2 knockdown-GBM cells (IGFBP-2 KD) of low density (A) and high density (B) are shown. Stable silencing of IGFBP-2 gene resulted in flattened cellular shape compared with control cells. (C) Effects of stable IGFBP-2 knockdown on in vitro growth. Cells were cultured in DMEM with 10% FBS, and growth curves were established. The knockdown cells (IGFBP-2 KD) showed decreased saturation density relative to control cells. Bars are mean ± SD. *p < 0.05. Phosphorylation levels of Erk and Akt at high cell density were also shown. Erk phosphorylation was suppressed by IGFBP-2 knockdown, while Akt phosphorylation was not altered. The total levels of Akt protein were also unchanged (not shown).

Fig. 4. Effects of stable IGFBP-2 knockdown on cellular motility, invasion, and tumorigenicity of GBM. (A) Monolayer wounding assay. IGFBP-2-knockdown (IGFBP-2 KD) U251 cells showed significantly reduced motility with alteration of cellular shape compared with control cells with scrambled shRNA (control). Similar, but less evident effects were observed in YKG-1 cells. (B) Effect of IGFBP-2 knockdown on Matrigel invasion. IGFBP-2 knockdown resulted in significantly reduced Matrigel invasion of GBM cells. 1 × 10^5 cells were placed in the upper compartment. cont., control cells; KD, IGFBP-2-knockdown cells.

Fig. 5. Effect of stable IGFBP-2 knockdown on tumorigenicity in intracranial transplantation model in nude mice. (A) Knockdown of IGFBP-2 resulted in improved survival. Forty percent (5/13) of mice with control U251 cells died (indicated as *) and 5 out of 8 remaining mice showed decreasing body weight in the last 2 weeks, indicating 77% of the control mice were symptomatic. None of the mice with IGFBP-2-knockdown U251 cells showed notable weight loss. (B) The long-term knockdown of IGFBP-2 suppressed the tumorigenicity of U251 cells. In tissue sections, the expression of endogenous IGFBP-2 was confirmed in control U251 cells immunohistochemically. On the other hand, viable tumor cells were hardly visible in most cases of the transplantation of knockdown cells.

Fig. 6. (A) Quantitative real-time RT-PCR for IGFBP-2 and CD24 mRNAs. The stable knockdown of IGFBP-2 resulted in simultaneous reduction of CD24 mRNA level. (B) RT-PCR for the expression of CD24 (30 cycles) in GBM tissues and cell lines. CD24 mRNA was identified in all GBM tissues and concomitant expression of IGFBP-2 and CD24 was observed in 6 out of 9 GBM cell lines. The data of GAPDH (30 cycles) is also shown. (C) Methylation specific PCR for CD24 and IGFBP-2. The promoter region of CD24 was hypermethylated in KS-1 and U87 cells, both of which did not express CD24 mRNA. Methylation of the IGFBP-2 promoter was suggested in A172 and U87.
Fig. 7. (A) Effect of IGFBP-2 knockdown on the transcriptional activity of CD24. Constructs for the luciferase assay are indicated in the upper panel, and possible binding sites for transcription factors assessed by TRANSFAC 7.0 public database are indicated. The knockdown of IGFBP-2 resulted in reduced promoter activity of CD24. (B) Engineered re-expression of IGFBP-2 in IGFBP-2-knockdown (IGFBP-2 KD) U251 cells. Transient overexpression of IGFBP-2 in IGFBP-2 knockdown cells resulted in reversion of CD24 promoter activity (upper figures). Invasive capability was also restored as judged by Matrigel assay (lower figures). $1 \times 10^5$ cells were placed on the filter and representative photos of migrated cells of mock-transfected (+ pcDNA mock) and IGFBP-2-transfected (+ pcDNA IGFBP-2) IGFBP-2 KD U251 cells are shown. (C) Effect of stable overexpression of IGFBP-2 on CD24. A172 cells were stably transfected with pcDNA-IGFBP-2 (IGFBP-2), and the expression of CD24 was compared with non-treated (parent) and mock-transfected (mock) cells.

Fig. 8. (A) Reversion of invasive capability of IGFBP-2 knockdown (IGFBP-2 KD) cells by engineered overexpression of CD24. Transient transfection with CD24 expression plasmid vector in stable IGFBP-2-knockdown GBM cells showed reversion of CD24 expression (upper panel, RT-PCR analysis; 30 cycles of amplification) and regain of invasive capabilities (lower panel, Matrigel invasion assay). Photos of representative results of invasion assay are also shown. (B) Effect of stable knockdown (U251 and YKG-1) or stable overexpression (A172) of CD24 on Matrigel invasion of GBM cells. $1 \times 10^5$ cells (U251 and YKG-1) or $5 \times 10^5$ cells (A172) were placed in the upper compartment. RT-PCR analyses for the level of CD24 expressions (30 cycles) were also indicated. Silencing of CD24 gene suppressed Matrigel invasion of GBM cells in U251 and YKG-1 cells. Although A172 cells showed low invasive activity through Matrigel with low CD24 level, overexpression of CD24 in A172 resulted in enhanced invasion. cont., control.
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A control IGFBP-2 KD

B control IGFBP-2 KD

C U251 YKG-1

Days in culture

Cell number (X 10^5)

9th day

10th day

p-Akt p-Erk total Erk

IGFBP-2 KD

control KD

control KD

*
Fig. 4

A

U251

control

IGFBP-2 KD

YKG-1

control

IGFBP-2 KD

B

U251

cont.

KD

Number of cells

YKG-1

cont.

KD

Number of cells

p < 0.05

p < 0.05
Fig. 5

A. Body weight (% of initial weight) of control (n=13) and IGFBP-2 KD (n=13) groups over 8 weeks after implantation.

B. Histological images of U251 control and U251 IGFBP-2 KD groups showing differences in tumor formation.

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A  IGFBP-2 mRNA levels

B  CD24 mRNA levels

C  IGFBP-2 mRNA levels

M: methylated primers
U: unmethylated primers

Fukushima et al., Fig. 6
Fukushima et al., Fig. 7

A

B

C

IGFBP-2 KD + pcDNA-IGFBP2

U251

YKG-1

IGFBP-2 KD

IGFBP-2 KD + pcDNA mock

IGFBP-2 KD + pcDNA IGFBP-2

control U251 cells

IGFBP-2 mRNA

luciferase activity

(control to β-actin)

number of cells / HPF

A172

IGFBP-2
CD24
GAPDH

289 bp
222 bp
300 bp
Fukushima et al., Fig. 8

A

U251 YKG1

CD24 IGFBP-2 GAPDH

222 bp 289 bp 300 bp

1 2 3 1 2 3
control + pCIneo mock IGFBP-2 KD + pCIneo mock IGFBP-2 KD + pCIneo-CD24

U251

YKG-1

Number of cells / HPF

1 2 3 1 2 3
control IGFBP-2 KD IGFBP-2 KD + mock IGFBP-2 KD + pCIneo-CD24

B

U251 YKG-1 A172

CD24 IGFBP-2 GAPDH

222 bp 222 bp

cont. KD cont. KD

cont. CD24 KD cont. CD24 KD mock pLNCX-CD24

Number of cells / HPF

cont. CD24 KD cont. CD24 KD mock CD24

p < 0.001 p < 0.001

p < 0.001 p < 0.001 p < 0.01 p < 0.001

p < 0.01 p < 0.01

p < 0.001 p < 0.01
Silencing of insulin-like growth factor binding protein-2 (IGFBP-2) in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24

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J. Biol. Chem. published online May 1, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M609567200

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