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Insulin-like Growth Factor Binding Protein 2 Enhances Glioblastoma Invasion by Activating Invasion-enhancing Genes[1]

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Abstract

Comparison of gene expressing profiles between gliomas with different grades revealed frequent overexpression of insulin-like growth factor binding protein 2 (IGFBP2) in glioblastoma (GBM), the most advanced stage of glioma. To determine whether IGFBP2 is involved in the proliferative and invasive nature of GBM, we established stable SNB19 GBM cell lines that overexpress IGFBP2. Although there was no marked difference in the cell growth between IGFBP2 overexpressing SNB19(BP2) lines when compared with the control cells, these clones showed significantly increased invasive rates when compared with the parental or vector transfected SNB19 cells. Total RNAs from controls and SNB19(BP2) clones were used for microarray analysis to detect IGFBP2-mediated alterations in gene expression. When compared with parental or vector-transfected control cells, SNB19(BP2) cells consistently showed 3–5-fold increase in the expression of matrix metalloproteinase-2 (MMP-2) as well as other invasion related genes. Increased MMP-2 expression in SNB19(BP2) cells was subsequently confirmed by real time reverse-transcription PCR, Western blotting, and gelatin zymography. Furthermore, consistent with increased MMP-2 expression in SNB19(BP2) cells, transient transfection of a MMP-2 promoter/luciferase reporter also resulted in 3–6-fold higher luciferase activity in SNB19(BP2) cells than in parental or vector-transfected control cells. Finally, tissue microarray analysis of 68 GBM tissue specimens showed a significant correlation between the overexpression of IGFBP2 and elevated MMP-2 expression. Taken together, our data provide evidence that IGFBP2 contributes to glioma progression in part by enhancing *MMP-2* gene transcription and in turn tumor cell invasion.

Introduction

Gliomas are the most common primary tumors of the central nervous system, accounting for 80% of adult primary brain tumors (1). The prognosis for patients with the advanced glioma, GBM,[3] is very poor, with a median survival of 8–10 months (1). Similar to many malignant tumors, GBM has a characteristically high proliferation index; however, the most devastating and thus far therapeutically intractable aspect of its biology is its highly invasive nature, which prevents complete tumor resection and causes significant neurological morbidity and, ultimately, mortality.

The acquisition of invasive potential through proteinase expression is an essential event in tumor progression. Among proteinases, MMP-2 and MMP-9 have been shown to be increased in GBMs and are thought to play a key role in facilitating the invasion of GBM cells through brain parenchyma (2, 3). Other molecules, such as secreted protein acidic and rich in cysteine and extracellular matrix protein fibronectin have also been implicated in GBM invasion (4, 5).

In previous studies, we searched for the gene activities associated with GBM using cDNA microarray profiling followed by tissue microarray validation and identified IGFBP2 overexpression as one of the most frequent molecular events characteristic of GBM (6–8). This finding has been subsequently confirmed independently by two different laboratories, and high levels of IGFBP2 expression have been shown to be associated with poor survival in patients with diffuse gliomas (9, 10). IGFBP2 is normally predominantly expressed in fetal tissues and is involved in brain development (11–14). After birth, IGFBP2 expression decreases significantly in glial cells (15, 16). The subsequent differential induction of IGFBP2 expression in the brain has been associated with a variety of pathological conditions, including hypoxia, regeneration, trauma, and tumors of the central nervous system (17). IGFBP2 is associated with other malignancies as well. In ovarian cancer, the increase in IGFBP2 have been found to correlate positively with the serum tumor marker CA125 (18). Similarly, increased serum levels of IGFBP2 have been proposed as a prognostic marker for prostate cancer (19). In addition, the overexpression of IGFBP2 increases the tumorigenicity of Y-1 adrenocortical tumor cells and epidermoid carcinoma cells (20, 21). However, the molecular mechanisms by which IGFBP2 enhances tumor cell growth and increases tumorigenicity remain undefined.

To elucidate the cellular pathways affected by elevated levels of IGFBP2 in high-grade gliomas, we established stable SNB19(BP2) clones that overexpress IGFBP2. Although IGFBP2 overexpression in these clones did not affect their growth rates, it enhanced cell invasion in an *in vitro* invasion assay. Comparison of gene expression profiles of the stable IGFBP2 expressing clones with parental cells or vector-transfected cells revealed increased expression of MMP-2, as well as several other invasion-related genes. Furthermore, IGFBP2 overexpression also correlated with MMP-2 overexpression in 68 glioblastoma tissues. Our results demonstrate for the first time that overexpression of IGFBP2 in glioma cells leads to increased MMP-2 expression and an increased invasion.

Materials and Methods

Cell Culture and Stable Transfection. The SNB19 human glioblastoma cells were generously provided by Dr. Alfred Yung (The University of Texas M. D. Anderson Cancer Center) and maintained in DMEM/F12 medium supplemented with 10% FBS in a humidified incubator containing 5% CO_2 at 37°C. To establish stable cell lines that overexpress IGFBP2, we transfected SNB19 cells with a pcDNA3 expression vector encoding IGFBP2 cDNA and a neoselectable marker using FuGENE6 reagent (Roche Diagnostics Corpo-

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[3] The abbreviations used are: GBM, glioblastoma multiforme; IGF, insulin-like growth factor; IGFBP2, insulin-like growth factor binding protein 2; MMP-2, matrix metalloproteinase-2; FBS, fetal bovine serum; RT-PCR, reverse-transcription PCR; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

ration, Indianapolis, IN). Transfected cells were subsequently selected in the presence of G418 (400 μg/ml) to establish the IGFBP2 overexpressing clones, which we labeled SNB19(BP2). The expression of IGFBP2 in SNB19(BP2) clones were determined by Western blotting of cell extracts and cell media with anti-IGFBP2 antibody (C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To establish stable SNB19(BP2) c4 subclones, SNB19(BP2) c4 cells, which express undetectable level of IGFBP2, were transfected with IGFBP2 cDNA with a puromycin selectable marker, and were subsequently selected for puromycin resistance at 4 μg/ml.

Determination of Cell Growth. A total of 5×10^4 cells in 5 ml of DMEM/F12 medium supplemented with 10% FBS were seeded into T25 tissue culture flasks. Every 24 h for 7 days, a set of three flasks were removed, rinsed with PBS, trypsinized, and resuspended. Three independent cell counts were obtained and the mean cell counts were calculated. The number of cells were plotted against time to obtain a growth curve for the cell population.

***In Vitro* Chemoinvasion Assay.** Chemoinvasion was measured using 24-well BioCoat Matrigel invasion chambers (Becton Dickinson Labware, Bedford, MA) with an 8-μm pore polycarbonate filter coated with matrigel. The lower compartment contained 0.6 ml of DMEM/F12 medium with 0.5% FBS as chemoattractants or serum-free DMEM/F12 medium as a control. In the upper compartment, 5×10^4 cells/well were placed in triplicate wells and incubated for 24 h at 37°C in humidified incubator with 5% CO_2. After incubation, the cells that had passed through the filter into the lower wells were stained with Giemsa (Fisher Scientific, Orangeburg, NY) and counted under a microscope in five predetermined fields. All assays were repeated at least three times. The differences in the invasion rates between control cell lines and stable SNB19(BP2) lines were analyzed using a two-tailed Student's *t* test.

RNA Isolation and Microarray Analysis. Total RNA was isolated from SNB19 glioma cells and stable SNB19(BP2) cells using TRI reagent (MRC, Cincinnati, OH). Microarray was carried out as described previously (22, 23) using the Pathway array containing 1500 functionally well-characterized genes in duplicate.[4] Hybridized arrays were scanned with a GeneTAC LSIV scanner (Genomic Solutions, Ann Arbor, MI). The images were quantified using the imaging software ArrayVision from Imaging Research Inc. (Ontario, Canada), and the significantly differentially expressed genes were identified as described (24). For differentially expressed genes, a smoothed *T* value cutoff of 2.0 was used.

LightCycler Real-time Quantitative RT-PCR. Total RNA was extracted using TRI reagent. One microgram of total RNA was reverse transcribed under standard condition in a reaction mixture containing all four deoxynucleoside triphosphates, 100 unit of Superscript II Plus RNase H^- Reverse Transcriptase (Life Technologies, Inc., Grand Island, NY) and 100 μM random hexamer primers (Pharmacia Biotech, Buckinghamshire, United Kingdom). PCR reactions were performed with 4 μl of reverse transcriptase product serving as template DNA (synthesized cDNA) and 6 μl of master-mix. The master-mix of the following reaction components was prepared: 4 μl of water, 1 μl of primer mix (4 mM $MgCl_2$ and 0.4 μM forward and reverse primers), and 1 μl of LightCycler Fast DNA Master SYBR Green I (Roche Diagnostic, Mannheim, Germany). Capillaries were closed, centrifuged, and placed into the rotor. Real-time PCR procedures and the primer sequences of the internal control gene *GAPDH* were described in detail in Nakanishi *et al.* (25). The primer sequences for *MMP-2* were as follows: 5′-CAAAAACAAGAAGACATACATCTT-3′ and 5′-GCTTCCAAACTTCACGCTC-3′. The specific PCR condition for *MMP-2* was 95°C for 1 min, followed by 50 cycles at 95°C for 0 s, 60°C for 5 s, and 72°C for 10 s. Fluorescence was measured at 80°C for 2 s. Each quantification reaction was performed in triplicate, and the mean values were used to calculate the ratios of *MMP-2* to *GAPDH*, with a value of 1 used as the control. All assays were repeated at least three times.

Immunoblotting. Production of IGFBP-2 and MMP-2 by stable SNB19(BP2) cells was analyzed by Western blotting. Briefly, cells were incubated in serum-free medium for 24 h, after which time the medium was clarified by centrifugation and concentrated using spin columns (Amicon, Beverly, MA). Equal amounts of proteins in the concentrated samples were resolved by 10% SDS-PAGE, electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Chicago, IL), blocked in 5% skim milk in 1× TBS, and probed with the following primary antibodies: anti-IGFBP-2 polyclonal antibody C-18 (Santa Cruz Biotechnology, Inc.) and anti-MMP-2 monoclonal antibody Ab-3 (Oncogene Research Products, Cambridge, MA). Proteins were detected with an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Gelatin Zymography. Gelatin zymography was performed as described previously with minor modifications (26). Equal numbers of cells (1×10^6) were seeded onto 100-mm plates in DMEM/F12 containing 10% FBS and cultured overnight. Cells were then washed twice with PBS and incubated with serum-free DMEM/F12 medium for 24 h. The medium was collected after the removal of cell debris and concentrated using Amicon spin columns. Concentrated samples with equal amounts of proteins were mixed with SDS sample buffer without reducing agent and subjected to 7.5% SDS-PAGE containing 0.1% gelatin A. After electrophoresis, the gels were washed four times in 2.5% Triton X-100 for 1 h at room temperature to remove SDS and then incubated for 24–48 h at 37°C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The proteins in the gel were stained with Coomassie Brilliant Blue. Proteolytic activity in the gel was visualized as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Luciferase Reporter Assays. A luciferase reporter plasmid driven by 1716 bp of the human *MMP-2* promoter (27) and an internal *Renilla* luciferase control vector (pRL-TK) were used in this study. Stable SNB19(BP2) cells were seeded into 6-well tissue culture plates and the next day transfected with 0.5 μg of the *MMP-2* luciferase reporter construct and 0.5 μg of the pRL-TK control using the FuGENE 6 reagent. After 24 h, cells were lysed and cell extracts assayed in triplicate with an enhanced luciferase assay kit (PharMingen, San Diego, CA). The luciferase activity from the *MMP-2* vector was normalized to the *Renilla* luciferase activity.

Brain Tumor Tissue Microarray. A human glioma tissue microarray was constructed using formalin-fixed, paraffin-embedded archival tissue blocks as described previously (7). Duplicate samples were collected from different sites of the most representative tumor regions. The tissue microarray included tissues from 68 GBM patients. Expression levels of IGFBP2, and MMP-2 were evaluated by a standard indirect immunoperoxidase procedure (ABC-Elite; Vector Laboratories, Burlingame, CA). In brief, antigen retrieval was performed by treatment in a steamer for 25 min. Anti-IGFBP2 (C-18, Santa Cruz Biotechnology, Inc.) was used at a 1:1000 dilution at 4°C overnight. Anti-MMP-2 monoclonal antibody Ab-6 (Oncogene Research Products) was used at a 1:50 dilution at 4°C overnight. Secondary antibody incubation was performed at room temperature for 60 min. Mayer's hematoxylin nuclear staining was used as a counterstain. Tumors with none or few (<5% of the neoplastic cells) IGFBP2 or MMP-2 cytoplasmic staining were regarded as negative. Tumors with strong cytoplasmic staining in ≥5% of the neoplastic cells were regarded as positive. The statistical analysis was performed using Fisher's exact test.

Results

IGFBP2 Overexpression in Glioma Cells Enhances Invasion. We have examined several glioma cell lines for IGFBP2 expression to identify suitable cells for studying the role of IGFBP2 in tumor cell progression. Among them, SNB19 cells were found to have low endogenous IGFBP2 expression and were, therefore, chosen to generate stable cell lines. Stable SNB19(BP2) cell lines that express different amounts of IGFBP2 were generated by transfecting SNB19 cells with a pcDNA3-IGFBP2 expressing vector followed by selection with G418. The expression of IGFBP2 in the selected clones was evaluated by Western blotting using cell extracts with anti-IGFBP2 antibody (Fig. 1*A*). The SNB19(BP2) c1 clone showed a level of IGFBP2 protein similar to that of the parental or vector control cells. Very low IGFBP2 expression was detected in c4 clone, whereas high levels of IGFBP2 expression were detected in c5 and c8 clones. Despite the differences in the levels of IGFBP2, these clones did not show marked differences in their growth rates (Fig. 1*B*). We then tested whether IGFBP2 expression affected glioma invasion using a matrigel *in vitro* invasion assay, with 0.5% FBS used as chemoattractant to stimulate cells to penetrate through the matrigel and migrate through the filters. The matrigel contains laminin, collagen type IV,

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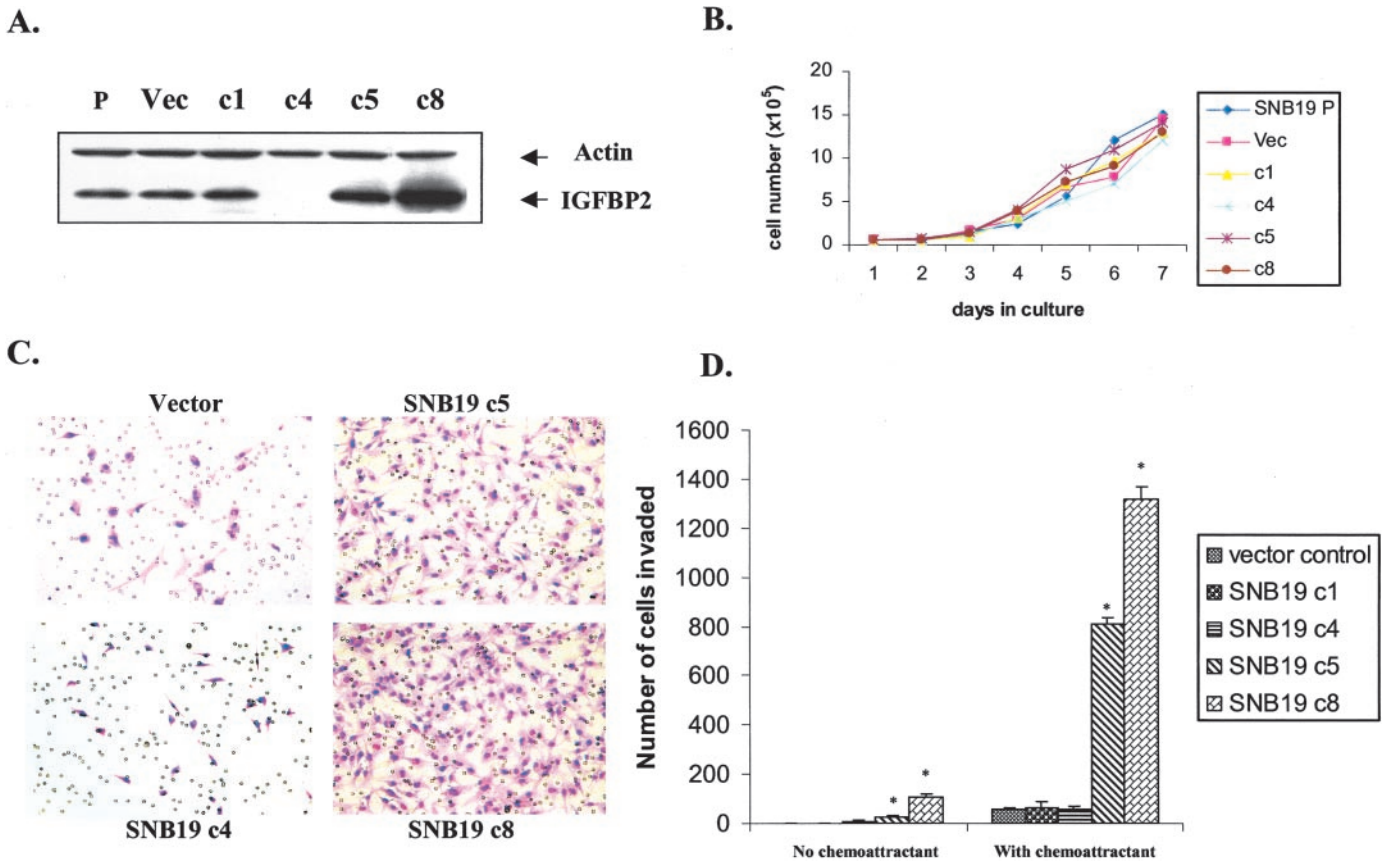

Fig. 1. Overexpression of IGFBP2 enhanced invasion potential of SNB19 cells. *A*, stable SNB19(BP2) clones expressing different levels of IGFBP2. SNB19 cells were transfected with the IGFBP2 expression plasmid or the empty vector followed by selecting the positive clones in G418-containing medium. Equal amounts of protein from cell extracts of the representative clones were subjected to Western blot with anti-IGFBP2 and anti-actin antibodies. *B*, comparison of *in vitro* growth of stable SNB19(BP2) cells. Equal number of cells (5×10^4) were incubated in DMEM/F12 + 10% FBS for different times, and cell numbers were quantified by direct cell counting. *C*, chemoinvasion was measured as described in "Materials and Methods." The cells that invaded through matrigel-coated *trans*-well inserts toward chemoattractant were stained with Giemsa. Photographs were taken at a magnification of ×200. *D*, the cells invading through the matrigel were counted under microscope in five predetermined fields at ×200. Each sample was assayed in triplicate, and assays were repeated at least twice. $*P < 0.01$.

heparin sulfate proteoglycan, entactin, which are extracellular matrix compositions of normal brain and gliomas. The invasion assay revealed prominent differences between IGFBP2 overexpressing c5 and c8 clones and the vector-transfected cells both with and without chemoattractant ($P < 0.01$; Fig. 1*D*). Significantly higher rates of invasion were observed in IGFBP2 overexpressing cells (c5 and c8 clones) when 0.5% FBS used as chemoattractant, they had a 15–24-fold higher invasion rate than that of the vector-transfected cells ($P < 0.01$). In contrast, there was no difference between the c1 and c4 clones and the vector- transfected cells ($P > 0.05$; Fig. 1, *C* and *D*). Because clone c4 had essentially undetectable IGFBP2 expression, it provided an additional independent system to verify the observed effects of IGFBP2 on cell invasion. SNB19(BP2) c4 cells were, therefore, transfected with IGFBP2 expressing vector and selected with puromycin for subclones that express IGFBP2. Three subclones were derived from this selection, named SNB19(BP2) c4.1, SNB19(BP2) c4.6, and SNB19(BP2) c4.10. The levels of IGFBP2 expression in these clones were evaluated by Western blotting using cell medium with anti-IGFBP2 antibody. As shown in Fig. 2, SNB19(BP2) c4.1 and SNB19(BP2) c4.10 subclones expressed high levels of IGFBP2, whereas SNB19(BP2) c4.6 subclone expressed only moderate levels of IGFBP2. Invasion assays were carried out to compare these subclones with the parental SNB19(BP2) c4 clone. Again, significantly higher rates of invasion were observed between IGFBP2 overexpressing c4.1 and c4.10 subclones and the parental cells both with and without chemoattractant ($P < 0.01$; Fig. 2). When

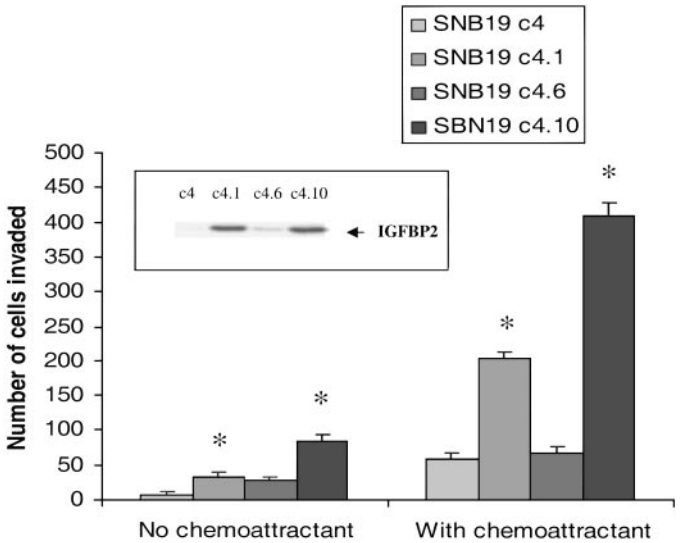

Fig. 2. Overexpression of IGFBP2 enhanced invasion potential of SNB19(BP2) c4 cells. Stable SNB19(BP2) c4 subclones expressing different levels of IGFBP2 are shown in the inset. SNB19(BP2) c4 cells were transfected with the IGFBP2 expression plasmid with puromycin selectable marker or the empty vector followed by selecting the positive clones in puromycin-containing medium. Equal amounts of protein from concentrated cell medium from the representative clones were subjected to Western blotting with anti-IGFBP2 antibody. Chemoinvasion was determined as described in Fig. 1. The cells invading through the matrigel were counted under microscope in five predetermined fields at ×200. Each sample was assayed in triplicate and assays were repeated at least twice. $*P < 0.01$.

0.5% FBS was used as chemoattractant, 4–7-fold higher rates were observed in IGFBP2 overexpressing cells (c4.1 and c4.10) that invaded through the membrane than in parental cells ($P < 0.01$); in contrast, there was no difference between the c4.6 subclone and the parental cells ($P > 0.05$; Fig. 2). Thus, the results consistently, in two independently derived cell lines, demonstrated that increased IGFBP2 expression in human SNB19 cells could facilitate tumor cell invasion *in vitro*.

Microarray Analysis of IGFBP2 Regulated Genes in Stable SNB19(BP2) Cell Lines. To gain insight into the molecular basis for the enhanced invasion function in IGFBP2 overexpressing cells, examine the global effects of IGFBP2 overexpression, and correlate gene activities with cellular phenotypic behaviors, we performed gene expression analysis on IGFBP2 overexpressing SNB19 cells (clones c5 and c8) and parental cells using the pathway microarray generated in M. D. Anderson Cancer Genomics Core Laboratory. This microarray contains 1500 functionally well-characterized genes involved in various important cellular processes, including cell adhesion and cell migration, apoptosis, cell cycle, DNA repair, and transcription. The microarray experiments were repeated three times. Data analysis identified 28 common significantly induced and 12 common repressed genes in several different functional categories in the two IGFBP2 high expressing clones (c5 and c8) compared with the parental cells. The representative results of these comparisons are reported in Table 1. *IGFBP2*, as expected, is one of the genes induced in the high expressing clones (see growth factor section in Table 1). The altered genes, which are consistently associated with the changes in cell invasion (Figs. 1 and 2), included invasion related genes *MMP-2, fibronectin 1, thrombospondin 2, integrin α 5* and *6.* Cell cycle inhibitor *p21* gene was also induced, although we did not see major growth alterations in the IGFBP2 overexpressing clones.

High Level of MMP-2 Expression and Activity in High IGFBP2 Expressing Clones. Considering the established role of type IV metalloproteinase in brain tumor invasion, we, therefore, focused to characterize on IGFBP2-mediated MMP-2 induction in IGFBP2 overexpressing cells. We performed real time RT-PCR using IGFBP2 overexpressing clones and IGFBP2 low expressing clones and confirmed that MMP-2 RNA levels in SNB19(BP2) c5 and c8 were 3–5-fold higher than those in vector-transfected control cells (Fig. 3*A*). In contrast, there was no difference in MMP-2 RNA levels between the clones expressing low levels of IGFBP2 (c1 and c4) and vector-transfected control cells. We also observed a consistent 3–5-

Table 1 *Functional grouping of genes whose expression were altered by IGFBP2*[a]

Genes	c5		c8	
	T Value[b]	Fold change	*T* Value	Fold change
Cell adhesion, motility and invasion				
MMP-2	3.2	5.0	4.8	3.0
Fibronectin 1	4.0	3.2	13.8	10.2
Thrombospondin 2	4.2	3.4	11.8	8.0
Integrin, α5	2.4	2.1	11.2	7.5
Integrin, α6	2.6	2.4	3.2	1.8
Osteoblast specific factor 2 (fasciclin I-like)	3.9	6.0	11.1	38.0
Fibroblast activation protein, α	4.6	4.5	9.0	15.9
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	4.7	4.5	6.7	3.2
Coagulation factor II (thrombin) receptor	3.0	2.3	5.8	2.5
Vinculin	2.1	1.8	5.5	2.5
Membrane metallo-endopeptidase (CD10)	3.1	2.6	3.7	3.3
Filamin A, α (actin-binding protein 280)	−2.9	0.4	−3.2	0.6
Growth factors, binding proteins, receptors, and cytokines				
IGFBP2	11.4	25.1	19.5	36.0
Interferon-induced transmembrane protein 3	2.1	1.8	8.5	4.3
Tumor necrosis factor receptor superfamily, member 6	2.9	6.1	6.8	6.3
Melatonin receptor 1A	2.1	1.8	4.9	2.3
Receptor tyrosine kinase-like orphan receptor 1	2.4	2.8	4.5	9.9
Bradykinin receptor B2	2.5	2.4	4.1	5.9
Transforming growth factor, β receptor II	3.5	4.6	3.6	1.9
Endothelial cell growth factor 1 (platelet-derived)	−2.6	0.4	−3.0	0.5
Interferon-inducible double-stranded RNA-dependent inhibitor	−3.1	0.2	−3.7	0.2
Oncogenes, tumor suppressors, apoptosis, and others				
Centaurin, γ1	4.2	3.3	7.4	3.3
Protein tyrosine phosphatase, nonreceptor type 13	3.0	2.9	7.2	5.9
RAB7, member *RAS* oncogene family-like 1	2.2	1.8	5.2	2.3
BCL2	2.6	2.3	4.5	5.3
Protein tyrosine phosphatase, receptor type R	−3.1	0.3	−3.1	0.4
Protein phosphatase 1, catalytic subunit, beta isoform	−2.6	0.4	−3.2	0.5
Bcl-2 binding component 3	−2.0	0.5	−3.5	0.2
JAG1	−6.5	0.1	−10.7	0.1
Cell cycle and DNA repair				
X-ray repair complementing defective repair 2 (XRCC2)	3.0	2.3	6.2	2.7
Cyclin-dependent kinase inhibitor 1A (P21, Cip1)	2.3	2.1	5.0	2.9
CDC28 protein kinase 2	2.2	2.0	3.5	1.8
Transcription factors and unknown				
HIF1A	2.7	1.2	4.8	2.2
Zinc finger protein 91	3.8	2.9	6.7	3.0
Zinc finger protein 36	2.9	2.3	4.3	2.0
Zinc finger protein 132	−2.0	0.5	−3.2	0.3
Zinc finger protein 134	−2.1	0.5	−4.0	0.5
Zinc finger protein 239	−2.8	0.5	−4.3	0.5
HLX1 like protein	−2.3	0.5	−5.1	0.4
Zinc finger protein 205	−3.6	0.4	−6.6	0.3

[a] For differentially expressed genes, a smoothed *T* value cutoff of 2.0 was used and corresponding fold changes are shown. The smoothed *T* value is computed by log ratio divided by the estimated standard deviation across the entire array. The smoothed *T* value gives the level of significance of the differentially expressed genes. The fold change is the ratio of the normalized signal from the two channels. See "Materials and Methods" for further details.

[b] Positive smoothed *T* values indicate induction in IGFBP2 overexpressing clones; negative smoothed *T* values indicate repression in IGFBP2 overexpressing clones.

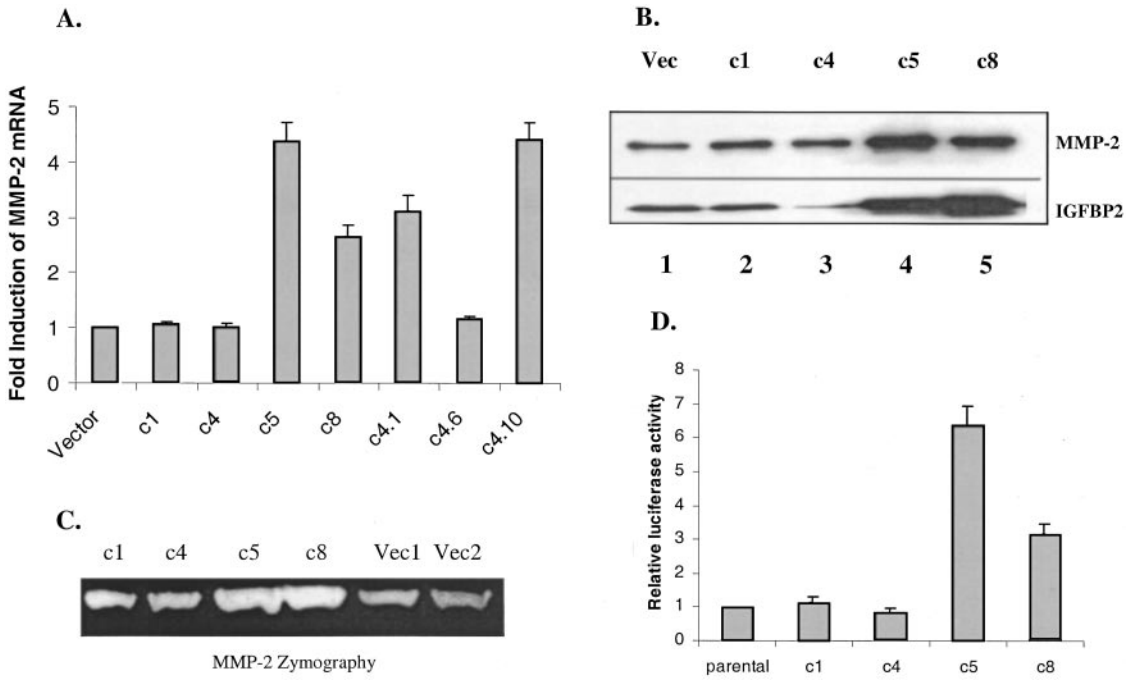

Fig. 3. Effects of IGFBP2 on *MMP-2* expression. A. Quantification of *MMP-2* mRNA of SNB19 vector-transfected control cells, stable SNB19(BP2) clones, and stable SNB19(BP2) c4 subclones by real time quantitative RT-PCR. The means (SD) of three independent experiments each run in triplicate normalized to GAPDH are shown. See "Materials and Methods" for further details. *B*, Western blot analysis of MMP-2 in conditioned media of SNB19 vector-transfected control cells *(Lane 1*) and stable SNB19(BP2) clones *(Lanes 2–5*). Equal amounts of samples were electrophoresed on SDS-PAGE gels. *Top*, samples probed with MMP-2 antibody; *bottom*, samples probed with IGFBP2 antibody. *C*, zymographic assay demonstrating increase in MMP-2 enzyme activities in the IGFBP2 overexpressing SNB19(BP2) clones over vector-transfected control cells. Equal numbers of cells from the indicated cell lines were plated in DMEM/F12 serum-free medium and cultured for 24 h. Culture media were collected, concentrated, and subjected to protein SDS-PAGE (with gel containing 1.5% gelatin) without reducing agent. After electrophoresis, the gel was washed, incubated in reaction buffer at 37°C, stained with Coomassie Brilliant Blue, and destained. *D*, SNB19 parental cells or stable SNB19(BP2) clones were transiently transfected with an MMP-2 promoter reporter construct, and luciferase activity was determined after 24 h as described in "Materials and Methods." The means (SD) of three independent experiments, each run in triplicate normalized to the *Renilla* luciferase activity, are shown.

fold increase in the *MMP-2* mRNA levels in the IGFBP2 overexpressing SNB19(BP2) c4.1 and SNB19(BP2) c4.10 clones (Fig. 3*A*). Only a slightly increased *MMP-2* mRNA level was observed in the SNB19(BP2) c4.6 clone, which had low IGFBP2 expression (Fig. 3*A*). To ascertain that the increase of MMP-2 mRNA levels in IGFBP2 high expressing clones was not attributable to a general increase mRNA expression by IGFBP2 overexpression and was a specific phenomenon, we examined the expression levels of platelet-derived growth factor receptor (PDGFR) in those clones by real time RT-PCR. We did not observe significant differences in platelet-derived growth factor receptor expression between clones expressing high (c5 and c8) and low (vector-transfected control, c1, and c4) levels of IGFBP2 (data not shown), indicating that the effect of IGFBP2 on *MMP-2* expression was specific.

We also investigated whether the effect of IGFBP2 on *MMP-2* mRNA levels could ultimately lead to increased MMP-2 protein and enzymatic activity by Western blotting and gelatin zymography using conditioned medium from each cell line. As shown in Fig. 3*B*, MMP-2 protein was significantly higher in IGFBP2 overexpressing clones (c5 and c8) than in vector-transfected control. In contrast, there was no difference in MMP-2 protein level between IGFBP2 low expressing clones (c1 and c4) and vector-transfected control. Consistently, gelatin zymography assay showed a stronger lytic zone at the molecular mass of 72 kDa, demonstrating the elevated MMP-2 enzyme activity in IGFBP2 overexpressing clones (Fig. 3*C*).

To investigate whether the increase in MMP-2 expression in SNB19(BP2) cells might be attributable to increased *MMP-2* gene transcription, we examined the activities of an *MMP-2* promoter-luciferase reporter construct after transient transfection into SNB19(BP2) cells and compared the activities with those in parental cells. MMP-2/luciferase activities were 3–6-fold higher in SNB19(BP2) c5 and c8 than in parental control cells, but there was no such difference between clones expressing low level of IGFBP2 (c1 and c4) with vector-transfected control cells (Fig. 3*D*). Taken together, these results support a role for IGFBP2 in transcriptionally up-regulating *MMP-2* gene expression.

IGFBP-2 Overexpression Correlated with MMP-2 Expression in GBM. To further confirm the significance of the *in vitro* observations showing that IGFBP2 regulates *MMP-2* gene expression, a human GBM tissue microarray containing 68 GBMs was constructed using formalin-fixed, paraffin-embedded archival tissue blocks. The expression of IGFBP2 and MMP-2 was evaluated by immunohistochemistry under identical standardized conditions for all patient samples on consecutive tissue sections cut from the array block. The immunohistochemistry staining for IGFBP2 and MMP-2 was successful in all 68 GBMs and the duplicate cores from the same case had identical expression pattern. Examples of both positive and negative staining are shown in Fig. 4, *A–D*. Quantification of the staining showed that among GBMs that were positive for IGFBP2, 87.5% (49 of 56) also showed an increase in MMP-2 expression and only 12.5% (7 of 56) did not show MMP-2 expression. In contrast, GBMs that were negative for IGFBP2 staining, 42% (5 of 12) did not have MMP-2 expression (Fig. 4*E*). Strong IGFBP2 immunopositivity was associated with MMP-2 immunopositivity ($P < 0.05$; Fisher's exact test). These data further support the notion that IGFBP2 contributes to glioma progression in part by enhancing *MMP-2* gene transcription and glioma cell invasion.

Discussion

Enhanced expression of IGFBP2 was recently found to be one of the most frequent alterations in advanced stage of gliomas, GBMs (6,

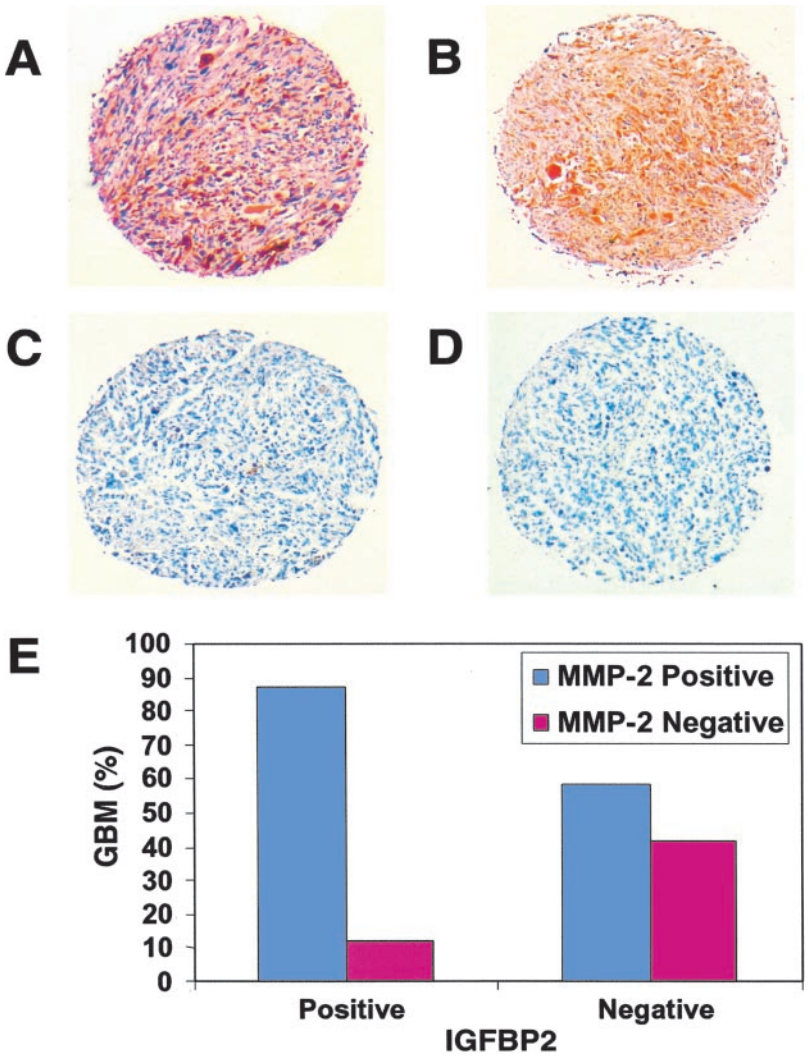

Fig. 4. IGFBP2 overexpression correlates with up-regulation of MMP-2 in human GBMs. *A* and *B*, a representative GBM case showing the concordant expression of IGFBP2 and MMP-2. *C* and *D*, GBM case showing negative immunostaining for both markers. *Photographs* were taken at a magnification of ×100. *E*, the concordant expression of IGFBP2 and MMP-2 in human glioma tissue microarray consisting of 68 GBMs is shown ($P < 0.05$).

9, 10). The fact that IGFBP2 is overexpressed only in the advanced stage of gliomas and other types of advanced tumors suggests that IGFBP2 affects the functional pathways that are crucial in determining the phenotypes of advanced stage of cancers, such as increased cell proliferation and invasion. Therefore, understanding IGFBP2 signaling pathway holds promise in understanding the fundamental molecular processes responsible for cancer progression.

Using stable SNB19 cell lines that express different levels of IGFBP2, we found that increased IGFBP2 had no effect on cellular proliferation, but did result in significantly increased cellular invasion in the *in vitro* cell invasion assay. Our microarray studies showed that IGFBP2 overexpressing clones overexpressed MMP-2 as well. MMP-2 is thought to play a key role in tumor progression by degrading the of extracellular matrix, thereby facilitating tumor cell invasion into surrounding normal tissues (28). We then further validated this finding by real time RT-PCR, Western blotting, and enzymatic assays, which confirmed that the MMP-2 protein level and the protease activity were both elevated in IGFBP2 overexpressing clones. Furthermore, tissue array and immunohistochemistry analysis showed a significant correlation between the overexpression of IGFBP2 and elevated MMP-2 expression in 68 GBMs. Therefore, our studies for the first time provided a molecular mechanism through which *IGFBP2* functions as an important invasion-promoting gene.

Our data also showed a positive correlation between IGFBP2 expression and the *MMP-2* promoter activity. This transcriptional regulation is not likely attributable to a direct action of IGFBP2, because we did not detect IGFBP2 staining in the nucleus by immunohistochemistry (data not shown). This is contrast to the behavior of IGFBP5, which can translocate to the nucleus by a nuclear localization signal and may directly affect transcription (29). IGFBP2 may instead serve as an upstream signal regulator that activates downstream transcription factors important for the up-regulation of *MMP-2*. Underscoring this possibility is the fact that among the genes altered in IGFBP2 overexpressing cells are a number of transcription factors (Table 1). Potentially, those transcription factors may be responsible for IGFBP2 mediated *MMP-2* up-regulation.

Because IGFs are involved in tumor cell migration and invasion through their regulatory effects on *MMP-2* expression (30, 31), the effect of IGFBP2 on MMP-2 may be achieved through enhancement of IGF-dependent pathway, although we cannot exclude the possibility of IGF-independent effect of IGFBP2. It is noteworthy that both negative and positive effects of IGFBP2 on IGF-dependent cell proliferation have been reported (32, 33), and IGFBP2 has been shown to augment both IGF-dependent and IGF-independent anti-apoptotic functions in the developing mouse limb and brain (34, 35). In this regard, it would be important to understand whether the regulation of *MMP-2* by IGFBP2 is mediated through an IGF-dependent or independent pathway.

Tumor cell invasion involves a series of complicated steps, including the acquisition of cell motility and cell adhesion properties and the

production of extracellular protease. Tumor cell adhesion to the basement membrane is mediated by integrin, which is a member of a family of transmembrane receptor heterodimers with α and β subunits. Extracellular matrix proteins, such as fibronectin and thrombospondin, stimulate motility through integrin receptors (36). Therefore, it is interesting that cell adhesion molecule integrins and motility factors fibronectin 1 and thrombospondin 2 were also induced in IGFBP2 overexpressing clone (Table 1). This suggests that IGFBP2 affects multiple genes in the cell invasion pathways. This potentially robust action of IGFBP2 may explain the frequent induction of IGFBP2 in multiple types of advanced cancer.

Taken together, using cellular, genomics, and molecular approaches, we have documented a strong association between high levels of IGFBP2 expression, increased expression of invasion gene MMP-2, and an increased invasion potential. These results shed light on an important biological aspect of glioma progression and may provide new insights useful for the design of novel mechanism-based therapies for GBM.

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