Inhibition of transcription factor STAT5b suppresses proliferation, induces G1 cell cycle arrest and reduces tumor cell invasion in human glioblastoma multiforme cells

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ABSTRACT

Abnormalities in the signal transducer and activator of transcription 5 (STAT5) signaling are involved in the oncogenesis of several cancers. However, previous studies have not elucidated clear and distinct roles for each STAT5 gene in cancers. To investigate the role of STAT5a, -5b isoforms in human glioblastoma multiforme (GBM) progression, we depleted each STAT5 isoforms with siRNA. Our results demonstrate that STAT5b is involved in GBM cell growth, cell cycle progression, invasion and migration through regulation of gene expression, such as Bcl-2, p21waf1/cip1, p27kip1, FAK and VEGF. Moreover, immunohistochemical staining reveals that cytoplasm staining of STAT5b is markedly increased in GBM (57.1%) compared with that in normal cortex (22.2%) and diffuse astrocytoma (27.3%), suggesting that STAT5b could have important implications in astrocytoma biology. Therefore, our findings illustrate the biological significance of STAT5b in GBM progression, and provide novel evidence that STAT5b may serve as a therapeutic target in the prevention of human glioblastoma multiforme.

1. Introduction

Astrocytoma, arising from astrocytes and their precursors, is the most common primary tumors of the brain. Based on degree of malignancy, astrocytoma is graded into grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma multiforme, GBM). GBM is the most common type with the worst prognosis, and average post-operative survival is less than 2 years [1–5]. The conventional modalities of treatment for GBM, which include surgery, radiation, and chemotherapy, remain ineffective, because the tumor cells are highly invasive and resistant to radiation and chemotherapeutic agents [1–3,5]. In this regard, the recent emphasis on the development of targeted molecular therapies for GBM affords the possibility of more effective treatment options in the future.

Signal transducers and activators of transcription (STAT) is a family of transcription factors and is involved in a wide variety of cellular physiological processes, including differentiation, survival, or cell growth [6,7]. To date, seven STAT genes have been identified: STAT1, -2, -3, -4, -5a, -5b, and -6. Among these STAT genes, STAT1 and STAT5 have shown to play a role in tumor development and progression [8]. For example, recent studies have revealed that constitutive activation of STAT3 correlates with cell proliferation in breast carcinoma [9] and nonsmall-cell lung cancer (NSCLC) [10] and also inhibits apoptosis [11–13]. Aberrant activation of STAT3 has also...
been reported in GBM cells but not in normal human astrocytes [10, 14, 15]. Moreover, knockdown of STAT3 expression by RNAi induces apoptosis in astrocytoma cells [16, 17]. Like STAT3, STAT5 has been shown to regulate proliferation and inhibition of apoptosis in several cancer cells [18]. For instance, STAT5 activation has been shown primarily in hematopoietic malignancies, which are associated with Bcl-Abl fusion protein [19]. Later, Li and coworkers reported that STAT5 was correlated with aggressiveness of prostate cancer [20]. However, thus far, there have been no data on the role of STAT5 in glioma. In addition, a distinct role of STAT3 genes (STAT5a and STAT5b) in human glioblastoma multiforme has not been elucidated yet.

STAT5 is a latent cytoplasmic protein, which is comprised two highly homologous isoforms, 94 kDa STAT5a and 92 kDa STAT5b [21]. Although these two STAT proteins share considerable functional overlap, gene-disruption experiments have revealed that STAT5a and STAT5b are functionally not redundant [22–24]. STAT5a knockout experiments have demonstrated that the product of this gene mediates prolactin signaling along with mammary gland development [23, 25, 26], whereas disruption of STAT5b abrogates sexually dimorphic liver gene regulation and is associated with loss of male characteristic body growth rates [22]. On the other hand, their expression patterns vary in different cancer. Some reports have shown that STAT5a is implicated in breast cancer [27], whereas STAT5b is correlated with prostate cancer progression [28]. These studies indicate that there are differences between STAT5a and STAT5b, and the above intriguing data raised our interest in examining the biological significance of each STAT5 gene in GBM. To directly assess the biological significance of each STAT5 gene in GBM, we investigated the changes in cell viability, apoptosis, cell cycle progression and cell invasive capability. We also explored the changes in cell viability, apoptosis, cell cycle progression and cell invasive capability. We also investigated the changes in cell viability, apoptosis, cell cycle progression and cell invasive capability. We also investigated the changes in cell viability, apoptosis, cell cycle progression and cell invasive capability. We also investigated the changes in cell viability, apoptosis, cell cycle progression and cell invasive capability.

2. Materials and Methods

2.1. Cell culture and transient transfection of STAT5a, -5b siRNA

Two human glioblastoma multiforme cell lines (U251 and U87) were used in this study, cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere.

Commercial STAT5a and STAT5b small interfering RNA (siRNA) were obtained from Dharmacon Inc. (USA) and used to target human STAT5a (GenBank Accession No. NM003152) and STAT5b (GenBank Accession No. NM012448), respectively. Cells were transfected with STAT5a or STAT5b siRNA (50 nM) by using the DharmaFECT™1 siRNA transfection reagent according to the manufacturer's instructions. Nonspecific siRNA (Dharmacon Inc.) was used as a negative control and the selective silencing of STAT5a, -5b was confirmed by Western blot.

2.2. Western blot and antibodies

Whole-cell lysates were prepared from cancer cell lines, and standard Western blot analysis was performed. Proteins were detected using the enhanced chemiluminescence detection kit (SuperSignal West Femto Substrate, Pierce). For loading control, the membrane was probed with monoclonal antibody for GAPDH.

Antibodies used in this study were purchased from Cell Signaling Technology Inc. (USA), unless otherwise specified: STAT5a and STAT5b (Chemicon, USA), Bcl-2 (R&D, USA) and GAPDH (Kangchen, China). All primary antibodies were used at a 1:1000 dilution.

2.3. ELISA analysis of VEGF, MMP2 and MMP9 release

Cells (1 × 10^5), respectively, transfected with STAT5a or STAT5b siRNA (50 nM) were maintained in serum-free medium for 48 h. The medium was collected, and the concentrations of vascular endothelial growth factor (VEGF), matrix metalloproteinases 2 (MMP2) and matrix metalloproteinases 9 (MMP9) in the medium were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, USA) according to the manufacturer's instruction.

2.4. Quantitative real-time PCR assay to detect Bcl-2, p21waf1/cip1, p27kip1, FAK and VEGF mRNA

Quantitative PCR was carried out using FastStart SYBR Green Master (Roche) in the Prism 7900HT sequence detection system (Applied Biosystems). The real-time PCR primers of each gene are as follows: for Bcl-2, 5'-TGCTGGAGAATCTTCCAGG-3' (Forward), 5'-TCCGAATCTCAAGGAACCAA-3' (Reverse); for p21waf1/cip1, 5'-CTGGAAGACTCTCAGGTCCGA-3' (Forward), 5'-GGATTAGGGCTTCCTCTTGG-3' (Reverse); for p27kip1, 5'-CGTCGGGTGTACCGAGGACG-3' (Forward), 5'-GATTCTTCTTCCGAACACAGAG-3' (Reverse); for FAK, 5'-GGTTGGGCTGAGGATACCTCTCCTGG-3' (Forward), 5'-CGTGGAGGAGTCAAGGAC-3' (Reverse); and for VEGF, 5'-CGTGGAGACATCTCTCAGAGGAC-3' (Forward), 5'-GGAGCTCACTCTCGTGGGTC-3' (Reverse). The PCR cycles were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was performed in triplicate and analyzed individually, relative to GAPDH (a normalization control), calculated using the 2^-ΔΔCt method [29]. Thereafter, data for transcript expression levels were
expressed as fold difference relative to that of negative control cells.

2.5. Cell viability assay

Cell viability was assessed by a tetrazolium salt (WST-8)-based colorimetric assay in the Cell Counting Kit 8 (CCK-8, Dojindo, Japan) [30,31]. Briefly, control and treated GBM cells were seeded onto 96-well plates at an initial density of 5 × 10^3 cells/well. At specified time points, 10 μl of CCK-8 solution was added to each well of the plate. Then the plate was incubated for 1.5 h. Cell viability was determined by scanning with a microplate reader at 450 nm. Data were expressed as the percentage of viable cells as follows: relative viability (%) = \( \frac{A_{450(\text{treated})} - A_{450(\text{blank})}}{A_{450(\text{control})} - A_{450(\text{blank})}} \times 100\% \).

2.6. Cell cycle analysis using propidium iodide and flow cytometry

Approximately 5 × 10^5 cells were removed at specified time points, washed twice with PBS and fixed in cold ethanol for 30 min, and then incubated with propidium iodide for 30 min. Thereafter, cells were analyzed by a flow cytometer (BD, USA).

2.7. Detection of apoptosis

Apoptosis was determined by flow cytometry analysis, and annexin-V FITC/propidium iodide (PI) double stain assay was performed in accordance with the manufacturer's protocol (Biovision, USA). Briefly, both floating and trypsinized adherent cells (5 × 10^5) were collected and resuspended in 500 μl of binding buffer containing 5 μl of annexin-V FITC and 5 μl of PI, then incubated for 5 min in the dark at room temperature. Analysis was immediately performed using a flow cytometer (BD, USA).

2.8. In vitro invasion assay

Cell invasion assay was performed as described by Hecht and coworkers [32]. In brief, cells were transfected with siRNA (50 nM) for 48 h. And then cells (1 × 10^5) in 250 μl of serum-free medium were seeded into the upper chamber, whereas medium supplemented with 10% fetal bovine serum was applied to the lower chamber as a chemo-attractant to induce invasion. Cells transfected with nonspecific siRNA were used as the negative control. After incubation for 24 h, nonmigrated cells on the upper chamber of the filter were removed with a cotton swab. Migrated cells on the bottom surface of the filter were fixed, stained and counted.

2.9. Tissue microarray and immunohistochemical staining

The glioma cases included in this study were 22 diffuse astrocytoma (grade II; 12 males, 10 females; age range 21–69 years, mean = 47.1 years), and 14 glioblastoma multiforme (GBM; grade IV; 8 males, 6 females; age range 23–71 years, mean = 50.5 years). The protocol had the approval of the ethics committee of the Fourth Military Medical University and the research was carried out according to the provisions of the Helsinki Declaration of 1975. None of the patients received preoperative treatments such as radiotherapy or chemotherapy. Meanwhile, nine specimens of normal cortex, taken from patients without glioma (6 males, 3 females; age range 16–57 years mean = 42.2 years), were used as a negative control. Tissue microarray (diameter, 1.0 mm; depth, 4 μm) was prepared by Chao-Ying Biotech (Xi’an, China) using standard techniques [33].

The tissue microarray sections were deparaffinized in xylene and rehydrated using a graded series of ethanol. A three-step streptavidin–biotin–horseradish peroxidase method was used, and the expressions of STAT5a and STAT5b were examined with the primary antibodies (STAT5a, dilution 1:150; STAT5b, dilution 1:100) using the LSAB+ kit (DakoCytomation, Denmark) according to the manufacturer’s instructions.

The slides were examined independently by two investigators blinded to both clinical and pathologic data. Protein expression was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells; graded on a scale of 0 to 4: 0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >75%) and the intensity of staining (graded on a scale of 0–3: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). For further analysis, we used a product of grades of the extent and intensity of staining to define the cutoff value for high expression of the proteins, and the protein expression was classified into two categories: high (grades 12–4) and low (grades 0 and 3).

2.10. Statistical analysis

Results were expressed as means ± SD. The data were analyzed for significance by ANOVA. Analyses comparing the expression of STAT5a and STAT5b were performed with \( \chi^2 \) test, and results were considered significant if \( P < 0.05 \).

3. Results

3.1. Targeting STAT5b, but not STAT5a, inhibits GBM cell growth and induces G1 cell cycle arrest

As detected by the CCK-8 assay, RNAi-induced STAT5b deficiency, but not STAT5a deficiency, inhibited GBM cell growth (Fig. 1A). To further explore the reason for the decrease in cell viability, we examined the effects of STAT5a-, -5b on cell cycle progression and apoptosis. As illustrated in Fig. 1B, pretreatment of GBM cells with STAT5b siRNA blocked the cell cycle in G1 phase. In U251 cells, for example, the G0/G1-phase fraction increased from 46.7% (untreated) to 68.4% at 72 h post-transfection with 50 nM of STAT5b siRNA. And the overall pattern was similar for U87 cells (Fig. 1B). At 72 h post-transfection, the G0/G1-phase fraction increased from 38.2% (untreated) to 64.1%. However, no significant change in cell cycle arrest was seen in both cells treated with STAT5a RNAi. Meanwhile, to evaluate whether the decrease in cell viability might also have occurred due to apoptotic cell death, we also performed flow cytometry analysis. However, there was no significant increased apoptosis in STAT5a and STAT5b transfected cells, when compared with the negative control cells (Fig. 1C). Therefore, our results indicate that STAT5-induced decrease in cell viability can be attributable at least in part to the occurrence of G1 cell cycle arrest after inhibition of STAT5b expression.

Besides, the molecular basis for cell growth inhibition and cell cycle arrest in GBM was also investigated. And the expression of various apoptosis- and cell cycle-regulatory proteins known to be downstream targets...
Fig. 1. The functional role of STAT5 isoforms on GBM cell growth and cell cycle progression. (A) Cell viability as determined by the CCK-8 assay of GBM cell treated with STAT5a and STAT5b siRNA. Cells transfected with nonspecific siRNA were used as the negative control, and the data were expressed as the percentage of viable cells as follows: relative viability (%) = \( \frac{A_{450\text{ (treated)}} - A_{450\text{ (blank)}}}{A_{450\text{ (control)}} - A_{450\text{ (blank)}}} \times 100\% \). STAT5b siRNA inhibited GBM cell growth and this suppression lasted for 72 h. The results represent means ± SD of three experiments. (B) Cell cycle analysis was performed and treating GBM cell with STAT5b siRNA blocked the cell cycle in G1 phase at 72 h after transfection. (C) Cell apoptosis was detected by flow cytometric analysis. However, there was no significant increased apoptosis in STAT5a and STAT5b transfected cells, when compared with the negative control cells.
of the STATs pathway was examined. We depleted STAT5a and STAT5b with siRNA (Fig. 2A). At 72 h post-transfection, RNAi-induced STAT5b deficiency, but not STAT5a deficiency, induced down-regulation of Bcl-2 simultaneously with up-regulation of p21<sub>waf1/cip1</sub> and p27<sub>kip1</sub> in both GBM cells (Fig. 2B). However, no significant change in survivin was seen in cells treated with either STAT5a or STAT5b RNAi. Furthermore, we also performed quantitative real-time PCR assay to measure mRNA, and the results correlated with those of Western blot. In U251 cells, for example, after 72 h post-transfection with 50 nM of STAT5b siRNA, Bcl-2 mRNA was decreased to 0.156, when compared to cells transected with nonspecific siRNA. However, the expression levels of p21<sub>waf1/cip1</sub> and p27<sub>kip1</sub> mRNA were approximately 6.98- and 7.19-fold higher than those of untreated U251 cells (Fig. 3D). These results demonstrate that STAT5b may be involved in transcriptional modification of Bcl-2, p21<sub>waf1/cip1</sub> and p27<sub>kip1</sub> in GBM cell.

3.2. Disruption of STAT5a and STAT5b by RNAi inhibits GBM cell invasion

We next addressed the functional role of STAT5a, -5b in the invasion of the cell into the surrounding tissue. As shown in Fig. 3A, both STAT5a and STAT5b siRNA suppressed GBM cell invasion. And notably, the numbers of migrated cells was more markedly suppressed by STAT5b siRNA treatment than by STAT5a siRNA treatment (*<i>P</i> < 0.05). For example, at 48 h post-transfection, the number of STAT5a siRNA and STAT5b siRNA treated U251 cells (Fig. 3D). These results demonstrate that STAT5b may be involved in transcriptional modification of Bcl-2, p21<sub>waf1/cip1</sub> and p27<sub>kip1</sub> in GBM cell.

**Fig. 2.** RNAi induces down-regulation of STAT5 signaling in GBM cell. (A) At 72 h post-transfection, Western blot analysis showed that STAT5a and STAT5b siRNA induced down-regulation of STAT5a and STAT5b in GBM cell, respectively. (B) STAT5b siRNA induced alterations of part but not all of the STATs downstream targets in GBM cell at 72 h after treatment. Bcl-2 was down-regulated, simultaneously associated with up-regulation of p21<sub>waf1/cip1</sub> and p27<sub>kip1</sub>, whereas survivin showed no detectable change. The data shown are representative of three separate experiments. GAPDH was used for the loading control. Quantification of the target protein bands relative to GAPDH is shown in right panel.
treated U87 cells that migrated through the filter decreased to 73.5% and 57.3%, respectively, when compared to cells transfected with nonspecific siRNA. Also, the overall pattern was similar for U251 cells.

To better understand the mechanisms of STAT5a, -5b on GBM cell invasion and to reveal downstream events of STAT5 signaling that are involved in the regulation of cell invasion, we examined the expression of various migration and invasion regulatory proteins by real-time PCR, Western blot and ELISA analyses. As shown in Fig. 3B, although both STAT5a and STAT5b deficiency induced down-regulation of FAK and VEGF in GBM cell at 72 h post-transfection. (C) Effects of STAT5a and STAT5b siRNA on the secretions of VEGF, MMP2 and MMP9. At 48 h post-transfection, the concentrations of VEGF was decreased compared to that of untreated cells (P < 0.05). Additionally, FAK and VEGF were more markedly down-regulated by STAT5b siRNA treatment than by STAT5a siRNA treatment. The experiment was performed three times with consistent findings. (D) Quantitative real-time PCR analysis for Bcl-2, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, FAK and VEGF in GBM cells. Results are expressed as relative expression compared with untreated cells. Each value is the mean ± SD.

3.3. STAT5b is up-regulated in GBM relative to normal cortex and diffuse astrocytoma

STAT5a and STAT5b showed predominantly cytoplasm localization. High level expression of STAT5b was detected in 57.1%, 27.3% and 22.2% of the GBM samples, diffuse astrocytoma samples, and normal cortex samples, respectively. High level expression of STAT5a was found in 22.2% of the normal cortex tissue samples, 18.2% of the diffuse astrocytoma samples and 28.6% of the GBM samples (Table 1, Fig. 4). Thus, GBM showed higher expression of STAT5b than normal cortex and diffuse astrocytoma (P < 0.05). Additionally, our results suggest that up-regulation of STAT5b in GBM could have important implications in astrocytoma biology.

4. Discussion

Although constituent activation of STAT5 has been reported in hematopoietic malignancies, prostate and breast cancer [19,20,27], previous studies have generally not distinguished the key functional role of each STAT5 isoforms. Studies of knockout mice revealed the functional difference between these two closely linked STAT proteins. Moreover, STAT5a was found to play an important role in breast cancer [27], whereas STAT5b activation contributes to carcinogenesis of prostate cancer [28]. Due to such contrasting data, it is of great importance to determine the distinct role of each STAT5 isoforms in cancer. In the present study, we provide experimental and mechanistic evidences that STAT5b contribute to tumor progression in human GBM cell.

Table 1

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<th>STAT5a expression (%)</th>
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<td>High</td>
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<tr>
<td>Normal cortex</td>
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<td>2 (22.2)</td>
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<tr>
<td>Diffuse astrocytoma (grade II)</td>
<td>22</td>
<td>4 (18.2)</td>
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<td>GBM (grade IV)</td>
<td>14</td>
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We first evaluated the biological significance of STAT5a and STAT5b in the pathogenesis of GBM cell. Although both originated from glioblastomas, the U251 and U87 cell lines are not isogenic (e.g., U251 contains mutant p53 and U87 contains WT p53), and these two cell lines have different genetic and epigenetic background. Therefore, two different GBM cell lines were chosen in our studies. Our results indicated that down-regulation of STAT5b, but not STAT5a, was associated with a gradual decrease in viable cells. Moreover, the decrease in cell viability can be attributed to the occurrence of G1 cell cycle arrest after inhibition of STAT5b in GBM cell. And these observations are consistent with up-regulation of p21waf1/cip1 and p27kip1 expression, followed by suppression of STAT5b, suggesting that the STAT5b is involved in cell cycle regulation. Additionally, despite the decrease in Bcl-2, we did not detect significant apoptotic cell death by suppression of each STAT5 gene. This result can at least be partially explained by the persistent expression of survivin. And our data indicate that STAT5 is essential but not sufficient in the survival process in GBM cell.

GBM exhibits a high degree of diffuse local invasion of normal nervous tissue. GBM cells often infiltrate beyond any obviously defined tumor margin and contribute to the high incidence of recurrence [34]. Therefore, it is of great value to study the molecular mechanism of GBM invasiveness. The Matrigel invasion assay showed that an inverse relationship between the invasiveness of GBM cell and inhibition of both STAT5 genes. Of note, GBM cell invasion was more markedly suppressed by STAT5b deficiency. And these observations are consistent with more markedly down-regulation of FAK and VEGF by STAT5b siRNA treatment than by STAT5a siRNA treatment. Thus, our data suggest that STAT5 may be involved in the regulation of the expressions of FAK and VEGF in GBM; STAT5 signaling may affect GBM metastasis by multiple mechanisms including angiogenesis, adhesion and migration; and STAT5b might play a more important role in such process. Furthermore, immunohistochemical staining showed that STAT5b expression is markedly increased in GBM (57.1%) compared with expression in normal cortex (22.2%) and diffuse astrocytoma (27.3%). Thus, STAT5b may play a significant role in astrocytoma progression and could be a potential therapeutic target for astrocytoma treatment. In addition, each STAT5 protein showed predominantly cytoplasm localization. These results support the concept that a large part of oncogenic STAT5 activity might not only involve a nuclear function but also a cytoplasmic function [35]. For example, a recent report documented STAT5 was present in the PI3K immunoprecipitates in leukemic bone marrow cells, suggesting crosstalk between STAT5 signaling and PI3K pathway in the development of myeloid leukemias [35]. Thus, we presume that STAT5 protein may play an integral part as cytoplasmic signaling effectors via its association with various cytoplasmic signaling molecules in the development of astrocytoma. However, further studies are needed to validate this hypothesis.

In conclusion, this study is the first to have examined in detail the mechanistic role of each STAT5 isoforms in GBM tumorigenesis and progression. Our work suggests that blocking STAT5b activity in GBM is a potential novel therapeutic approach, since it is implicated in many areas of tumor progression, including cell growth, cell cycle regulation, invasion and migration. Intervention in STAT5b function by agents, such as siRNA, may have potential therapeutic value in the prevention of GBM. Thus, our findings not only provide a molecular basis for the role of STAT5b in GBM but also suggest a novel therapeutic target for the treatment of GBM.

Conflict of interest

The authors have declared that no conflict of interest exists.
References


