Glioma is the most common primary tumor of the CNS in humans. Of the various types of glioma, GBM is the most frequent and aggressive, and is characterized by highly malignant features. Because of their diffuse infiltrative nature, most gliomas are not curable by resection, and they are extremely resistant to radiotherapy and/or chemotherapy. Those characteristics make GBMs extraordinarily lethal.\(^{15,30}\)

Recent studies have demonstrated that the administration of temozolomide in addition to radiotherapy in cases of newly diagnosed GBM resulted in significant survival benefit, but the median survival was still only 14.6 months.\(^{29,41}\) Furthermore, the efficacy of the combination of bevacizumab (a humanized monoclonal antibody against VEGF) and irinotecan has been reported to be superior to that of temozolomide in the treatment of recurrent GBM.\(^{1,35,36}\) However, despite these extensive clinical studies, outcomes in patients with malignant gliomas remain very poor. New alternative strategies for treatments are thus urgently needed.\(^{25}\)

Recent studies have shown that glioma cells produce IL-6 and its expression may play an important role in gliom-
Interleukin-6 is a pleiotropic cytokine that regulates immune response, inflammation, hematopoiesis, and diverse physiological functions, including the developmental differentiation of lymphocytes and astrocytes, cell proliferation, survival, and amelioration of apoptotic signals. Accordingly, it is involved in numerous disease states physiologically. Consequendy, modulating IL-6 may be an innovative therapeutic strategy in those diseases. It has been reported that IL-6 expression or an increase in plasma IL-6 level may play important roles in neoplasms such as leukemia, multiple myeloma, prostate cancer, ovarian cancer, gastrointestinal cancer, metastatic renal cell carcinoma, and Kaposi sarcoma. Interleukin-6 also plays a crucial role in promoting tumor growth and survival in several organ systems and has a growth stimulatory effect and increases angiogenesis via upregulation of VEGF expression in several types of cancer. Recent studies have revealed that IL-6 is highly produced in glioma as well as several other cancers and promotes autocrine cell growth in the human GBM cell line U87MG. Moreover, the level of IL-6 gene expression has been found to increase with the grade of malignancy in glioma. Despite the importance of IL-6 in various types of tumors, however, the biological role of IL-6 in glioma cells still remains to be elucidated.

In this study, we evaluated the involvement of IL-6 signaling in glioma and the effect of inhibiting IL-6 signaling on glioma tumor progression. We studied the expression of IL-6R in glioma tissues by means of immunohistochemistry and also determined the involvement of IL-6 signaling in GBM U87MG cell proliferation. In addition, to examine the inhibitory effect of IL-6 signaling on glioma cell proliferation, we investigated the effects of tocilizumab, a clinically developed humanized anti-human IL-6R antibody in U87MG cells.

Methods

Tocilizumab was donated by Chugai Pharmaceutical Co. Ltd. Mouse monoclonal anti–IL-6R antibody was obtained from Chemicon. Antibodies against phosphorylated STAT3 (pSTAT3 Tyr705) and STAT3 were purchased from Cell Signaling Technology, Inc. The monoclonal anti–α-tubulin antibody was obtained from Sigma-Aldrich Co. The JAK inhibitor AG490 was purchased from Calbiochem.

Patients and Tissue Specimens

Tissue specimens from GBMs obtained at resection (6 tumors, 1 from a male and 5 from female patients; age range 27–73 years, mean 51.8 years, median 50.5 years) and normal brain obtained at autopsy (7 specimens, 4 from male and 3 from female cadavers; age range at death 42–73 years, mean 55.3 years, median 53.0 years) from the Department of Neurosurgery and Neurology of Kumamoto University Hospital were used in immunohistochemical analysis. All protocols were approved by the Internal Review Board of Kumamoto University, and prior informed consent was obtained from all patients. All histological diagnosis was confirmed by standard histological analysis of specimens as previously described.

Cell Culture

The human glioma cell line U87MG was obtained from the European Collection of Cell Cultures, and A172 and T98G were obtained from the American Type Culture Collection. All cells were grown in Dulbecco modified Eagle medium and Ham’s F-12 medium (Wako) supplemented with 10% fetal bovine serum, penicillin (35 mg/L), and streptomycin (30 mg/L) at 37°C in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry

Formalin-fixed specimens were embedded in paraffin, cut into 3-µm sections, and mounted onto slides. Sections were de-waxed in xylene and rehydrated in descending concentrations of alcohol. Endogenous peroxidase and nonspecific background staining was blocked by incubating slides with 3% hydrogen peroxide for 10 minutes. After slides were washed with PBS for 5 minutes, they were blocked by immersion in PBS containing 3% bovine serum albumin for 30 minutes, and then incubated overnight at 4°C with anti–IL-6Rα antibodies diluted 1:50 in PBS containing 3% bovine serum albumin. After slides were rinsed with PBS for 5 minutes, they were incubated for 1 hour with horseradish peroxidase–conjugated mouse IgG and then washed again. Chromagen was developed with 3,3-diaminobenzidine (Dako). All slides were lightly counterstained with hematoxylin for 1 minute before dehydration and mounting.

Cell Proliferation Assay

The cells were seeded in 96-well plates with 1 × 10³ cells per well in quintuplicates and allowed to adhere overnight. Then culture medium was replaced with control medium or medium containing various reagents at indicated concentrations following 24 hours of serum starvation. The antiproliferative effect of tocilizumab and AG490 against GBM cell lines was determined by the MTT dye uptake method as described previously. The absorbance was measured at 570 nm with a 96-well plate reader.

Western Blot

The cells were washed once in ice-cold PBS and then lysed by adding lysis buffer (25 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride [PMSF], 4 mmol/L NaVO₄, and 1 mmol/L DTT). Cell lysates were then centrifuged at 14,000 rpm for 10 minutes to remove insoluble material and fractionated on 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT3 or anti-pSTAT3 antibodies (1:1000) overnight at 4°C. The blot was washed, exposed to horseradish peroxidase–conjugated secondary antibodies for 1 hour, and visualized using the ECL (enhanced chemiluminescence) detection system from Amersham Biosciences.

Real-Time PCR

Total RNA was isolated from treated cells by TRIzol reagent (Invitrogen Corp.), according to the manu-
facturer’s instructions. Total RNA (0.5 μg) was reverse transcribed to cDNA by using ExScript RT reagent (Takara Bio, Inc.), according to the manufacturer’s protocol. Each PCR was performed with 2 μl of the cDNA and 0.2 μmol/L of each probe in a LightCycler System with SYBR Premix Ex Taq (Takara Bio, Inc.). Polymerase chain reaction primer sequences were as follows: for IL-6, forward primer 5′-AAGCCAGAGCTGTCAGATGATGTA-3′, reverse primer 5′-TGTCCTGCAAGCACTGGTTT-3′; for IL-6Rα, forward primer 5′-TGGCAGAGGAGATACCG GGCTGAAC-3′, reverse primer 5′-CGTCTGGAGATGAC ACAGTGATG-3′; for B2M forward primer 5′-CGGGCAT TGAGCTCAGATATCG-3′, reverse primer 5′-GGATGGGATG AAACCCAGACACATAG-3′. Each reaction (20-μl samples) was performed under the following conditions: initialization for 10 seconds at 95°C, and then 45 cycles of amplification, with 5 seconds at 95°C for denaturation, and 20 seconds at 60°C for annealing and elongation. Beta-2-microglobulin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin (“actin, beta,” ACTB) were tested as our potential reference genes. Because changes in expression of B2M, of the 3 genes, were minimum (data not shown), as previously described,7 we selected B2M as the reference gene. Data that were below the limit of detection sensitivity were set at 0.00. A standard curve was plotted for each primer probe established by using a serial dilution of pooled cDNA from cells. All standards and samples were analyzed in triplicate.

Small Interfering RNA Transfections

Small interfering RNA for IL-6, IL6R, and negative control was purchased from Applied Biosystems (catalog nos. AM16708, AM4611). The following siRNA sequences were used: for IL-6, sense 5′-GGACAGAAGAGUCUCACUCUC UCtt-3′, antisense 5′-GAGAUGAGUUGCAUGUCGtg-3′; and for IL-6Rα, sense 5′-CGACUCUGGAAACAUUC Att-3′, antisense 5′-UGAAUAGUUCUCCAGAGCtg-3′. We cultured U87MG cells on 12-well plates. A final concentration of 200 nM siRNA was transfected into 80% confluent cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

Statistical Analysis

The Student t-test and analysis of variance were used for statistical analysis. Probability values < 0.05 were regarded as statistically significant.

Results

Expression of IL-6R in GBM

We first performed immunohistochemical studies with 6 GBM and 7 normal brain specimens using a monoclonal anti–IL-6Rα antibody (Fig. 1A). Positive immuno-reactivity for IL-6R protein was limited in the cytoplasm of both malignant cells and normal astrocytes. In all GBM samples, the reactivity was predominantly found in the cytoplasm of endothelial cells in newly formed vessels. In contrast, no immuno-reactivity was observed in vessels of normal brain specimens. We next examined the relative expression of IL-6 and IL-6R mRNA in several types of glioma cell lines, such as U87MG, A172, and T98G using real-time PCR. As shown in Fig. 1B, U87MG, one of the most common glioblastoma cell lines, exhibited higher expression of both IL-6 and IL-6R mRNA than other cell lines did. These data suggest that IL-6R downstream signaling pathway may play an important role in glioma tumor progression.

Involution of the IL-6R Signaling Pathway in Glioma Cell Proliferation

We next sought to determine whether the IL-6R signaling pathway was involved in glioma cell proliferation using IL-6– and IL-6R–specific siRNA. The IL-6– and IL-6R–specific siRNA efficiently reduced endogenous protein expression levels of IL-6 and IL-6R, respectively (data not shown). As shown in Fig. 2A and B, knockdown of both IL-6 and IL-6R by siRNA suppressed the proliferation of U87MG cells, indicating that the IL-6 signaling pathway may regulate glioma cell proliferation.

Effect of AG490 on Glioma Cell Proliferation

It has been reported that the IL-6 signaling pathway
regulates tumor cell growth by activating JAK-STAT3 pathway. To elucidate the inhibitory effect of the IL-6R signaling pathway on glioma cell proliferation further, we used AG490, a specific inhibitor for JAK2 phosphorylation that consequently inhibits STAT3 activation. As shown in Fig. 3A, AG490 significantly inhibited the proliferation of U87MG cells in a dose-dependent manner. Concomitantly, while STAT3 was constitutively phosphorylated in Fig. 2, AG490 inhibited cell proliferation of U87MG glioma cells. Cell number was evaluated by MTT assay. ‡p < 0.01, Student t-test.

Fig. 2. Influence of IL-6 and IL-6R in glioma cell proliferation. A: Graph showing that siRNA for IL-6 and IL-6R inhibits cell proliferation of U87MG glioma cells. Cell number was evaluated by MTT assay. ‡p < 0.01, Student t-test. B: Photomicrographs showing cell morphology after transfection with siRNA for IL-6, IL-6R, or control. Original magnification × 40.

Fig. 3. Influence of the JAK-STAT3 pathway in glioma cell proliferation. A: Graph showing that AG490 inhibited proliferation of U87MG cells in a dose-dependent manner. Data were obtained after the cells were treated for 72 hours with JAK2-specific inhibitor AG490. p < 0.01, Student t-test. B: Photomicrographs showing the morphological changes in U87MG cells after 72 hours’ incubation with AG490. Original magnification × 100. C: Immunoblot showing that AG490 inhibited phosphorylation of STAT3.
Antiproliferative effect of tocilizumab on glioma cells

U87MG cells, AG490 (50 μM) treatment strongly reduced its phosphorylation (Fig. 3C). These results suggest that the IL-6 signaling pathway may play an important role in glioma cell proliferation and could be a potential target for the treatment of glioblastoma.

**Effect of Tocilizumab on Glioma Cell Proliferation**

Tocilizumab, a clinically developed humanized anti-human IL-6R antibody, specifically blocks IL-6 signaling pathways and ameliorates diseases characterized by IL-6 overproduction.\(^2^1,^2^4\) We evaluated whether tocilizumab had an antiproliferative effect on U87MG cells. The antibody inhibited U87MG cell proliferation in a dose- and time-dependent manner (Fig. 4A–C). In this experiment, we incubated the cells under serum-free conditions during treatment with tocilizumab to eliminate influences of IL-6 and soluble IL-6R in serum. We speculate that such influences may be why the cell proliferation ratio decreases 24 hours after administration of tocilizumab. Accordingly, tocilizumab also inhibited the phosphorylation of STAT3, as AG490 did (Fig. 4D). Taken together, these results indicate that tocilizumab exerts an antiproliferative effect on glioblastoma via the IL-6R–dependent JAK-STAT3 pathway.

**Discussion**

In our study we demonstrated evidence that the IL-6 signaling pathway played an important role in glioma cell proliferation and that tocilizumab exerted an antitumor effect in U87MG glioma cells by inhibiting the IL-6R-depen-
dent JAK-STAT3 pathway. The present results are consistent with the those of previous studies: neutralization of IL-6 signaling by anti–IL-6 antibody inhibits cell proliferation and induces apoptosis.\textsuperscript{6,8,25} Tocilizumab, a clinically developed humanized anti–human IL-6R antibody, specifically blocks the IL-6 signaling pathway and ameliorates various diseases that involve IL-6 overproduction.\textsuperscript{21,24} Nishimoto and Kishimoto\textsuperscript{21} reported that tocilizumab was therapeutically effective for rheumatoid arthritis, systemic juvenile idiopathic arthritis, and Castleman disease, all of which are characterized by IL-6 overproduction. Now this antibody is an accepted treatment for these diseases in Japan. Therefore, this antibody also could be used as therapeutic strategy for glioma. However, several issues still remain to be elucidated. First, it is necessary to determine whether tocilizumab can cross the BBB. Since it is thought that tumor might possibly alter BBB permeability,\textsuperscript{2} it seems not unlikely that tocilizumab might be delivered to the tumor by intravenous administration. Moreover,\textsuperscript{10} several clinical trials of Aβ immunotherapy are currently underway in patients with Alzheimer disease (studying, for example, bapinezu- zumab). Results of these trials are showing that antibodies against β-amyloid can accumulate at brain β-amyloid deposits, and even small amounts of antibodies penetrating the BBB may be sufficient to cause therapeutically relevant brain concentrations over time.\textsuperscript{22} It is suggested that similar mechanism can be observed in tocilizumab. In addition, several studies have reported that treatment with the anti-VEGF antibody bevacizumab and irinotecan is effective for recurrent GBM.\textsuperscript{1,5,26} Although it is not yet clear whether bevacizumab can cross the BBB sufficiently, we speculate that it might be able to. As to other methods of delivery, it has been reported that local implantation of drug-releasing biodegradable microspheres or wafers is an efficient tool for drug delivery into brain tumor.\textsuperscript{16–18,40} Even if tocilizumab could not cross the BBB sufficiently, we suspect that the similar methods can be applicable. Further investigations regarding delivery of this antibody are required. Second, although we also examined the effect of tocilizumab using glioma cell lines A172, U87MG, and T98G, the antiproliferative effect of tocilizumab was observed in only U87MG cells (data not shown). Interestingly, of these cell lines, only U87MG has both wild type p53 expression\textsuperscript{13,27} and high levels of IL-6 expression (Fig. 1B). Because STAT3 inhibits p53 expression,\textsuperscript{21} p53 status may affect the effect of tocili- zumab. Third, immunohistochemical analysis showed IL-6R expressed highly in the cytoplasm of endothelial cell of intratumoral new vessels. Interestingly, previous studies have shown that IL-6 signaling may play important roles in tumor angiogenesis. It is well documented that IL-6 promotes the release of VEGF from glioblastoma cells\textsuperscript{3} and astrocytes\textsuperscript{4} and leads to tumor angiogenesis. Therefore, we consider it possible that tocilizumab may inhibit angiogenesis by inhibiting both IL-6 signaling and following transcription of VEGF. In fact, several studies also support the idea that IL-6 is a potent pro-angiogenic cytokine and a role for IL-6 in the neovascularization that accompanies physiological tissue remodeling.\textsuperscript{20} It is possible that IL-6 might be involved in intratumoral proliferation of new vessels and tumor growth. Therefore, it is necessary to investigate whether tocilizumab inhibits angiogenesis.

Conclusions

Our results show that the IL-6 signaling pathway plays an important role in glioma cell proliferation and that tocilizumab exerts antitumor effect in U87MG glioma cells. These findings may lead to trials of tocilizumab inhibition of IL-6 signaling. Study of IL-6 signaling may bring new insight concerning the molecular pathogenesis of glioma and may lead to new therapeutic interventions.

Disclosure

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Antiproliferative effect of tocilizumab on glioma cells


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