

Increased expression of CCAAT/enhancer binding protein β correlates with prognosis in glioma patients

JUMPEI HOMMA, RYUYA YAMANAKA, NAOKI YAJIMA, NAOTO TSUCHIYA,
NOBUYUKI GENKAI, MASAKAZU SANO and RYUICHI TANAKA

Department of Neurosurgery, Brain Research Institute, Niigata University, 1-757 Asahimachi-dori, Niigata 951-8585, Japan

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Abstract. C/EBP β (CCAAT/enhancer binding protein β) is a transcriptional factor that belongs to the basic region-leucine zipper class DNA-binding proteins and plays a role in cell differentiation and inflammatory reactions. Although high tissue levels of inflammatory cytokines, such as interleukin (IL)-6, IL-8 and transforming growth factor- β , have been observed in glioma patients, the mechanisms underlying this phenomenon remain to be elucidated. C/EBP β induces a variety of cytokines and thus may play a role in the pathogenesis of glioma. In this study, we investigated the relationship between C/EBP β expression, tumor histology, and prognosis in glioma. The expression of C/EBP β mRNA was examined with quantitative real-time PCR and protein expression was examined with immunohistochemical techniques in 47 glioma tissue samples. Expression of C/EBP β mRNA and protein was markedly increased in high grade glioma compared with low grade glioma. Patients whose expression of C/EBP β mRNA and protein in tumor tissue was lower survived longer than those whose expressions were higher. *In vitro*, C/EBP β siRNA inhibited glioma cell proliferation and invasion. Moreover, IL-8 production by glioma cells was inhibited by C/EBP β siRNA transfection. These data suggest that increased expression of C/EBP β may contribute to the promotion of tumor invasiveness and progression. The data imply that the comparison of C/EBP β expression could be a prognostic marker for patients with glioma.

Introduction

Glioblastoma is an ordinary solid tumor with poor prognosis; even with recent advances in cancer diagnostic methodology and treatment, the prognosis of glioblastoma worldwide has not improved (1,2). This poor prognosis is attributable to the difficulty of early detection and a high recurrence rate during post-initial treatment observation periods. This is at least partly due to the lack of reliable tumor markers for, and molecular targets against, glioblastomas.

The CCAAT/enhancer binding proteins (C/EBPs) comprise a family of structurally related transcription factors which play a role in normal tissue development, cellular proliferation and differentiation (3). The typical C/EBP protein consists of a N-terminal activation domain, a basic DNA binding region, and a leucine zipper dimerization domain that enables dimer formation with other C/EBP proteins as well as with transcriptional factors of the AP-1, NF- κ B (3-5). All of these distinct interactions result in the differential transcriptional activity of the C/EBP isoforms. At present, six members (C/EBP α , β , γ , δ , ϵ , and ζ) with different tissue-specific expression patterns have been characterized (3,6-14). C/EBPs exert pleiotropic effects based on tissue- and stage-specific gene expression, alternative translation of various protein isoforms, interaction with other transcription factors, and variable DNA-binding specificities. C/EBPs are reported to play an important role in the control of, and as essential transducers of intracellular responses to extracellular control signals (15). The expression of these proteins is influenced by a variety of external stimuli such as growth factors, hormones or cytokines (3,16). In addition, by using different start codons, the translation of C/EBPs results in two or more protein products which differ in their biological activities (3,12,17,18). As an additional level of regulation, post-translational phosphorylation influences DNA binding and transactivation properties (3,19).

C/EBP β is ubiquitously expressed and is induced by such inflammatory cytokines as IL-1, IL-6 and TNF- α (20). C/EBP β was originally identified and named NF-IL-6 as a DNA-binding protein responsible for IL-1-stimulated IL-6 induction (8). It was recently reported that C/EBP β induces IL-8 and TNF- α expression (21,22). IL-8, an angiogenic cytokine secreted from glioma cells, is thought to be a causative cytokine of hypervascularity in malignant glioma (23). Thus, C/EBP β may play a role in the tumor progression of glioma.

Correspondence to: Dr Ryuya Yamanaka, Department of Neurosurgery, Brain Research Institute, Niigata University, Asahimachi-dori 1-757, Niigata City 951-8585, Japan
E-mail: ryaman@bri.niigata-u.ac.jp

Abbreviations: C/EBP, CCAAT/enhancer binding protein; IL, interleukin; MEM, minimum essential medium; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline

Key words: CCAAT/enhancer binding protein β , glioma, interleukin-8, siRNA

Table I. Patient characteristics.

Pathological diagnosis	No. of cases	Gender (male/female)	Age (mean \pm SD)
Astrocytoma	9	4/5	28.0 \pm 18.0
Anaplastic astrocytoma	11	5/6	38.6 \pm 12.5
Glioblastoma	27	17/10	52.0 \pm 16.9
Total	47	26/21	44.23 \pm 18.6

An increased expression of C/EBP β is reported in breast cancer (24), ovarian cancer (25), colorectal cancer (26) and renal cell carcinoma (27). In contrast, down-regulated expression has been reported in squamous cell carcinoma (28) and hepatocellular carcinoma (29). In this study, we investigated the expression of C/EBP β in 47 glioma tissue specimens of different grades, and whether or not an increased expression of C/EBP β correlated with patient prognosis.

Materials and methods

Cell lines and culture. All glioma cell lines were cultured in MEM (Nissui Pharmaceutical Inc., Tokyo, Japan) supplemented with 10% FBS (fetal bovine serum). The T98G, GI-1 and U251 cell lines were purchased from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan).

Patient characteristics and tissue samples. A total of 47 glioma tissue samples were investigated that were surgically removed in the Department of Neurosurgery, Brain Research Institute, Niigata University. Tumor tissue and normal brain tissue specimens were dissected and immediately frozen at -80°C . The patient data are shown in Table I. After surgical resection of their tumor, patients with high grade glioma had a course of external beam radiation therapy (standard dose, 40 Gy to the tumor with 3-cm margins, 20 Gy boost to the whole brain) and nitrosourea based chemotherapy during the course of the disease. Survival was determined from the date of diagnosis to death or last visit. These diagnoses were based on the World Health Organization (WHO) criteria (30). This study was approved by the Ethics Committee of Niigata University. Informed consent was obtained from all patients.

Extraction of proteins. Stored samples were crushed in liquid nitrogen and homogenized in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% desoxycholate, 150 mM NaCl, 1 mM EGTA, and 0.2% sodium dodecyl sulfate (SDS)] containing the following kinase, phosphatase, and protease inhibitors: 1 mM NaVO_3 , 1 mM NaF, 1 mM Na_2MoO_4 , 10 nM okadaic acid, 1 $\mu\text{g}/\text{ml}$ benzamide and aprotinin (all Sigma, Tokyo, Japan) and incubated on ice for 15 min. The supernatants were taken and stored at -80°C .

IL-8 determination by ELISA. The cells were cultured in triplicate in MEM containing 10% FBS medium for 48 h.

Cell-free culture supernatants were analyzed for IL-8 protein content by ELISA using a commercial kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Immunoblotting. The extracted protein (100 μg) with sample buffer containing 2-mercaptoethanol was separated on 5-20% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with 5% skim milk in Tris-buffered saline overnight followed by incubation with primary antibodies for C/EBP β (Santa Cruz Biotechnology, Santa Cruz, CA). Anti- β -actin mouse monoclonal antibody (Santa Cruz Biotechnology) was used as an internal control. Immunodetection was performed using a horseradish peroxidase detection system with ECL plus (Amersham, Tokyo).

Immunohistochemistry. Five-micron sections from formalin-fixed, paraffin-embedded tissue specimens were deparaffinized in xylene and dehydrated in graded ethanols, followed by PBS. Antigen was retrieved by incubation at 121°C for 10 min in 10 mM sodium citrate (pH 6.0) followed by an incubation with 0.3% H_2O_2 to quench the endogenous peroxidase activity. The slides were blocked in 10% goat serum and incubated with rabbit polyclonal anti-C/EBP β antibody (dilution 1:50; Santa Cruz Biotechnology) for 24 h at 4°C . After washing, the slides were incubated with an avidin-biotin-peroxidase system (Vectastain ELITE ABC kit, Vector Laboratories, Burlingame, CA). Finally, the sections were exposed for 10-20 min to 0.01% 3,3-diaminobenzidine (DAB) (Sigma) and PBS containing 0.01% hydrogen peroxide. A total of ≥ 500 tumor cells were examined at an original magnification of $\times 400$ by light microscopy, and the intensity of positive cells was recorded for each immunostained specimen. At the time, observers were blind as to case numbers. The expression of C/EBP β was evaluated by assigning an immunohistochemical score, defined as the intensity (0, none; 1, weak; 2, moderate; 3, strong) of the expression.

RNA isolation and quantitative PCR. Total RNA was isolated with Isogen (Nippongene, Toyama, Japan) from the frozen samples. First-strand cDNA was prepared with RNA from the specimens, oligo(dT) primers and reverse transcriptase (SuperScript II RNase H, Life Technologies, Grand Island, NY) according to the manufacturer's instruction. Quantitative PCR was performed by using real-time PCR with a LightCycler (Idaho Technology, Salt Lake City, UT). PCR reagents contained 1X LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), 0.5 μM of each primer, 3 mM MgCl_2 , and 2 μl of cDNA template. PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 5 sec, and 72°C for 10 sec. The 195 bp reaction product was then subjected to a post-PCR melting cycle. The intensity of fluorescence was calculated at each cycle, and the standard curve was constructed with 3-fold serial dilutions of cDNA obtained from glioma cell lines. The primer sequences for PCR amplification were as follows: C/EBP β sense, 5'-ACAGCGACGAGTACAAGATCC-3'; and C/EBP β antisense, 5'-GCAGCTGCTTGAACAAGTTC-3'.

siRNA treatment and cell proliferation assay. Specific siRNA directed against human C/EBP β was purchased from Santa Cruz Biotechnology. The siRNAs were introduced into glioma cell lines by cytofectin-mediated transfection according to the manufacturer's instruction (Qiagen, Tokyo). Cells were cultured in 96-well plates in 100 μ l of serum-enriched medium. When 80% confluence was reached, 25 μ l of 100 nM siRNA in cytofectin was added drop-wise to the cell culture. The number of viable cells was evaluated at 48 h of culture by incubation with Tetra color one (Seikagaku Co., Tokyo) and the values obtained were compared with those of controls. Control experiments were done with Cy3-labeled siRNA (Qiagen) directed against an unrelated mRNA (Luciferase; siRNA_{LUC}: Qiagen). Transfection efficiency was confirmed with Cy3-labeled siRNA_{LUC} in each assay. All proliferation experiments were repeated as independent experiments at least twice. The results represent the mean \pm standard deviation (SD) of two independent experiments.

Cell invasion of Matrigel. A transwell with an 8 μ m diameter pore membrane (Becton-Dickinson, Tokyo, Japan) was coated with 500 μ l of Matrigel (Becton-Dickinson) at 100 μ g/ml. Cells were left untreated, treated with control, and C/EBP β siRNA, transfected as described above. After 24 h of incubation, the cells were detached with cell dissociation solution (Sigma), washed twice with PBS, and resuspended in MEM containing 10% FBS. In the case of the siRNA, this corresponds to a second transfection 24 h after the first. In each case, 2×10^5 cells were seeded into the upper, Matrigel-coated chamber of the Transwell. The lower chamber was filled with MEM with 10% FBS. After 24 h of incubation at 37°C, the non-migrated cells in the upper chamber were gently detached by scraping, and the adherent cells present on the lower surface of each insert were stained by Giemsa; 10 fields were counted by light microscopy at x200 magnification. Results were calculated with reference to control values observed after incubation in untreated control for control and C/EBP β siRNA.

Statistics. The differences in the C/EBP β expression between the subgroups of glioma were tested for statistical significance using the Mann-Whitney U test. Statistical significance was determined at the $P < 0.05$ level. The survival curves were estimated according to the method of Kaplan and Meier and the curves were compared using the generalized Wilcoxon's test. The log-rank test was used to assess the strength of the association between survival time and single variables corresponding to factors thought to be prognostic for survival. Survival was determined from the date of diagnosis to death or last visit.

Results

Immunohistochemical analysis of C/EBP β expression in glioma tissue. Immunohistochemistry was used to detect the localization of C/EBP β proteins. The immunostaining pattern of glioma tissue is shown in Fig. 1. The C/EBP β expression occurred in the nucleus of the tumor, showing activated transcription factors translocated into the nucleus. We analyzed 25 available specimens and the correlation was investigated

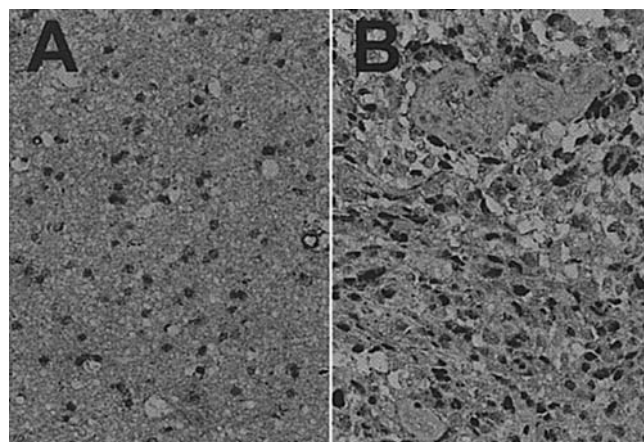


Figure 1. Representative immunohistochemical staining pattern of anti-C/EBP β . (A) Astrocytoma classified as showing low C/EBP β expression. (B) Glioblastoma multiforme classified as showing high C/EBP β expression. Magnification is x200.

Table II. Correlation of immunohistochemical pattern and pathological diagnosis.

	0: none	1: weak	2: moderate	3: strong
Astrocytoma	0	3	3	0
Anaplastic astrocytoma	2	2	2	0
Glioblastoma	1	2	7	3

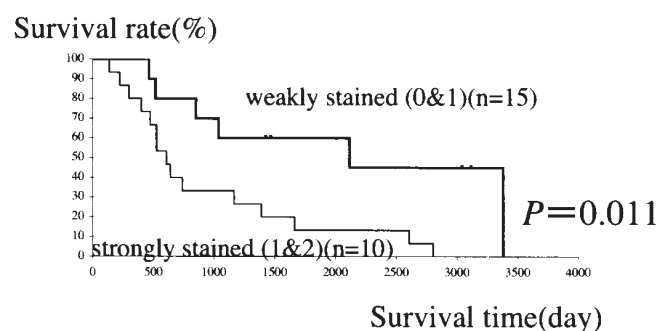


Figure 2. C/EBP β protein expression and patient survival. Kaplan-Meier survival curves for patients who had undergone surgical resection of glioma tissue, stratified according to the level of C/EBP β expression in their tumors. Comparison of protein expression and overall survival of patients with glioma (low C/EBP β staining, 0-1 score; high C/EBP β staining, 2-3 score; log-rank test, $P=0.011$).

between the C/EBP β staining, patient survival, and histological grading. Glioblastoma patients (13 cases) showed strong staining in comparison with grade 2 and 3 patients (12 cases) (Table II, $P=0.045$). When comparing the high C/EBP β group with the low C/EBP β group, increased C/EBP β expression correlated with patient survival (log-rank test, $P=0.011$; Fig. 2).

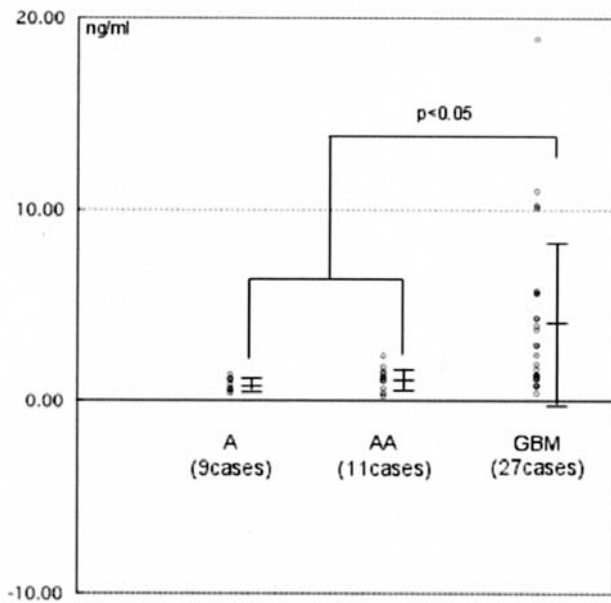


Figure 3. C/EBP β gene expression compared to histological grading. High expression of C/EBP β mRNA was significantly associated with glioblastoma multiforme (GBM) compared to astrocytoma (A) and anaplastic astrocytoma (AA) ($P < 0.05$).

Increased C/EBP β gene expression in glioma correlates with the prognosis of the patients. We compared the expression level of the C/EBP β genes in 47 samples with quantitative real-time PCR. C/EBP β gene expression was compared with histological grading. High expression of C/EBP β mRNA was significantly associated with glioblastoma multiforme (GBM) compared to astrocytoma (A) and anaplastic astrocytoma (AA) (Fig. 3, $P = 0.027$). The survival curves of the patients, grouped according to the level of C/EBP β gene expression in their tumors, are shown in Fig. 4. The high C/EBP β expression group (> 1.5 ng/ml) had a significantly poorer prognosis than the low C/EBP β expression group (< 1.5 ng/ml) (log-rank test, $P = 0.043$).

Glioma cell proliferation and invasion are inhibited by anti-C/EBP β siRNA transfection. C/EBP β overexpression was linked with the aggressiveness of the glioma in our analysis. In order to determine whether down-regulation of endogenous C/EBP β would suppress the proliferation and invasive behavior of glioma, we synthesized siRNAs that, when transfected into cells, targeting C/EBP β mRNA for degradation, thus reducing the expression of the C/EBP β protein. We analyzed the efficacy of the siRNA-mediated inhibition of C/EBP β synthesis in U251, GI-1 and T98G cells by Western blotting. Because the results obtained were virtually identical in the three cell types, only one is presented here. As shown in Fig. 5A, when U251 cells were transfected with the corresponding siRNA, C/EBP β proteins were down-regulated 48 h later. Furthermore, β -actin protein levels, which serve as a control, were not modified by exposure to these siRNAs. Finally, transfection with a related control siRNA also failed to modify C/EBP β protein expression.

After transfection with anti-C/EBP β siRNA, U251 cell counts in the ensuing 48 h were approximately 70% of

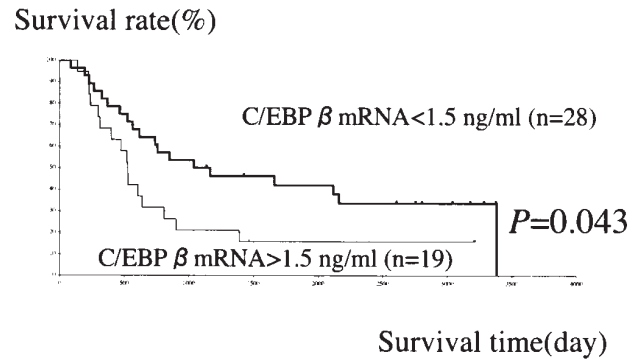


Figure 4. C/EBP β mRNA expression and patient survival. Kaplan-Meier survival curves for patients who had undergone surgical resection of glioma tissue, stratified according to the level of expression of C/EBP β in their tumors. Comparison of mRNA expression and overall survival of patients with glioma (low C/EBP β mRNA, 0-1.5 ng/ml; high C/EBP β mRNA, ≥ 1.5 ng/ml are detected; log-rank test, $P = 0.043$).

untreated or control-siRNA-treated cells during this same period of time (Fig. 5B, $P < 0.05$). Cell proliferation was significantly suppressed by siRNA against C/EBP β , as reflected in the observed reduction of protein expression.

For the invasion assays, the transfectants were seeded onto Matrigel-coated invasion chambers and incubated for 24 h and the total number of cells on the underside of each filter was determined. As shown in Fig. 5C, transfections of U251 cells with anti-C/EBP β siRNA inhibited cell invasion through the Matrigel by $> 80\%$, whereas the control siRNA had no effect ($P < 0.01$). Invading cells were significantly suppressed by siRNA against C/EBP β , as reflected in the observed reduction of protein expression.

IL-8 secretion on glioma cell lines. IL-8 secretion was determined by ELISA. IL-8 production was detected in T98G, U251 and GI-1 cells by 100 IU/ml of IL-1 β stimulation after 24-48 h. IL-8 production was significantly suppressed by siRNA against C/EBP β , as reflected in the observed reduction of IL-8 production (Fig. 6, $P = 0.0073$).

Discussion

Malignant gliomas are the most common primary brain tumors and generally have very poor prognosis. Glioblastoma multiforme, pathologically the most aggressive form, has a median survival time of just 9-12 months (1,2). Advances in the basic knowledge of cancer biology, as well as surgical techniques, chemotherapy and radiotherapy, have led to little improvement in survival rates for glioblastoma multiforme (31). There is thus a need to venture along previously unexplored avenues in order to devise more effective therapeutic approaches, to reveal more clearly the biological features of glioblastoma, and to discover a novel target molecule.

Cytokine secretion has been suggested to be related with the clinical features of glioma, such as positive inflammatory reactions and hypervascularity (23). IL-6, IL-8 and TGF- β are the most frequently detected cytokines in glioma (23).

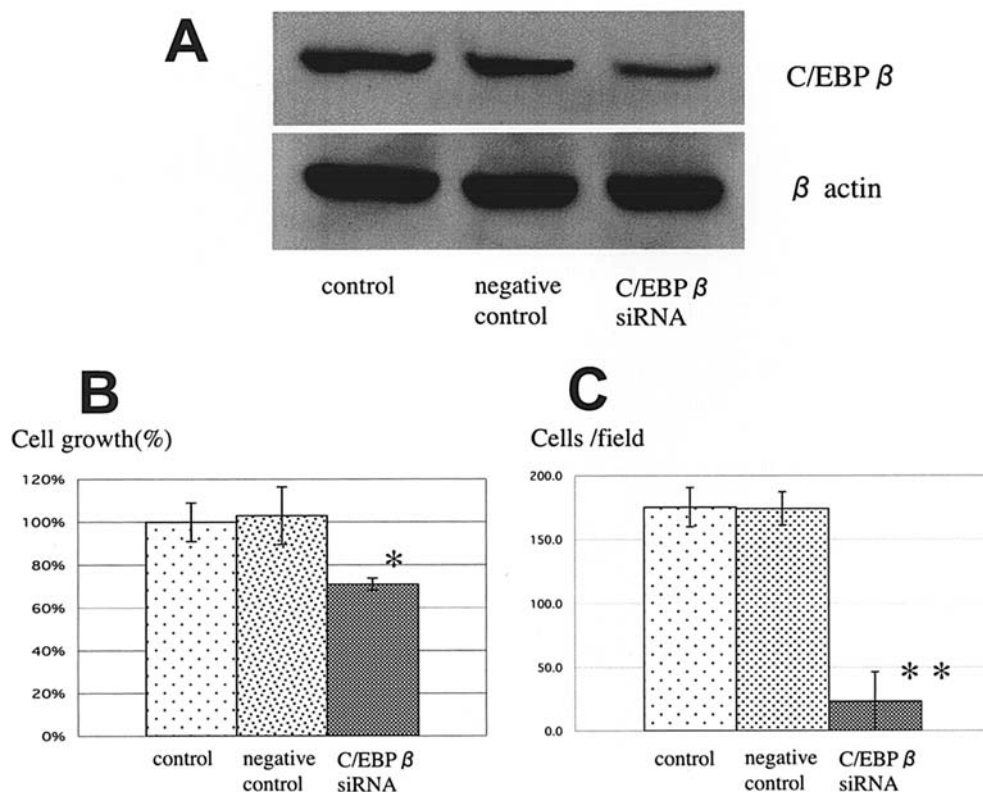


Figure 5. Effects of C/EBP β knockdown by RNA interference on the proliferation and invasiveness of human glioma cell lines. U251 cells were transiently transfected with short interfering RNAs and subjected to Western blot analysis, MTT assay or Matrigel invasion assays. (A) Reduction of C/EBP β protein expression by siRNA against C/EBP β was determined by Western blot analysis. Transfection with C/EBP β siRNA significantly reduced C/EBP β , whereas transfection with a siRNA targeted to an unrelated mRNA had no effect on C/EBP β expression. (B) Cell proliferation assay. Cells were cultured in 96-well plates in 100 μ l of serum-enriched medium. When 80% confluence was reached, 25 μ l of 100 nM siRNA in cytofectin was added drop wise. The number of viable cells was evaluated at 48 h of culture by incubation with Tetra color one and the values obtained were compared with those of controls. After transfection with anti-C/EBP β siRNA, U251 cell counts in the ensuing 48 h were approximately 70% of untreated or control-siRNA-treated cells during this same period of time. * $P < 0.05$ compared with both control groups. (C) For the invasion assays, the transfectants were seeded onto Matrigel-coated invasion chambers and incubated for 24 h and total number of cells on the underside of each filter was determined. Invading cells were significantly suppressed by siRNA against C/EBP β , as reflected in the observed reduction of protein expression. ** $P < 0.01$ compared with both control groups.

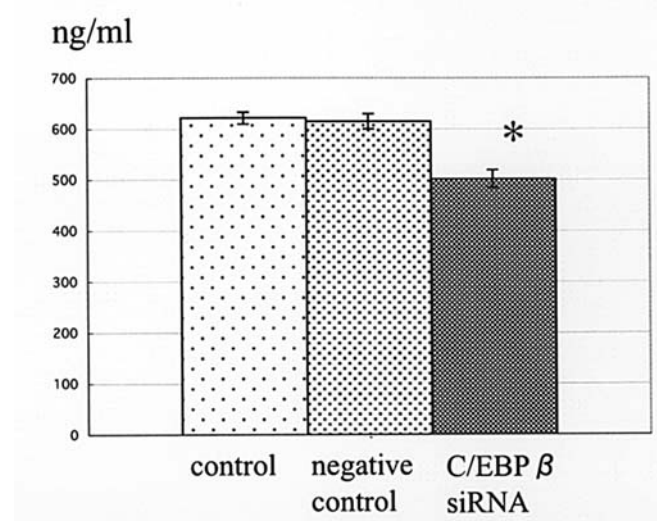


Figure 6. IL-8 level detected in U251 cells by 100 IU/ml of IL-1 β stimulation after 24-48 h by ELISA. The results represent the mean \pm SD of cell-free culture supernatants per milliliter. IL-8 production was significantly suppressed by siRNA against C/EBP β , as reflected in the observed reduction of IL-8 production. * $P < 0.01$ compared with both control groups.

Notably, IL-6 and IL-8 are inducible by the transcription factor C/EBP β , and the expression of C/EBP β can be stimulated by these cytokines (20). Therefore, C/EBP β may play a role in the autocrine growth stimulation by IL-6 and IL-8. IL-8, an angiogenic cytokine secreted from glioma cells, is thought to be a causative cytokine of hypervascularity in malignant glioma (23), thus contributing to the glioma progression. On the other hand, IL-6 and IL-8 have been suggested to have a role in cancer cell invasion by the induction of MMPs (matrix metalloproteinase) (32-34). Degradation of collagen in the extracellular matrix mediated by MMPs has been suggested to play an essential role in cancer invasion (35). Collagenase-1, belonging to a subgroup of the MMP gene family, plays an important role in collagen turnover in most human tissues (36). C/EBP β regulates MMPs expression (37) and collagenase-1 induction (38). Thus C/EBP β may be related to the invasiveness of cancer.

In our study, an increased expression of C/EBP β was associated with a malignant phenotype of glioma. Therefore, the increased expression of C/EBP β may be related to the proliferation and invasiveness of glioma. Very few mutations in C/EBP β were identified in a survey of 381 cancers and

cell lines representing leukemias, lymphomas, and various solid tumors (39). Only 2 of the 381 samples had missense mutations and 2 others had silent mutations. In contrast, 7% of acute myeloid leukemia samples analyzed contained a mutated form of C/EBP α , resulting in a truncated dominant-negative isoform or a protein with decreased DNA-binding activity (40). Therefore, unlike C/EBP α , C/EBP β most likely contributes to tumorigenesis through increased mRNA or protein expression rather than somatic or germline mutations. Increased C/EBP β expression has been detected in breast cancer, ovarian tumors, colorectal tumors, and renal cell carcinoma (24-27). Conversely, C/EBP $\beta^{-/-}$ mice were refractory to tumorigenic agents applied to the skin, implying that C/EBP β is required for tumor progression in the epidermis (41).

On the basis of our data, C/EBP β may play a potential role in the proliferation and invasion of glioma. Thus, C/EBP β can be a novel molecular target of therapy as well as an important predictive marker for survival in patients with glioma.

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