Expression of CXCL12 on pseudopalisading cells and proliferating microvessels in glioblastomas: An accelerated growth factor in glioblastomas

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Abstract. CXCL12, an α-chemokine that binds to G-protein-coupled CXCR4, plays an important and unique role in the regulation of stem/progenitor cell trafficking. To elucidate the correlation between the CXCR4/CXCL12 axis and glioblastomas (GBs), the present study assessed CXCR4/CXCL12 expression in 44 astrocytic tumor tissues using immunohistochemical analyses. Several cell lines of brain tumors were also analyzed by RT-PCR analyses. Although low-grade, astrocytic tumors were rarely positive for CXCL12 immunohistochemically, all GBs showed moderate to intense immunostaining with CXCL12, with particularly intense immunostaining being observed in the pseudopalisading cells and the proliferating microvessels. Regarding CXCR4, widespread positive immunoreactivity was noted in the tumor cells in almost all cases of GBs. In contrast, RT-PCR analysis showed low expression of CXCR4/CXCL12 in the aerophilic condition of GB cells and high expression of CXCR4/CXCL12 in the hypoxic condition of GB cells. Taken together, these results suggest that secretion of CXCR4/CXCL12 by hypoxic pseudopalisading and proliferating microvascular cells contributes to an outward migration of tumor cells away from hypoxia, creating a peripherally moving wave and subsequent microvascular proliferation. Pseudopalisades and proliferating microvessels are also considered to be associated with accelerated growth in GBs. These results indicate that expression of CXCL12 is an accelerated growth factor in GBs

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Introduction

Chemokines, small pro-inflammatory chemoattractant cytokines which bind to specific G-protein-coupled seven-span transmembrane receptors present on plasma membrane target cells, are the major regulators of cell trafficking (1,2). They and their receptors were initially associated with the trafficking of leukocytes in physiological immune surveillance and with inflammatory cell recruitment in various diseases. In addition, they have been shown to play an important role in hematopoietic development by regulating migration, proliferation, differentiation and survival of hematopoietic stem and progenitor cells (1).

Based on the relative position of the four cysteine residues, chemokines are classified in two main subfamilies: CXC(α) and CC(ß). There are two other subfamilies of chemokines for which the prototypes are fractalkine: a membrane-bound glycoprotein, in which the first two cysteines are separated by three amino acid residues (CXXXC), and lymphotactin, which has only two cysteines. The α-chemokines activate the CXC receptors (CXCR) 1 to 6, while ß-chemokines signal the CC receptors (CCR) 1 to 10, and fractalkine exerts its effects through the CX3CR1 receptor (3). Recently, evidence indicates that cancer cells may employ several mechanisms involving chemokine-chemokine receptor axes during metastasis which also regulate the trafficking of normal cells (4-8). Of all the chemokines, CXCL12, an α-chemokine that binds to G-protein-coupled CXCR4, plays an important and unique role in the regulation of stem/progenitor cell trafficking. Since CXCR4 is expressed on several tumor cells, these CXCR4-positive tumor cells may metastasize to the organs that secrete/express CXCL12. Furthermore, the CXCR4/CXCL12 axis has been shown to play a role in the development of peritoneal carcinomatosis from gastric carcinoma (4). Regarding gliomas, data show that CXCR4 and CXCL12 mRNAs are co-localized in glioblastomas and that their expression increases with tumor grade and is associated with regions of necrosis and angiogenesis (9). Other investigators have demonstrated that CXCR4/CXCL12 is involved in normal and malignant glial cell proliferation *in vitro* (2,3,10-15).

Table I. PCR primers used for RT-PCR.

Gene		Sequence	Product (bp)
CXCR4	Sense	GGCCCTCAAGACCACAGTCA	352
	Antisense	TTAGCTGGAGTGAAAACTTGAAG	
SDF-1	Sense	ATGAACGCCAAGGTCGTGGTC	276
	Antisense	CTTGTTTAAAGCTTTCTCCAGGTACT	
Flt-1	Sense	GTCACAGAAGAGGATGAAGGTGTCTA	414
	Antisense	CACAGTCCGGCACGTAGGTGATT	
VEGF	Sense	ATGAACTTTCTGCTGTCTTGGG	444 (VEGF121)
	Antisense	TCACCGCCTCGGCTTGTCACAT	576 (VEGF165)
ß-actin	Sense	TCGTGATGGACTCCGGTGAC	376
	Antisense	TCGTGGATGCCACAGGACTC	

The aim of the present study was to examine the potential association between expression of the CXCR4/CXCL12 axis and the clinicopathological characteristics in glioblastomas.

Materials and methods

Cells and culture conditions. U87-MG and U138-MG human glioblastoma cell lines, the Daoy human medulloblastoma cell line and the CHP-212 human neuroblastoma cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). U87-MG, U138-MG and Daoy cells were maintained in DMEM, and CHP-212 cells were grown in DMEM/Ham's F-12. The CCF-STTG1 human astrocytoma cell line was obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in RPMI-1640. Media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown in a humidified incubator at 37˚C and 5% CO_2.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from different human glioma cells and human brain tumor tissues by the acid guanidinium phenol chloroform method using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. RNA was quantified based on its absorbance at 260 nm. cDNA was reverse-transcribed from 5 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) and was primed by oligo(dT) primer (GE Healthcare). Three microliters of cDNA was subjected to PCR reaction using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). PCR primers for CXCR4, SDF-1, Flt-1, VEGF and ß-actin are listed in Table I. The cycling conditions were: 95˚C for 10 min, followed by 35 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min, with a final extension at 72˚C for 10 min. The amplified products were evaluated in 2% agarose gel and visualized by ethidium bromide staining under ultraviolet light. The cDNA quality was monitored using RT-PCR with ß-actin primers. cDNAs yielding a 376-bp product for ß-actin mRNA without contamination with the 471-bp genomic amplification product were used for the amplification experiment.

Tissue samples. Surgical tissue samples (n=44) from 44 patients with astrocytic tumors were used for the present study. All tumor specimens were retrieved from the archives of Kurume University and its affiliated hospitals between 2002 and 2007. Clinical information for the 44 patients was also retrieved from these archives. This study was carried out in accordance with the principles of the Helsinki Declaration and was approved by the ethics committee of our institution.

Histologic and immunohistochemical studies. The tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and then processed conventionally for histology and immunohistochemistry. The sections (5-μm) were stained using hematoxylin and eosin (H&E) for histological evaluation. The remaining serial unstained sections were used for immunohistochemistry. All specimens were then histologically diagnosed according to the World Health Organization criteria for tumors of the central nervous system (16). The immunohistochemical studies were performed using peroxidase avidin-biotin methods (LASB Kit, Dako Corp., Carpinteria, CA, USA) on paraffin sections, following pretreatment with proteinase K (CXCR4) or heat-induced antigen retrieval (CXCL12, VEGF). Primary antibodies were directed toward CXCL12 (dilution 1:100; R&D Systems, Minneapolis, MN, USA), CXCR4 (dilution 1:200; R&D Systems) and vascular endothelial growth factor (VEGF, dilution 1:10; R&D Systems).

The immunohistochemical studies were evaluated as follows: (-), no staining; (1+), 0-5% positive cells; (2+), 5-50% positive cells; (3+), >50% positive cells. The immunohistochemical evaluation was performed by two observers (Y.S. and H.K.) in independent readings. Any cases that varied significantly between the readers were re-evaluated in order to arrive at a consensus.

Statistical analysis. Statistical analyses were performed using the Stat Mate III for Windows Version software package (ATMS, Tokyo, Japan). The survival rates were computed using the Kaplan-Meier method. Patients were censored on loss-to-follow-up at the time of analysis. Survival curves were computed between subgroups of patients using the Cox-Mantel test. All tests of significance were two-sided. The

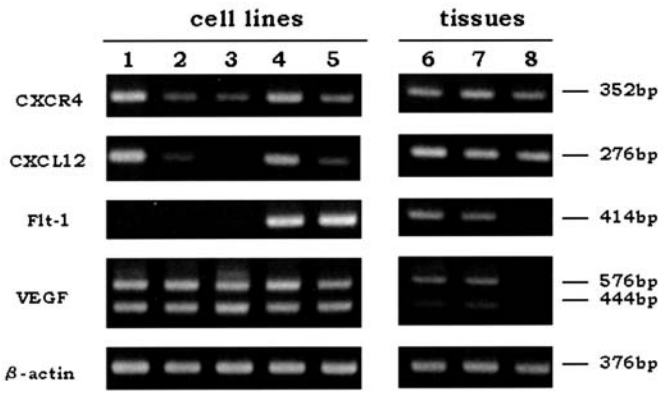

Figure 1. Regarding CXCR4, sharp intense bands are noted in an astrocytoma cell line (lane 1) and a medulloblastoma cell line (lane 4). Sharp bands are noted in a neuroblastoma cell line (lane 5), a pilocystic astrocytoma tissue (lane 6) and glioblastoma tissues (lanes 7 and 8). Vague weak bands are noted in glioblastoma cell lines (lanes 2 and 3). Regarding CXCL12, sharp intense bands are noted in an astrocytoma cell line (lane 1), a medulloblastoma cell line (lane 4), a pilocystic astrocytoma tissue (lane 6) and glioblastoma tissues (lanes 7 and 8). A vague weak band is observed in a glioblastoma cell line (lane 2). No band is detected in a glioblastoma cell line (lane 3). Regarding Flt-1, sharp intense bands are noted in a medulloblastoma cell line (lane 4) and a neuroblastoma cell line (lane 5). Vague weak bands are noted in a pilocystic astrocytoma tissue (lane 6) and a glioblastoma tissue (lane 7). No bands are noted in an astrocytoma line (lane 1), glioblastoma cell lines (lanes 2 and 3) and a glioblastoma tissue (lane 8). Regarding VEGF, sharp intense bands are noted in an astrocytoma line (lane 1), glioblastoma cell lines (lanes 2 and 3), a medulloblastoma cell line (lane 4) and a neuroblastoma cell line (lane 5). Vague weak bands are observed in a pilocystic astrocytoma tissue (lane 6) and in a glioblastoma tissue (lane 7). No bands are detected in a glioblastoma cell tissue (lane 8).

data are presented as means + SD, and a level of $P<0.05$ was considered significant.

Results

RT-PCR. RT-PCR analysis of the mRNA showed the expression of CXCR4, CXCL12, Flt-1 and VEGF in five human glioma cell lines (lanes 1-5) and tumor tissues (lanes 6-8), respectively (Fig. 1). Regarding CXCR4, sharp intense bands of ~352 bp were detected in an astrocytoma cell line and a medulloblastoma cell line. Sharp bands were detected in a neuroblastoma cell line, a pilocystic astrocytoma tissue and glioblastoma tissues. Vague weak bands were detected in glioblastoma cell lines. Regarding CXCL12, sharp intense bands of ~276 bp were detected in an astrocytoma cell line, a medulloblastoma cell line, a pilocystic astrocytoma tissue and glioblastoma tissues. A vague weak band was detected in a glioblastoma cell line. No band was detected in a glioblastoma cell line. Regarding Flt-1, sharp intense bands of ~414 bp were detected in a medulloblastoma cell line and a neuroblastoma cell line. Vague weak bands were detected in a pilocystic astrocytoma tissue and a glioblastoma tissue. No bands were detected in an astrocytoma line, glioblastoma cell lines and in a glioblastoma tissue.

Regarding VEGF, sharp intense bands of ~576 and 444 bp were detected in an astrocytoma line, in glioblastoma cell lines a medulloblastoma cell line and a neuroblastoma cell line. Vague weak bands were detected in a pilocystic astrocytoma tissue and a glioblastoma tissue. No bands were detected in a glioblastoma cell tissue.

Clinical features. The clinical features of the 44 patients are summarized in Table II.

Immunohistochemical features of astrocytic tumors. The histological characteristics of the 44 astrocytic tumors are summarized in Table II. CXCL12 expression was detected in 32 of 44 cases; of these, 7, 20 and 5 were scored as 3+, 2+ and 1+, respectively. CXCR4 expression was detected in 43 of 44 cases; of these, 20, 16 and 7 were scored as 3+, 2+ and 1+, respectively. CXCL12 immunoreactivity was observed in 24 of 24 (100%) glioblastomas, 7 of 9 (78%) anaplastic astrocytomas, and 1 of 11 (9%) astrocytomas or oligoastrocytomas. CXCR4 immunoreactivity was observed in 24 of 24 (100%) glioblastomas, 9 of 9 (100%) anaplastic astrocytomas, and 10 of 11 (91%) astrocytomas or oligoastrocytomas (Table II).

In the glioblastomas, all tumors showed scores of 2 to 3 for immunostaining with CXCL12, with particularly intense

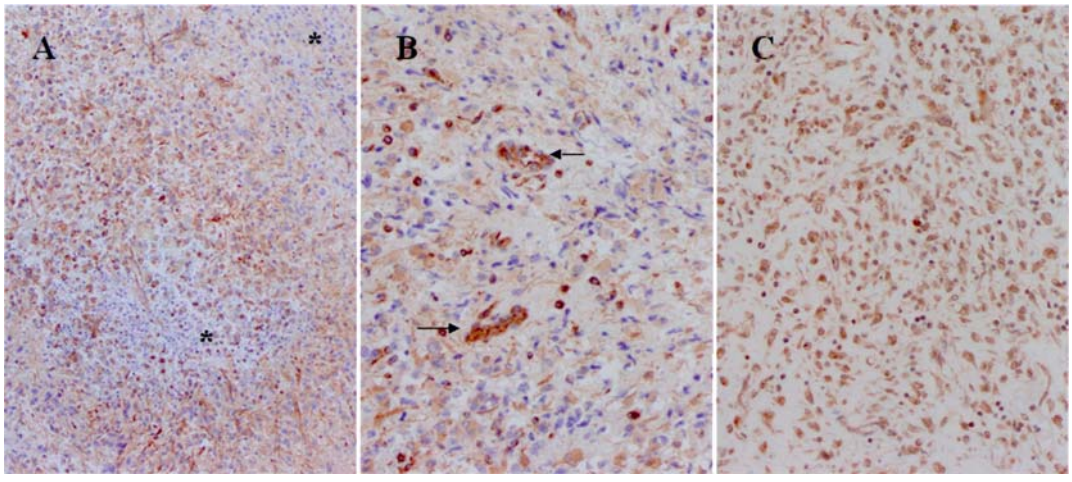

Figure 2. Immunohistochemical distribution of CXCL12 in glioblastoma (case 6). (A) Intense immunostaining with CXCL12 is noted in the pseudopalisading cells (score, 3; *, necrosis). (B) Intensive immunostaining with CXCL12 is observed in the proliferating microvessels (arrows). (C) Immunohistochemical distribution of CXCR4 in the glioblastoma (case 6). Widespread positive immunoreactivity for CXCR4 is noted diffusely in the cytoplasm of the glioblastoma cells (score, 3).

Table II. Summary of clinicopathological data.

Case no.	Age/gender	Location of tumor/histopathology	CXCL12	CXCR4	Follow-up
1	72/M	Lt. frontal-parietal lobe/GB	2+	1+	ANED, 16 months
2	70/F	Lt. cerebellum/GB	3+	1+	DOD, 13 months
3	73/M	Rt. temporal lobe/GB	2+	3+	DOD, 13 months
4	67/M	Lt. temporal lobe/GB	2+	1+	ANED, 3 months
5	79/M	Lt. cerebellum/GB	3+	3+	DOD, 6 months
6	38/M	Rt. temporal lobe/GB	2+	3+	DOD, 21 months
7	39/F	Rt. temporal lobe/GB	2+	2+	DOD, 18 months
8	36/F	Rt. corpus callosum/GB	2+	3+	DOD, 20 months
9	61/F	Lt. frontal-parietal lobe/GB	2+	2+	ANED, 42 months
10	64/F	Rt. frontal lobe/GB	3+	2+	ANED, 20 months
11	50/M	Rt. frontal-parietal lobe/GB	2+	3+	DOD, 23 months
12	69/F	Rt. frontal lobe/GB	2+	3+	ANED, 12 months
13	51/M	Rt. frontal lobe/GB	2+	3+	DOD, 23 months
14	57/M	Rt. frontal lobe/GB	2+	2+	ANED, 7 months
15	54/M	Lt. temporal lobe/GB	2+	3+	DOD, 14 months
16	66/F	Rt. temporal lobe/GB	2+	3+	DOD, 15 months
17	72/F	Lt. temporal lobe/GB	2+	3+	ANED, 7 months
18	63/F	Rt. frontal lobe/GB	3+	2+	DOD, 4 months
19	68/F	Lt. cerebellum/GB	2+	3+	DOD, 19 months
20	56/F	Rt. frontal-parietal lobe/GB	2+	2+	DOD, 6 months
21	69/F	Lt. frontal lobe/GB	3+	3+	DOD, 12 months
22	53/M	Rt. frontal lobe/GB	2+	2+	ANED, 17 months
23	72/F	Lt. temporal lobe/GB	2+	2+	ANED, 4 months
24	65/M	Lt. frontal lobe/GB	2+	3+	DOD, 11 months
25	31/M	Lt. frontal lobe/AA	1+	1+	ANED, 48 months
26	74/F	Lt. frontal lobe/AA	2+	2+	ANED, 8 months
27	18/F	Rt. frontal lobe/AA	3+	2+	DOD, 34 months
28	71/M	Rt. parietal lobe/AA	2+	1+	ANED, 40 months
29	52/M	Rt. frontal lobe/AA	0	3+	ANED, 24 months
30	41/M	Rt. temporal lobe/AA	0	3+	ANED, 16 months
31	43/M	Lt. frontal lobe/AA	1+	3+	ANED, 6 months
32	53/M	Lt. frontal lobe/AA	1+	2+	ANED, 17 months
33	33/M	Lt. frontal lobe/AA	1+	2+	ANED, 3 months
34	68/M	Lt. frontal lobe/DA	0	3+	DOD, 18 months
35	58/M	Rt. frontal lobe/OA	0	2+	ANED, 64 months
36	14/M	Rt. frontal lobe/DA	0	1+	ANED, 14 months
37	35/F	Rt. frontal lobe/OA	0	2+	ANED, 9 months
38	11/M	Rt. parietal lobe/OA	0	3+	ANED, 58 months
39	64/M	Lt. frontal lobe/OA	0	0	DOD, 12 months
40	52/M	Rt. frontal lobe/DA	1+	2+	ANED, 24 months
41	41/F	Rt. frontal lobe/DA	0	3+	ANED, 60 months
42	38/M	Rt. temporal lobe/DA	0	3+	ANED, 45 months
43	33/M	Rt. frontal lobe/OA	0	1+	ANED, 36 months
44	35/M	Rt. temporal lobe/OA	0	2+	ANED, 48 months

M, male; F, female. GB, glioblastoma; AA, anaplastic astrocytoma; DA, diffuse astrocytoma; OA, oligoastrocytoma. (-), no staining; (1+), 0-5% positive cells; (2+), 5-50% positive cells; (3+), >50% positive cells. ANED, alive and no evidence of disease; DOD, deceased due to disease.

immunostaining either in the pseudopalisading cells or in the proliferating microvessels (Fig. 2A and B). Regarding CXCR4, widespread positive immunoreactivity was noted in the tumor cells in almost all cases of glioblastomas (Fig. 2C). In the anaplastic astrocytomas, CXCL12 expression was detected in 7 cases; of these, 1, 2, and 4 were scored as 3+,

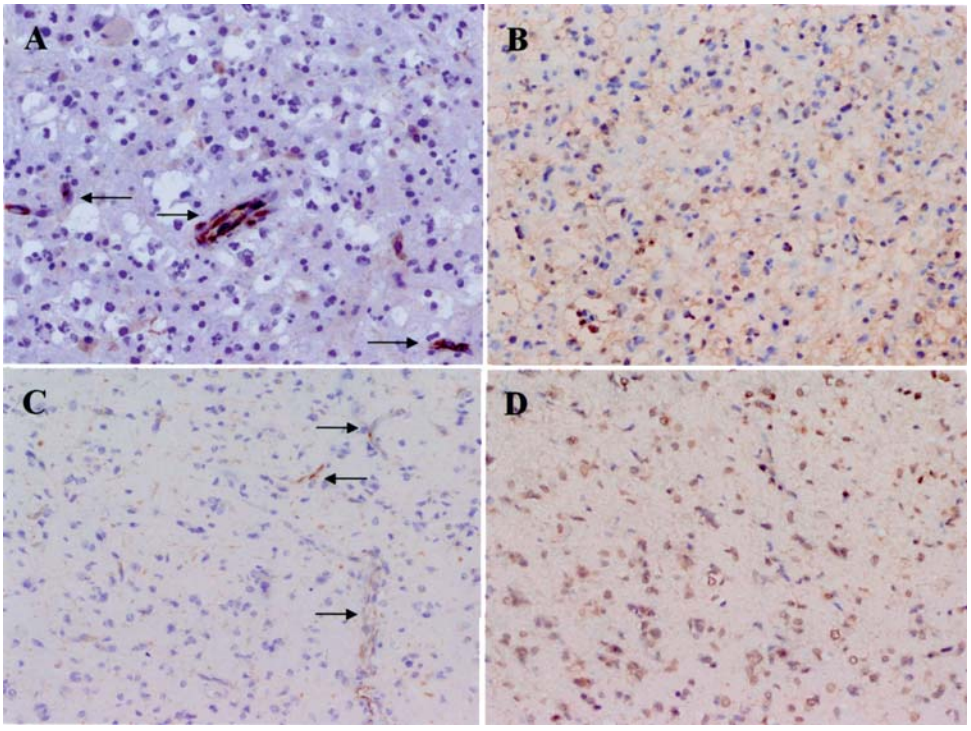

Figure 3. Immunohistochemical findings of the astrocytic tumors. (A) Ten percent of the tumor cells showing positive CXCL12 staining (score, 2). Intensive immunostaining with CXCL12 is also noted in the proliferating microvessels (arrows) (anaplastic astrocytoma, case 28). (B) Tumor cells (40-50%) showing positive CXCR4 staining (score, 2) (anaplastic astrocytoma, case 28). (C) Less than 5% of the tumor cells showing positive CXCL12 staining (score, 1). However, moderate immunostaining with CXCL12 is also noted in the proliferating microvessels (arrows) (diffuse astrocytoma, case 41). (D) More than 50% of the tumor cells showing positive CXCR4 staining (score, 3) (diffuse astrocytoma, case no. 41).

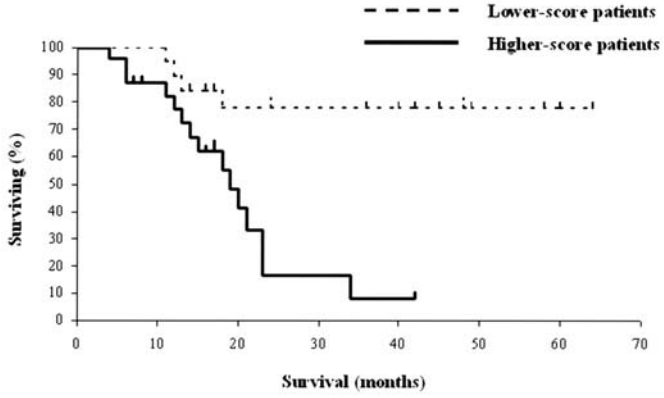

Figure 4. Survival curves (Kaplan-Meier methods) for patients with (A) low CXCL12 score (score, <1) (n=17) and (B) high CXCL12 score (score, >2) (n=27). Significant differences were observed in the 5-year survival rate between these two groups (P<0.05).

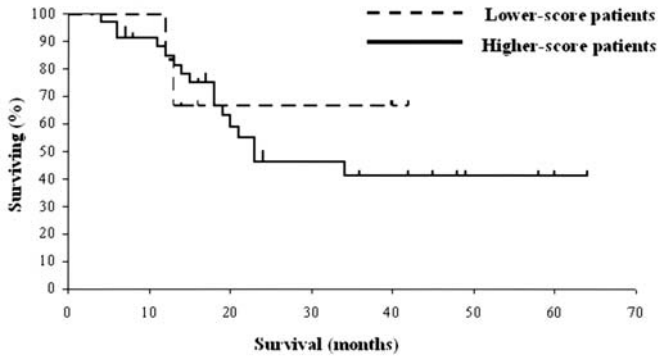

Figure 5. Survival curves (Kaplan-Meier methods) for patients with (A) low CXCR 4 score (score, <1) (n=8) and (B) high CXCR4 score (score, >2) (n=36). Significant differences were not observed in the 5-year survival rate between these two groups (P=0.73).

2+, and 1+, respectively. In 3 cases, the proliferating microvessels showed moderate to intense immunostaining (case no. 26, 27 and 28) (Fig. 3A). Regarding CXCR4, widespread positive immunoreactivity was noted in the tumor cells in almost all cases of anaplastic astrocytomas (Fig. 3B). In the astrocytomas or oligoastrocytomas, CXCL12 expression was detected in only case no. 41, which was scored as 1+. In this case, the proliferating microvessels showed mild immunostaining (Fig. 3C). Regarding CXCR4, widespread positive immunoreactivity was observed in the tumor cells in almost all cases of astrocytomas or oligoastrocytomas (Fig. 3D).

To gain a better understanding of the relationship between the CXCL12/CXCR4 axis and the prognosis of astrocytic tumors, statistical analyses were performed.

According to the CXCL12 or CXCR4 scores, patients were classified into lower-score (scores <1) and higher-score patients (scores >2). When CXCL12 was used as a marker, the survival rate between these two groups demonstrated a significant difference (P<0.05). (Fig. 4), while no significant difference (P=0.73) (Fig. 5) was observed when CXCR4 was used as a marker. Upon multivariate analysis, high expression of CXCL12 demonstrated an independent prognostic impact (risk ratio=15.1, P=0.037). However, high expression of

Table III. Multivariate analysis of prognostic factors for astrocytic tumors (n=44).

Variables	Risk ratio	P-value
CXCL 12		
<2	1	0.037
≥2	15.1	
CXCR 4		
<2	1	0.467
≥2	0.314	
Age		
<60 years	1	0.094
≥60 years	0.08	
Gender		
Male	1	0.744
Female	1.22	
Chemotherapy		
Yes	1	0.579
No	0.581	

CXCR4 showed no independent prognostic impact (risk ratio=0.31, P=0.47) (Table III).

Discussion

Many studies of the CXCR4/CXCL12 signaling system have been carried out, primarily in the field of immunology and infection (1). However, in recent years, many investigators have shown that CXCR4 is expressed more highly in various cancer tissue than in normal tissue, and that CXCL12 is expressed in many organs, including lymph nodes, bone marrow and lungs along with corresponding CXCR4 (1,4-8,17). In addition, CXCL12 was found to be a highly conserved gene localized to chromosome 10q11.1, and its mRNA was predominant in all tissue (18). Accordingly, many researchers have begun to take an interest in the role of the CXCR4/CXCL12 signaling system in tumorigenesis. For example, Jiang *et al* showed that CXCL12-CXCR4 interactions up-regulate tumor necrosis factor and integrin ß1 in ovarian tumor cell lines and promote angiogenesis, lymph node metastasis and chemoattractant migration and invasion, which might facilitate migration and invasion of tumor cells resulting in tumor progression (6). Singh *et al* also showed that the cellular and molecular mechanisms of CXCR4/CXCL12 mediated prostate cancer cell migration and invasion (5). Several reseachers found hypoxia-induced CXCR4 expression in tumor cells, which would sensitize tumor cells to CXCL12 signals and promote tumor metastasis (19,20).

Regarding brain tumors, Bajetto *et al* demonstrated that human meningiomas express CXCR4 and CXCL12. They also described that CXCL12 induces proliferation in primary meningioma cell cultures through the activation of ERK1/2 (8). To note, both CXCR4 and CXCL12 are overexpressed in glial tumor cells *in vivo*, suggesting a role for this chemokine in tumor growth. Rempel *et al* demonstrated using immunohistochemical analysis of different glioblastomas that CXCR4 and CXCL12 do not co-localize with regions highly expressing the proliferation marker MIB-1, but are present in tumoral regions characterized by necrosis and angiogenesis (9). They therefore speculated that CXCL12 promotes neoangiogenesis to supply nutrients to sustain tumor growth and/or modulation of the immune response. In the present study, we demonstrated that tumor cells and tumor cells associated with neighboring vessels expressed CXCL12 in astrocytomas, anaplastic astrocytomas and glioblastomas. We also detected CXCR4 expression in the cytoplasm in astrocytomas, anaplastic astrocytomas and glioblastomas. We thus speculate that CXCR4/CXCL12 interactions play an important role in growth, angiogenesis, and in sustaining the microenvironment in astrocytic tumors by an autocrine and paracrine mechanism. However, there were obvious differences in the intensity and distribution of CXCL12 expression in the subtypes of astrocytic tumors.

In tissue sections, the pathologic features that distinguish glioblastomas from astrocytomas include necrotic foci, usually with evidence of peripheral cellular pseudopalisading and micovascular proliferation. It is also well known that numerous vascular changes occur at the transition of anaplastic astrocytomas to glioblastomas (21).

Recent studies have shed light on the initial events of pseudopalisade formation in glioblastomas. A dramatic shift in biological behavior occurs following the transition from anaplastic astrocytomas to glioblastomas. The latter is characterized by pseudopalisading necrosis and increased levels of angiogenesis; features that are pathophysiologically linked and mechanistically instrumental to disease progression (21,22). Pseudopalisade formation results from the following sequence: i) vascular occlusion, related to endothelial apoptosis and associated with intravascular thrombosis; ii) hypoxia in regions surrounding vascular pathology; iii) outward migration of glioma cells away from hypoxia, creating a peripherally moving wave; iv) death of non-migrated cells leading to central necrosis; v) an exuberant angiogenic response creating microvascular proliferation in regions peripheral to central hypoxia; and vi) enhanced outward expansion of infiltrating tumor cells toward a new vasculature (22). In the present study, we showed that tumor cells associated with pseudopalisades and neighboring vessels show more intense and diffuse CXCL12 expression in glioblastomas. Investigators have demonstrated that CXCL12 regulates the cell growth and migration of hematopoietic stem cells but may also play a central role in brain development (2,3). More specifically, CXCL12 activates CXCR4 receptors expressed in a variety of neural cells, and this signaling results in diverse biological effects. It enhances migration and proliferation of cerebellar granule cells, chemoattracts microglia, and stimulates cytokine production and glutamate release by astrocytes. It was also shown that the tumorigenesis of neuroepithelial tumors reflects architectural changes in the developing brain (23). In addition, we demonstrated the existence of messenger RNA of CXCR4/CXCL12 and vascular growth factor in various cell lines of various neuroepithelial tumors using RT-PCR. Although our RT-PCR analysis of the mRNA showed low expression of CXCR4/CXCL12 in the glioblastoma cell

line, it showed high expression of CXCR4/CXCL12 in the glioblastoma tissue. In other words, these results showed low expression of CXCR4/CXCL12 in the aerophilic condition of glioblastoma cells and high expression of CXCR4/CXCL12 in the hypoxic condition of glioblastoma cells. Taken together, these results suggest that secretion of CXCR4/CXCL12 by hypoxic pseudopalisading and proliferating microvascular cells participates in outward migration of glioma cells away from hypoxia, creating a peripherally moving wave and subsequent microvascular proliferation.

We also showed that the survival rate of the lower-expression CXCL12 patients was significantly higher than that for the higher-expression CXCL12. In multivariate analysis, CXCL12 emerged as an independent prognostic factor in glioblastoma patients. In contrast, when CXCR4 was used as a marker, there was no significant difference in survival rates between these two groups. In addition, CXCR4 did not emerge as an independent prognostic factor in glioblastoma patients. These findings seem to be inconsistent with a previous report that CXCR4 staining is associated with an unfavorable prognosis in malignant neoplasms (6). Instead, our results indicated that CXCR 4 should play an important role in tumor growth of both low- and high-grade astrocytic tumors. We thus consider that interactions of CXCR4/CXCL12 are necessary for the growth of all low- to high-grade astrocytic neoplasmas and that additional high expression of CXCL12 from glioblastoma cells themselves and the ensuing microvessels due to hypoxia promote tumorigenesis of primary glioblastomas *de novo* or the transition from astrocytomas to glioblastomas. Actually, pseudopalisades and the ensuing microvascular proliferation are considered to be associated with accelerated growth in glioblastoma (21,22). As such, expression of CXCL12 in glioblastomas could be a reliable and independent prognostic factor in patients with glioblastomas.

It was also suggested that CXCL12 induces up-regulation of several other chemokines in glioma cells such as MCP-1 and interleukin 8, which appear to be involved in vascular endothelial cell proliferation and tumor neovascularization. Yang *et al* also demonstrated that activation of CXCR4 in malignant glioma cells promoted the production of vascular endothelial growth factor in an *in vitro* study (15). Recently, investigators reported that CXCL12 stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinase 1/2 and Akt (14,17). Based on the data, they suggested the role of CXCL12 in the regulation of glioblastoma growth *in vitro*,which likely occurs through an autocrine/paracrine mechanism. In addition, Zhang *et al* demonstrated that CXCL12-CXCR4 interaction leads to the selective increase of MT2-MMP, which mediates the invasiveness of glioma cells *in vitro* and glioma growth *in vivo* (11).

In the present study, we also showed using RT-PCR analysis that CXCR4/CXCL12, VEGF and Flit-1 expression occurs in glioma tissues and cell lines.

In particular, in our immunohistochemical study, CXCR4/CXCL12 expression increased with tumor grade in glioma tissues. Yang *et al* pointed out that CXCR4 might contribute to high levels of VEGF produced by malignant glioma cells and constitutes a therapeutic target of anti-angiogenesis (15). These findings, together with the observed overexpression of CXCR4/CXCL12 staining in glioblastomas in the present study, could possibly lead to future therapeutic trials with selective CXCR4/CXCL12 inhibitors for glioblastomas.

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