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Laboratory Investigation

Up-regulation of CC chemokine, CCL3L1, and receptors, CCR3, CCR5 in human glioblastoma that promotes cell growth

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Summary

Human CC ligand 3-like protein 1 (CCL3L1), a member of the CC chemokine family, that induces MCP1 and RANTES, exhibits a variety of proinflammatory activities including chemotaxis, and functional and proliferative activation of leukocytes, lymphocytes and macrophages. Its signal is transmitted through transmembrane receptors, CC chemokine receptors, CCR1, CCR3 and CCR5. To examine gene expression of chemokine, CCL3L1, and its receptors, CCR1, CCR3 and CCR5, we analyzed tumor tissues from 21 patients with several types of primary gliomas. CCL3L1, CCR3 and CCR5 gene exhibited over-expression in 70% (7/10), 60% (6/10), and 60% (6/10) of glioblastoma, in comparison with lower frequencies seen in lower-grade gliomas. Transfection of CCL3L1-expression vector to glioblastoma cell line enhanced proliferation of the tumor cells. These data suggest that increased expression of the CCL3L1, CCR3 and CCR5 chemokine-receptors system is involved in brain tumorigenesis, especially in the progression of glioblastoma.

Introduction

Chemokines are secretary chemoattractive proteins that are classified into four subtypes, C, CC, CXC, CX3C. Different tissues produce different repertoires of chemokines to attract appropriate subsets of effector cells into the tissue to respond to the particular type of tissue damage [1]. Chemokines transmit signals through transmembrane receptors, such as CC chemokine receptors (CCRs). CC ligand 3-like protein 1 (CCL3L1) belongs to a member of chemokines, called as macrophage inflammatory protein 1A (MIP-1A), chemokines that induce wide variety of immune cells, particularly $CD8^+$ T cells and immature dendritic cells (DCs), and, exert its effect in anti-tumor immunity [2]. The MIP-1A proteins bind to CC chemokine receptors (CCRs) 1, 3 and 5, with variable affinity [3]. Expression levels and types of the receptors (CCRs) varies depending on types of cells, i.e., monocytes, T cells, macrophages, DCs, neutrophils and eosinophils. MIP-1A was described to inhibit proliferation of astrocytes *in vitro* [4]. Transduction of MIP-1A *in vivo* was described to suppress tumor growth in rat tumor due to secondary immune response [5]. On the other hand, another report showed that transfection of MIP-1A into a plasmacytoma model leads to a strong CTL response, but not to remarkable differences in tumor growth [6].

Malignant astrocytoma (anaplastic astrocytoma and glioblastoma) is the most frequent malignant tumor in central nerve system among adults. Five-year survival rate of patients bearing those tumors remains around 20% for over 20 years in spite of advancement of clinical care. Malignant gliomas are highly invasive and difficult to be cured by surgical procedure alone. Thus, advancement of multidisciplinary treatment including chemotherapy, radiotherapy and immunity therapy is expected. Modulation of immune system, such as targeted treatment using cytokines to control

tumor cells or immune cells may prove to be a effective treatment of human malignant gliomas.

In the present work, we studied mRNA expression of the gene product for MIP-1A isoform, CCL3L1 and its receptors, CCR1, CCR3, and CCR5 in human astrocytic brain tumors and non-tumorous brain tissues. Our data tend to implicate aberrant expression of CCL3L1, CCR3, and CCR5 in human brain tumorigenesis, suggesting that they may serve as a target molecule for treatment of brain tumors.

Materials and methods

Isolation of RNA from tissue samples

Tumor tissues were obtained from 21 patients who underwent surgery for primary gliomas containing six diffuse astrocytomas, five anaplastic astrocytomas and ten glioblastomas at Nippon Medical School Hospital from 1997 to 2002. All the patients hadn't be treated with neither chemotherapy nor irradiation before the surgery. In addition to them, we obtained a small piece of injured cerebral cortex from a patient who underwent head injury and removal of subdural hematoma. Dissected samples were frozen immediately and stored at −80 °C. Total RNA was extracted from each specimen using TRIzol (Life Technologies, Inc.) as previously described [7]. Total RNAs were purified with RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacture's instructions. The integrity of total RNAs and purified RNAs were verified by electrophoresis in 3% NuSieve GTG agarose gel (BMA, Rockland, ME USA). All the tissues and RNAs were stored at −80 °C until use. Informed consent was obtained prior to surgery from all participating patients. Three distinct batches of normal brain RNA were obtained from CLONTECH Laboratories, Inc.

Semiquantitative RT-PCR

A 5μg aliquot of each total RNA was treated with DNase I (Epicentre Technologies) and reverse-transcribed to single-stranded cDNA using oligo$(dT)^{12-18}$ primer with Reverscript II reverse transcriptase (Wako Pure Chemical Industries, Ltd., Osaka). Each single-stranded cDNA was diluted for subsequent PCR amplification by monitoring beta-actin as a quantitative control. PCR amplification for CCL3L1, CCR3 and CCR5 was performed in 30 μl of reaction mixture containing 0.05 U/μl AmpliTaq Gold (Roche, Indianapolis, USA), 0.2 mM each dNTPs, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 1 μM each forward and reverse primers and 50 mM KCl. Ten microliters of the reaction mixture for CCR1 contained 5 μl of 2 × GC buffer I (TaKaRa Bio. Shiga, Japan), 0.05 U/μl Taq DNA Polymerase, (TaKaRa Bio), 0.2 mM each dNTPs and 1 μM each forward and reverse primers. All PCRs were carried out in the GeneAmp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences of the primers used for RT-PCR were as follows: CCL3L1, (forward) 5′-TCT GCA ACC AGG TCC TCT CT-3′ and (reverse) 5′-TTT CTG GAC CCA CTC CTC AC-3′; CCR1, (forward) 5′-ACA AAG ACT TCA CGG ACA AAG-3′ and (reverse) 5′-GCC AAT GAC AAA TAC CAA GGA G-3′; CCR3, (forward) 5′-ACC ATC TTC TGT CTC GTT CTC C-3′ and (reverse) 5′-AGA GAA GGA TAG CCA CAT TGT-3′; and CCR5, (forward) 5′-ATG GCC TCT GCT AAG CTC AA-3′ and (reverse) 5′-TGA TGT CTT TTC AAG GGT TTC TC-3″; beta-actin (forward) 5′-CCT CGC CTT TGC CGA TCC-3′ and (reverse), 5′-GGA TCT TCA TGA GGT AGT CAG TC-3′. PCR amplification was performed on the following conditions: for CCL3L1, 94 °C for 10 min, then 31 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, then 72 °C for 3.5 min; for CCR1, 94 °C for 2 min, then 34 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, then 72 °C for 3.5 min; for CCR3, 94 °C for 10 min, then 36 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, then 72 °C for 3.5 min; for CCR5, 94 °C for 10 min, then 36 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, then 72 °C for 3.5 min. PCR products were electrophoresed in 2% agarose gels.

In vitro *transfection of CCL3L1-expression vector to cell line*

The coding region of this gene (nucleotides 1186–2949, refer to NCBI homepage; link to nucleotide D90145) was amplified by RT-PCR against a Glioblastoma case cDNA using the primers as follows; (forward) 5′-CCA ATC ATG GAG

GTC TCC ACT-3′ and (reverse) 5′-AGC CCT GAA CAA AAG CAT CT-3′, and cloned into pCR3.1 vector (Invitrogen). pCR3.1 vector consists of 5060 nucleotides. It is driven by CMV promoter, and the transcription of the inserted gene starts after T7 promoter/priming site. The test gene is inserted within the multiple cloning site located just downstream of T7 promoter site. The vector contains SV40 replication origin, pUC/*E. Coli* replication origin, neomycin/kanamycin resistance gene and ampicillin resistance gene. The plasmid obtained was confirmed to be as our design by direct sequencing. Empty vector (pCR3.1 vector without the insertion) was used as control. U251 is a cell line from glioblastoma, and we found that this line expresses of CCR1, CCR3 and CCR5 to a certain level by RT-PCR as above, but CCL3L1 expression was not detected. 4×10^5 cells of U251 were spread in each wells of 6-well plates and cultured in 2 ml of Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% FBS, 100 unit/ml of penicillin G and 100 μg/ml of streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. The day after spread, transfection was performed using PolyFect Transfection Reagent (QIAGEN) according to manufacture's protocol for COS-7, NIH/3T3 and CHO cells. The day after transfection, cells were collected from each well and respread to 100 mm dishes. From the next day during cultrure, we washed cells twice with PBS and exchange media supplemented with 300 μg/ml of geneticin every 24 h. We collected CCL3L1-transfected and control cells from two dishes respectively at 1, 3, 5 and 7 days after beginning of cell selection with geneticin. The collected cells were counted with Burker–Turk cytometer. After counting, total RNA is derived from the collected cells of each dishes and reverse-transcribed to cDNA. We assessed expression of CCL3L1 in these cells with RT-PCR using the primers as above.

Results

Over-expression of CCL3L1, CCR3 and CCR5 mRNA in glioblastoma

To examine gene expression of CCL3L1, CCR1, CCR3 and CCR5 in types of gilomas, we analyzed fresh-frozen tumor tissues from 21 patients with primary gliomas for abnormalities in levels of CCL3L1, CCR1, CCR3 and CCR5 mRNA, using total RNAs for RT-PCR experiments. The concentration of each RT product was equalized to that of beta-actin, a house-keeping gene chosen as a quantitative control. Representative results are shown in Figure 1. Increased expression of CCL3L1 was observed in 1 of 6 diffuse astrocy-

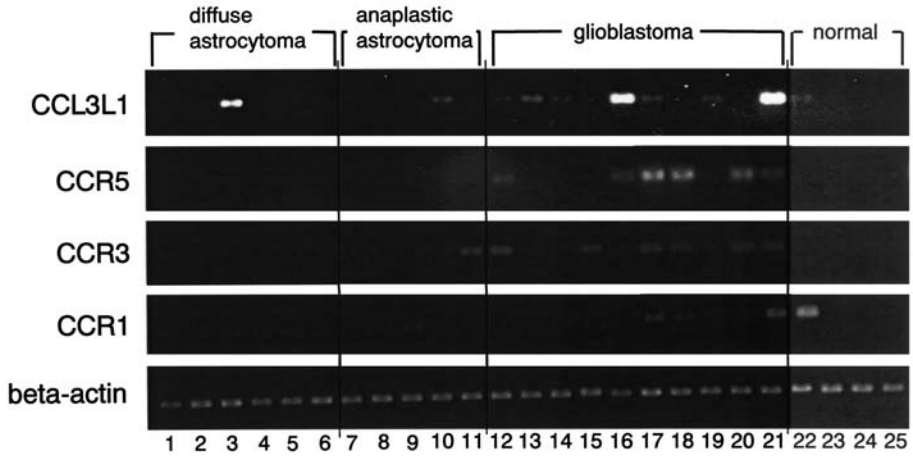

Figure 1. RT-PCR analysis showing expression of mRNA of CCL3L1, CCR1, CCR3 and CCR5. Beta-actin was used as a positive control. Lane 1–6: diffuse astrocytoma. Lane 7–11: anaplastic astrocytoma. Lane 12–21: glioblastoma. Lane 22–25: non-tumoral brain (normal). Lane 22 corresponds to the case of trauma. Lane 23–25 are based on the purchased RNA from CLONTECH. Lane 23, 24 and 25 correspond to lot.#1100831, lot.#1070762 and lot.#2020076, respectively.

tomas, 2 of 5 anaplastic astrocytomas, and 7 of 10 glioblastomas. Increased expression of CCR3 was observed in 1 of 6 diffuse astrocytomas, 1 of 5 anaplastic astrocytomas, and 6 of 10 glioblastomas. Low level expression was also detected in one of four normal controls. Increased expression of CCR5 was observed in none of diffuse astrocytomas, nor anaplastic astrocytomas, but 6 of 10 glioblastomas. These expression were not detected in normal brain tissue except one case expressing CCL3L1 at low level. Expression of CCR1 gene showed no remarkable correlation between frequency and malignancy. CCL3L1, CCR3 and CCR5 mRNA was predominantly over-expressed in two thirds of the glioblastomas examined, whereas over-expression of these gene were uncommon among lower grade gliomas.

In vitro *transfection of CCL3L1-expression vector resulted in promotion of cell proliferation*

From the results of RT-PCR as above, we supposed that the CCL3L1, CCR3 and CCR5 chemokine-receptors system involves in malignancy of glioma. To find tumorigenic effect of CCL3L1 , we firstly attempted *in vitro* transfection of CCL3L1-expression vector to brain tumor cell line, U251. CCL3L1-transfected cells grew twice as much compared to cells transfected with control empty vector 7 days after start of supplement of geneticin (Figure 2). Expression level of CCL3L1 in the cells of each dish is shown in the bottom panel of Figure 2. To confirm absence of expression of CCL3L1 in control-transfected cells, the concentration of each cDNA solutions was equalized by beta-action as a quantitative control. In this result, expression level of CCL3L1 in CCL3L1-transfected cells seems to be lower at day 5 and day 7. A cause of this result is transient effectiveness of transfection in this method. Another cause is decrease of the ratio of cells expressing CCL3L1 in each dishes. We equalized the concentration of cDNA solutions from each dishes despite of increase of cells with time, so in the latter half of this experiment expression level of CCL3L1 seems to be relatively lower.

We then supplemented recombinant CCL3L1 protein (recombinant human LD78-beta, PEPROTECH) to the culture medium for U251 cell line. We spread 2×10^4 U251 cells in 6-well plates and, later, exchange their medium with supplementation of recombinant CCL3L1 at various concentrations, i.e., 0, 0.1, 1, 10, and 100 ng/ml. It has been described that CCL3L1 protein is efficient as a chemoattractant at the concentration of between 0.1–10.0 ng/ml. Culture of cells in medium with recombinant CCL3L1 at concentration of 0.1, 1 and 10 ng/ml, displayed no difference in cell growth rate against culture without CCL3L1 supplementation, whereas those supplemented with 100 ng/ml of CCL3L1 enhanced proliferation of U251 cell line as shown in Figure 3.

Discussion

CCL3L1, CCR3 and CCR5 mRNAs were over-expressed in two third of the glioblastoma we examined.

Human CCL3L1 is a member of the CC chemokine family, which also induces MCP1 and RANTES [8]. MIP1 includes two isoforms, namely MIP1 alpha (MIP-1A) and MIP1 beta (MIP-1B), and three different human homologues of MIP-1A exist, namely LD78-alpha (CCL3), -beta (CCL3L1) and -gamma (a pseudogene). MIP1 exhibits a variety of proinflammatory activities including chemotaxis, and functional and proliferative activation of leukocytes, lymphocytes, macrophages [9]. Thus, it is thought to play an important role in response to pathogens and cell damages. MIP-1A mRNA was also detected in rat ischemic brain [10]. Human astrocytic tumor cells are capable of expressing and producting MIPs [11].

We here demonstrated over-expression of a chemokine, CCL3L1, and its receptors, CCR3 and CCR5 in human gliomas. The CCL3L1-receptors combination was predominantly up-regulated in malignant gliomas rather than lower-grade gliomas. Two hypotheses may be addressed in explanation for its up-regulation in malignant gliomas; (1) up-regulation of the CCL3L1-receptors system may exert its effect in autocrine system in proliferation of the gliomas; the system has been suggested to be involved in neoplastic transformation of hematopoietic cells [12]; (2) it may be over-produced to attract appropriate subsets of effector cells into the tissue to respond to tissue damage

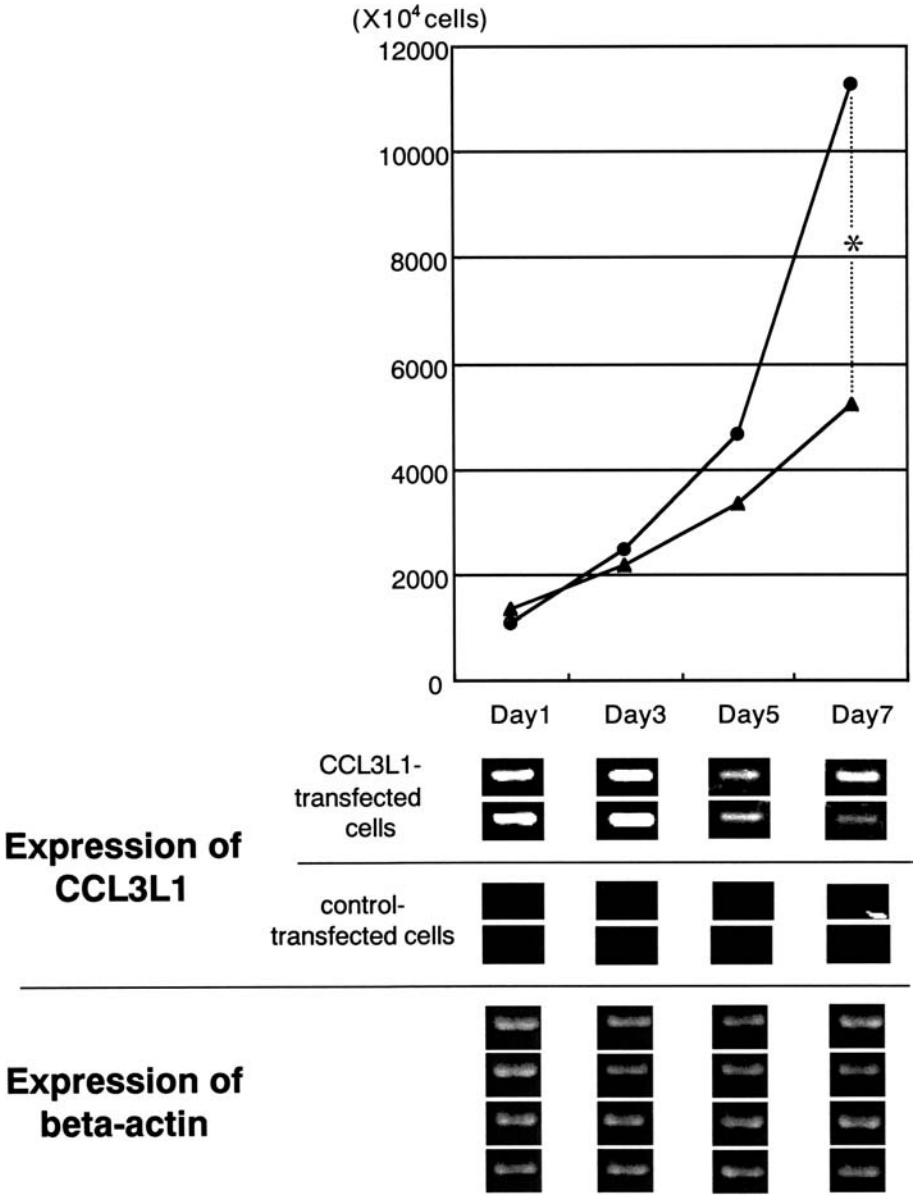

Figure 2. (Top) *In vitro* transfection with CCL3L1-expresion vector. Number of U251 cells after transfection of CCL3L1 or control. At each point of time, we collected CCL3L1-transfected and control-transfected cells from two dishes, respectively. Cells in a part of suspention of collected cells were counted using Burker–Turk cytometer. The longitudinal axis shows the mean number of cells from two dishes. The horizontal axis shows the Day after beginning of supplement of geneticin. ●: CCL3L1-vector transfected, ▲: empty vector transfected. $^{*}P = 0.0000339$. (Bottom) Expression level of CCL3L1 in the cells of each dish was analysed by RT-PCR.

associated with glioma progression, and exert its effect in anti-tumor immunity. Some described that MIP1A inhibits proliferation of astrocytes *in vitro* [4] as well as suppression of tumor growth by MIP-1a transduction *in vivo* in rat tumor [5], while others described that transfection of MIP1A into a plasmacytoma model lead to a strong CTL response, but not to remarkable differences in tumor growth [6], and, that MIP1A-transfected B16F10 melanoma cells after i.v. injection formed less

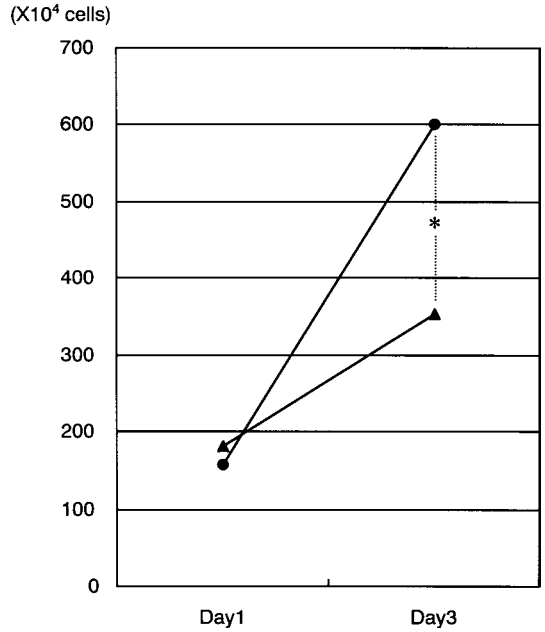

Figure 3. Culture of U251 cells with recombinant CCL3L1 protein. Number of U251 cells cultured in medium with 100 ng/ml or no recombinant CCL3L1. At each point of time, we collected 100 ng/ml or no recombinant-supplemented cells from three wells, respectively. Cells in a part of suspension of collected cells were counted using Burker–Turk cytometer. The longitudinal axis shows the mean number of cells from three wells. The horizontal axis shows the day after beginning of supplement of the recombinant. ●: recombinant CCL3L1 100 ng/ml, ▲: no recombinant CCL3L1. $*P = 0.073497$.

number but equal size of pulmonary foci compared with control melanoma cells [2].

Growth of tumor was not inhibited in a established MIP-1A-producing melanoma [2], the report described that MIP1A at low dose was chemoattractive for B cells and CTLs, but, the chemoattractive effect was diminished at high dose. This observation supports a hypothesis that tumor producing high level of MIP1A escaped the risk of tumor immunity. This observation is consists with our finding. In addition, it is possible from our result that over dose of CCL3L1 promotes proliferation of tumor cells, not confirmed *in vivo*. One of the histopathological characteristics of glioblastoma is perivascular lymphocytic cuffing. It is possible that these lymphocytes were induced by chemokines including MIP1A from tumor and gathered around blood vessels, but they couldn't invade inside the tumor because of too much concentration of chemokines. Growth of tumor may be involved with the balance of benefit and risk for tumor induced by various chemokines.

In conclusion, the results reported here suggest that up-regulation of the CCL3L1, CCR3 and CCR5 chemokine-receptors system is involved in brain tumorigenesis, especially in the progression of glioblastoma. This information might contribute to development of a molecular-targeted therapy for brain tumors that show aberrant expression of the CCL3L1, CCR3 and CCR5 chemokine-receptors system. On the other hand, further analysis for the characteristic of MIPs is required. The ways to utilize MIPs previously described, including transduction to tumor cells, should be discussed furthermore.

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