Effect of CCL2 siRNA on proliferation and apoptosis in the U251 human glioma cell line

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Abstract. Glioma is one of the most common types of tumor of the central nervous system. Increased expression of C-C motif chemokine 2 (CCL2) has previously been observed in various types of cancer. The effect of CCL2 small interfering (si)RNA on the proliferation, angiogenesis and apoptosis of the glioma cell line U251 was investigated in the present study. Data on CCL2 expression in glioma and normal tissues were obtained from The Cancer Genome Atlas. A total of 30 patients with glioma were enrolled in the present study. Cell proliferation was measured using a Cell Counting kit-8 assay, while cellular apoptosis and cell cycle distribution were examined using flow cytometric analysis. The reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to measure the expression levels of biological pathway-associated proteins caspase-3, caspase-7, tumor necrosis factor receptor superfamily member 10C (TNFRSF10C), growth regulated α protein (CXCL1), C-X-C motif chemokine 2 (CXCL2), C-X-C chemokine receptor type 2 (CXCR2), vascular endothelial growth factor (VEGF)A, VEGFB and VEGF. In addition, the mechanism of cellular apoptosis was analyzed by examining the phosphorylation of extracellular signal-related kinase (ERK)1/2 and p38 mitogen-activated protein kinase (p38) in cells treated with the C-C chemokine receptor type 2 inhibitor RS-102895. CCL2 was observed to be expressed in the glioma cell line U251 and was inhibited by CCL2 siRNA. Cells transfected with CCL2 siRNA exhibited inhibited cell proliferation, cell cycle arrest and increased cellular apoptosis. The expression levels of the apoptosis-associated proteins caspase-3, caspase-7 and TNFRSF10C were observed to be downregulated, in addition to those of the angiogenesis-associated proteins CXCL1, CXCL2, CXCR2, VEGFA, VEGFB and VEGF. The decrease in the rate of phosphorylation of ERK1/2 and p38 demonstrated the involvement of the mitogen-activated protein kinase/ERK pathway in apoptosis. In conclusion, CCL2 siRNA exhibited effective inhibition of cell proliferation and angiogenesis in the glioma cell line U251, which may provide a theoretical basis for the use of CCL2 in in vivo research and clinical treatment as a novel anticancer agent.

Introduction

Glioma is one of the most common types of tumor of the central nervous system, accounting for ~44.6% of cases (1). It was previously reported that the incidence of glioma is 3-10/100,000 people, among whom 70-80% are diagnosed with malignant glioma (2). Due to incomplete differentiation and infiltrative growth in deep brain tissue, it is rarely possible to remove the glioma completely through surgery. Although radiotherapy and chemotherapy are used post-operatively, patients exhibit a recurrence rate of ~98% and a median overall survival of <1 year (2). As the second most common cause of mortality in patients with cancer <34 years of age, and the third most common in patients between 35 and 54 years of age, malignant glioma leads to 180,000-600,000 annual mortalities in young and middle-aged people worldwide (2). Therefore, glioma-associated research is a global focus in neurosurgery, investigating the basic characteristics, biological behavior and treatment of glioma.

C-C motif chemokine 2 (CCL2), additionally termed monocyte chemoattractant protein 1, is a β-chemokine located on chromosome 17q11 (3). Human CCL2 was first cloned in the supernatant of a glioma culture in 1989 (4). CCL2 was initially considered to be a tumor-derived chemokine, and further research demonstrated abnormal expression of CCL2 in various types of cancer, including breast, ovarian, prostate, cervical, esophageal, gastric and nasopharyngeal cancer (5). Functional CCL2, combined with the surface receptor C-C chemokine receptor type 2 (CCR2), is able to activate phospholipase C and kinase C, resulting in the release of calcium ions (6). Among the primary functions of CCL2 is chemotaxis and the sequestration of monocytes and macrophages to contribute to the inflammatory response. In addition, it has been reported that CCL2-mediated chemotaxis and the induction of histamine release from basophils is associated with immune regulation (3).

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However, although the importance of the CCL2/CCR2 axis in the occurrence and development of cancer has been demonstrated in previous research, the biological function of CCL2 in glioma has rarely been investigated. Clinical research has exerted little impact on the recurrence rate, mortality rate and survival time of patients with glioma. In the present study, cellular and molecular biology methods were used to investigate the function and mechanism of the CCL2/CCR2 axis, in the recruitment of tumor-associated macrophages (TAMs), infiltrative growth of glioma and angiogenesis in tumors using *in vitro* and clinical data. The effect of CCL2 small interfering (si)RNA on the proliferation of the glioma cell line U251 was demonstrated using a Cell Counting kit-8 (CCK8) assay. Cell cycle distribution and cellular apoptosis were determined using flow cytometry. The expression levels of the biological pathway-associated proteins caspase-3, caspase-7, tumor necrosis factor receptor superfamily member 10C (TNFRSF10C), growth regulated α protein (CXCL1), C-X-C motif chemokine 2 (CXC2), C-X-C chemokine receptor type 2 (CXCR2), vascular endothelial growth factor (VEGF) A, VEGFB and VEGF were measured using western blotting and the reverse transcription-quantitative polymerase chain reaction (RT-qPCR), in order to investigate the biological functions involved in the CCL2/CCR2 axis. Glioma cells treated with the CCR2 inhibitor RS-102895 were used to investigate the mechanism underlying the apoptosis of the glioma cell line U251.

**Materials and methods**

**Patients and tissue samples.** The data on CCL2 expression levels were collected from The Cancer Genome Atlas database (TCGA; cancergenome.nih.gov). In order to investigate the mechanism involved in the pathogenesis of glioma through the CCL2 pathway, 30 patients with glioma (average age of 43.23±4.84; 14 male and 16 female) were recruited from Huzhou Central Hospital, with complete clinical and pathological follow-up data, and were enrolled in the present study. Additionally, 20 non-neoplastic brain tissue samples were obtained from surgical procedures for epilepsy. Ethical approval for the present study was provided by the independent ethics committee, Shanghai Tongren Hospital (Shanghai, China). Informed written consent was obtained from all patients or their advisers according to the guidelines of the ethics committee.

**Transfection.** U251 cells (5x10^5/well) were seeded into 6-well plates containing antibiotic-free DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) the day prior to transfection. The cells were transfected with 50 nmol/l CCL2 siRNA or the negative control using Lipofectamine™ 2000 transfection. The cells were transfected with 50 nmol/l CCL2 siRNA-transfected U251 cells were digested following culturing for 48 h using 0.25% trypsin, followed by suspension in PBS. The cells were fixed with 2% formaldehyde and incubated with 12% ethanol for 10 min. The membrane was blocked with 5% non-fat milk at 37˚C in 5% CO_2_ for 1 h. The cell viability was determined by the optical density at 450 nm, measured using a spectrophotometer.

**Cell cycle distribution and cellular apoptosis assays using flow cytometry.** CCL2 siRNA-transfected U251 cells were digested following culturing for 48 h using 0.25% trypsin, followed by suspension in PBS. The cells were fixed with 2% formaldehyde and incubated with 12% ethanol for 10 min. The cell viability was determined by the optical density at 450 nm, measured using a spectrophotometer.

**Western blot analysis.** Control and CCL2 siRNA-transfected U251 cells were washed with PBS. Besides, cultured U251 cells were pre-treated with 5 µM CCR2 inhibitor RS-102895 (MCE, USA) at room temperature. Cells were fully lysed in radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min and centrifuged at 12,000 x g for a further 10 min at 4˚C. A total of 35 µg supernatant was subjected to 15% SDS-PAGE following quantification using bicinchoninic acid protein assay reagent (Sangon Biotech Co., Ltd., Shanghai, China). A nitrocellulose filter membrane (Merck KGaA, Darmstadt, Germany) was used to transfer the blots following soaking in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS and 20% methyl alcohol) for 10 min. The membrane was blocked with 5% skimmed milk (BD Biosciences) at room temperature for 1 h. Anti-caspase-3 (Ab32351; 1:5,000; Abcam Cambridge, UK), caspase-7 (Ab32522; 1:1,000; Abcam), vascular endothelial growth factor (VEGF) A (Ab115961; 1:1,000; Abcam),
VEGFB (Ab185696; 1:500; Abcam), VEGF (Ab46154, 1:1,000; Abcam), CXCL1 (Santa, Sc-130316; 1:200), CXCL2 (Abcam, Ab139115, 1:100), CXCR2 (Abcam, Ab65968, 1:200; Abcam), TNFRSF10C (Sc-26462, 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p38 (9212; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-p38 (9211; 1:1,000; Cell Signaling Technology, Inc.), extracellular signal-related kinase (ERK)1/2 (4695; 1:1,000; Cell Signaling Technology, Inc.), p -ERK1/2 (4376; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (5174; 1:1,500; Cell Signaling Technology, Inc.) were diluted and added to blocking buffer (Merck KGaA), followed by incubation at 4˚C for 12 h. The membrane was subsequently incubated with Goat anti-rabbit (A0208; 1:1,000; Beyotime Institute of Biotechnology, Haimen, China) and anti-mouse (A0216; 1:1,000; Beyotime Institute of Biotechnology) secondary antibodies tagged with horseradish peroxidase at 37˚C for 1 h. The image was developed using enhanced chemiluminescence reagents (Merck KGaA) and Tanon-5200 (Tanon Science and Technology Co., Ltd., Shanghai, China).

**RT-qPCR.** The gene expression level of CCL2 in glioma cell lines T98G (Thermo Fisher Scientific, Inc.), U251, and SHG44 (Type Culture Collection of the Chinese Academy of Sciences), was measured using RT-qPCR. TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc.) was used to extract and quantify total mRNA from U251 cells. RT was performed to synthesize cDNA using the Thermoscript RT-PCR System (Thermo Fisher Scientific, Inc.) at a total volume of 25 µl (12 µl RNA-primer mix, 5 µl 5X RT reaction buffer, 1 µl 25 mM dNTPs, 1 µl 25 U/µl RNase inhibitor, 1 µl 200 U/µl M-MLV Rta, 1 µl Oligo(dT) 18 and 4 µl DNase-free ddH 2 O).

A total PCR mix (Thermo Fisher Scientific, Inc.) of 25 µl (12.5 µl SYBR-Green mix, 0.5 µl forward primer, 0.5 µl reverse primer, 9.5 µl ddH 2 O and 2 µl cDNA template) was amplified via 40 cycles of denaturing at 95˚C for 15 sec, annealing at 60˚C for 30 sec and elongation at 60˚C for 45 sec. Amplification kinetic curves were obtained and the data was analyzed using ABI Prism 7300 SDS built-in software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers used in the real-time PCR analysis are listed in Table I.

**Statistical analysis.** Data are expressed as the mean ± standard deviation and were analyzed using t-tests and analysis of variance. GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) was used to perform all statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Screening of glioma cell lines with high expression level of CCL2.** According to the data on CCL2 expression level obtained from TCGA, glioma tissue exhibited an increase in CCL2 expression compared with healthy tissue (Fig. 1A). RT-qPCR analysis was also performed to detect the gene expression level of CCL2 in glioma tissue and healthy tissue. Similarly, compared with healthy samples, CCL2 expression
level was significantly increased in the glioma tissue samples (Fig. 1B). Due to the significant alteration of CCL2 expression between glioma tissue and normal tissue, three glioma cell lines, T98G, U251 and SHG44, were used to detect the expression level of CCL2 using western blot analysis. As presented in Fig. 1C, CCL2 exhibited increased expression levels in U251 cells compared with the other cell lines. Therefore, the glioma cell line U251 was used for further investigation of the possible mechanism underlying siRNA interference in glioma.

**CCL2 siRNA inhibits cell proliferation.** The glioma cell line U251 was transfected with CCL2 siRNA and the results are presented in Fig. 2. The expression level of CCL2 was significantly decreased in U251 cells transfected with CCL2 siRNA compared with the control group, demonstrating the interference ability of CCL2 siRNA in glioma cell lines. Additionally, the data presented in Fig. 2D and E demonstrated that the proliferation of U251 cells transfected with CCL2 siRNA decreased in a time-dependent manner compared with the control group, measured using a CCK8 assay. Although a significant difference was observed between the negative control and control groups at 48 and 72 h post-transfection, it was hypothesized that this was due to over-proliferation and not the effect of the empty plasmid.

**CCL2 siRNA causes cell cycle arrest.** The cell cycle distribution of the glioma cell line U251 was detected 48 h following transfection with CCL2 siRNA using flow cytometry. The cell cycle of U251 cells was observed to be arrested at the S phase. As presented in Fig. 3A, the percentage of G0-G1 cells in the siRNA-treated U251 group decreased significantly from 64.52±1.47 to 42.16±1.80% (n=3; P<0.001) compared with the control group, while the percentage of cells in the S phase increased from 15.59±1.65 to 36.36±1.71% (n=3; P<0.001), compared with the control group. The significant increase in the percentages of cells in S phase indicated that the cell cycle
of U251 cells was arrested at S phase due to the treatment with CCL2 siRNA.

**CCL2 siRNA induces cellular apoptosis.** The effect of CCL2 siRNA on the cellular apoptosis of the glioma cell line U251 was evaluated using flow cytometry, following treatment with CCL2 siRNA for 48 h. As presented in Fig. 3B, the cellular apoptosis induced by CCL2 siRNA in U251 cells was detected visually using Annexin V FITC/PI double staining. The percentages of early apoptotic cells located in the lower right quadrant of the histograms were recorded as the apoptotic rate and are presented in Fig. 3B. The apoptotic rate of U251 cells transfected with CCL2 siRNA increased between 2.73±0.89 and 26.57±1.14% (n=3; P<0.001) compared with the control group, demonstrating that cellular apoptosis of U251 cells was induced by treatment with CCL2 siRNA. Additionally, western blot and RT-qPCR analyses were used to evaluate the alteration in cellular apoptosis-associated proteins caspase-3, caspase-7 and TNFRSF10C. The expression levels of caspase-3 and caspase-7 were upregulated in U251 cells compared with the control, while TNFRSF10C exhibited a significant decrease in expression level (Fig. 4A-C). Consistent with the results obtained in the western blot assay, the gene expression levels of VEGFA (P<0.01), and CXCL1, CXCL2, CXCR2, VEGFB and VEGF (all P<0.001) exhibited a decrease compared with the control group (P<0.001). Consistent with the results obtained using western blotting, the gene expression levels of VEGFA (P<0.01), and CXCL1, CXCL2, CXCR2, VEGFB and VEGF were observed to be significantly decreased compared with the control group (P<0.001). The downregulation of biological pathway-associated proteins demonstrated the inhibitory ability of CCL2 siRNA against the proliferation and invasion of the glioma cell line U251.

**Inhibition of CCL2/CCR2 signaling downregulates the MAPK/ERK signaling pathway.** The glioma cell line U251 was treated with the CCR2 inhibitor RS-102895, and the phosphorylation of p38 and ERK1/2 was measured using western blotting. The inhibition of CCR2 led to the downregulation of the expression of apoptosis-associated proteins p38 and ERK1/2. As presented in Fig. 4D and E, the phosphorylation of ERK1/2 and p38 decreased significantly compared with the control in U251 cells, due to the inhibition of CCR2 (P<0.001). The inhibition of CCR2 resulted in the downregulation of p38 and ERK1/2 expression levels, leading to cellular apoptosis in U251 cells and demonstrating the important role of the CCL2/CCR2 signaling pathway in the proliferation of the glioma cell line U251.

**Discussion**

Chemokines, which belong to the superfamily of small molecular weight secreted proteins, are cytokines with chemotactic abilities, containing 70-100 amino acids (7). The receptors of chemokines are G-protein-coupled receptors located in the cell membrane of immune cells, endothelial cells and tumor cells (7). It was previously reported that...
Chemokines and their receptors are involved in cell growth, differentiation, apoptosis and tissue damage in somatic cells, and cell proliferation and migration in tumor cells (7). Chemokines and their receptors have been demonstrated to exhibit a dual role in tumor biological behavior. Chemokines exhibit anticancer functions through chemotaxis and activation of immune cells, or the inhibition of angiogenesis; in addition, they promote growth, invasion and migration, through the stimulation of tumor cells and induction of chemotaxis (8). Therefore, research focused on chemokines and their receptors has investigated the tumorigenesis, progression and potential treatment of cancer (9,10).

Figure 4. Effect of CCL2 siRNA on the expression levels of caspase-3, caspase-7, TNFRSF10C, VEGFA, VEGFB, VEGF, CXCL1, CXCL2 and CXCR2. (A) Western blot analysis of the protein expression of caspase-3, caspase-7, TNFRSF10C, VEGFA, VEGFB, VEGF, CXCL1, CXCL2 and CXCR2 was performed using SDS-PAGE. (B) Quantification of the western blotting used to measure the protein expression level of apoptosis- and angiogenesis-associated proteins in the glioma cell line U251 following transfection with CCL2 siRNA for 48 h. (C) Gene expression of apoptosis- and angiogenesis-associated proteins in the glioma cell line U251 following transfection with CCL2 siRNA for 48 h was measured using the reverse transcription-quantitative polymerase chain reaction. (D) Western blot analysis of the protein expression of ERK1/2, p-ERK1/2, p38 and p-p38 was performed using SDS-PAGE. (E) Quantification of the western blotting used to detect the phosphorylation of ERK1/2, p-ERK1/2, p38 and p-p38. n=3; **P<0.01, ***P<0.001 vs. control; n=3. CCL2, C-C motif chemokine 2; siRNA, small interfering RNA; NC, negative control; TNFRSF10C, tumor necrosis factor receptor superfamily member 10C; VEGF, vascular endothelial growth factor; CXCL1, growth regulated alpha protein; CXCL2, C-X-C motif chemokine 2; CXCR2, C-X-C chemokine receptor type 2; ERK, extracellular signal related kinase; p38, p38 mitogen-activated protein kinase; p, phosphorylated.
CXCL1 and CXCL2, regulated by CCL2, have been demonstrated to be involved in the growth, proliferation, metastasis, invasion and angiogenesis of tumors via specific binding with the G-protein-coupled receptor CXCR2 (11). The expression of CXCL1 was previously observed in melanoma, colorectal, breast, bladder and epithelial ovarian cancer, while no CXCL1 was observed in healthy melanocytes (11). Additionally, melanocytes with continuously-expressed CXCL1 exhibit the potential to transform into tumor cells (12). In a previous study, mice carrying CXCL1-expressed melanocytes were treated with serum containing a CCR2 inhibitor. Tumor growth was observed to be inhibited with a decrease in angiogenesis, demonstrating the facilitating effect of CXCL1 in the proliferation and growth of tumor cells (13). In addition, the expression of metastasis-associated proteins including, matrix metalloproteinase 2 and integrin β1, were demonstrated to be upregulated in the cells with overexpressed CXCL1 (14). The invasive capability of five human uveal melanoma cell lines was observed to be promoted by CXCL1, interleukin 8, stromal cell-derived factor 1 and hepatocyte growth factor in vivo. CXCL1, and its receptor CXCR2, were observed to be expressed in all of the five cell lines with the highest invasion rates, indicating that invasion and metastasis of tumor cells is promoted by CXCL1 (15). Invasion and metastasis of tumor cells is an active and organized process with specific underlying mechanisms (16). The infiltrating growth of glioma is one of the most important factors influencing the surgical removal of glioma. The effect of the CCL2/CXCR2 axis on stem cells, including bone marrow stromal cells and neural stem cells, has been well-studied, while the effect of CCL2/CXCR2 on the chemotaxis and growth facilitation of tumor cells has rarely been investigated. The present study demonstrated the association between decreased expression of CXCL1 and inhibited cell viability of glioma cells due to CCL2 siRNA; exhibiting the positive regulatory effect of CXCL1 and CXCR2 on cell proliferation in tumor cells.

Increased angiogenesis and overexpressed CXCL1 has been observed in rat cornea tissue, and the angiogenic response was inhibited following treatment with anti-CXCL1 antibody (17). It was previously reported that CXCL1 regulates angiogenesis via epidermal growth factor and ERK1/2 in vitro, in addition to the recruitment of TAMs, which was regulated by VEGF (18). VEGF, also termed vascular permeability factor, is a type of specific heparin-binding growth factor which is expressed in vascular endothelial cells (19). The process of angiogenesis, including the proliferation and migration of endothelial cells, the degradation of the basement membrane and the formation of the lumen, is a complex process regulated by numerous positive and negative regulatory factors (20). Tumor angiogenesis factor serves a role in the generation of tumor vessels, the uncontrolled growth of which is an important characteristic of tumor cells. VEGF, VEGFα and VEGFβ were previously demonstrated to induce angiogenesis through the recruitment of TAMs, which serve an important role in the angiogenesis and growth of tumor cells in gastric cancer (21). The association between increased expression of VEGF and metastasis, angiogenesis and survival rate in rectal cancer has been previously investigated (22). VEGF promotes angiogenesis through direct induction of CCL2 protein expression. The capillary lumen was able to be formed in CCL2-treated umbilical vein endothelial cells, demonstrating the regulatory effect of CCL2 on the expression of VEGF, which results in an increased angiogenic response in tumor tissues (23).

In the present study, the phosphorylation levels of ERK1/2 and p38 were measured using western blotting, in order to investigate the mechanism underlying cellular apoptosis in U251 cells. Mitogen activated protein kinases, including p38, are involved in the activation of cellular apoptosis regulated by mitochondria, and induce the translocation of apoptosis regulator BAX to the mitochondria, followed by the release of cytochrome c. This process results in the activation of caspase-3 and the death of cells via the formation of apoptosisomes (24). ERK1/2, activated by dual specificity mitogen-activated protein kinase kinase, promotes the survival of cells by inducing the expression of protein C-ets-1, ETS domain-containing protein ELK1 and Myc proto-oncogene protein (25). In the present study, the phosphorylation levels of p38 and ERK1/2 were observed to be downregulated following treatment with the CCR2 inhibitor RS-102065, demonstrating that the involvement of the MAPK/ERK pathway in cellular apoptosis is induced by CCL2 siRNA, which was associated with the increased expression levels of caspase-3 and -7. Additionally, the downregulated expression level of TNFRSF10C was consistent with the result obtained in the western blot analysis.

In conclusion, the results of the present study demonstrated the regulatory effect of CCL2 on the proliferation and angiogenesis of the glioma cell line U251, by inhibiting the expression of CCL2 with CCL2 siRNA; The MAPK/ERK signaling pathway was identified in the cellular apoptosis of U251 cells. The present study may provide a theoretical basis for further in vivo research and clinical treatment for glioma.

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