p38MAPK activation mediates tumor necrosis factor-α-induced apoptosis in glioma cells

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Abstract. Gliomas are a type of heterogeneous primary central nervous system tumor, which arise from the glial cells; these types of tumor generally respond poorly to surgery, radiation and conventional chemotherapy. Tumor necrosis factor-α (TNF-α) has been suggested to produce an antitumor effect by binding to specific receptors on the tumor cell membrane to induce apoptosis. TNF-α is known to activate a number of signaling pathways, including extracellular signal-regulated protein kinase, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK), nuclear factor-κB and caspase cascades, depending on the cell type. However, the involvement of p38MAPK signaling in TNF-α-induced apoptosis in glioma cells remains unclear. In the current study, the role of p38MAPK in TNF-α-induced apoptosis in rat glioma C6 cells was investigated. TNF-α was observed to induce cell apoptosis and the phosphorylation of p38MAPK in C6 cells. In addition, the inhibition of p38MAPK markedly reduced TNF-α-induced apoptosis, while JNK inhibition did not affect apoptosis. Furthermore, p38MAPK transfection altered the cell cycle of glioma cells and increased the rate of apoptosis. It also led to an increase in the level of soluble TNF-α in the culture supernatant and membrane TNF receptor I levels in tumor cells. In conclusion, the results of the current study demonstrated that the activation of p38MAPK mediates TNF-α-induced apoptosis in glioma C6 cells, suggesting p38MAPK as a potential target for glioma therapy.

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Introduction

Phosphorylation/dephosphorylation by protein kinases is one of the most important mechanisms mediating signal transduction (1). The mitogen-activated protein kinase (MAPK) cascade is essential in the transduction of signals from the extracellular space to the nucleus (2). Four signaling pathways of the MAPK family have been identified in eukaryotic cells as follows: (i) The extracellular signal-regulated protein kinase (ERK); (ii) c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK); (iii) ERK5/big MAP kinase (BMK1); and (iv) p38MAPK pathways (3). p38MAPK is a kinase induced by stress signals and can also be referred to as p38αMAPK (4). Other isoforms exist in the p38MAPK family, including p38β, p38γ (ERK6/SAPK3) and p38δ (5). Among these isoforms, p38αMAPK has been widely demonstrated to function in a number of cellular functions, including differentiation, cell motility, developmental processes and survival (6,7). There are conflicting views on whether p38MAPK is a positive or negative regulator of apoptosis (8,9).

Tumor necrosis factor-α (TNF-α), a cytokine with high immunological competence, is implicated in cell proliferation and apoptosis (10). TNF-α has been demonstrated to serve a paradoxical role in the evolution and treatment of cancer. Certain studies have demonstrated that TNF-α inhibits cell viability and induces apoptosis by binding to its specific receptors on the tumor cell membrane (11-13), or by activating signaling pathways including ERK, JNK, p38MAPK, nuclear factor-κB (NF-κB) and caspase cascades (14). In rat fetal brown adipocytes, p38MAPK mediates TNF-α-induced apoptosis (15). However, studies have reported contradictory results; in murine fibroblasts, TNF-α-induced cytotoxicity was enhanced by p38MAPK inhibition (16), whilst in LNCaP prostatic cancer cells, p38MAPK was demonstrated to protect against TNF-α-induced apoptosis (17). However, little is known regarding the p38MAPK signaling involved in TNF-α-induced apoptosis in glioma cells.

In the current study, it was hypothesized that the activation of p38MAPK mediates TNF-α-induced apoptosis in glioma C6 cells, suggesting p38MAPK as a potential target for glioma therapy.

Materials and methods

Cell culture. Rat glioma C6 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Gibco-BRL). The cell lines were maintained at 37˚C in a humidified air atmosphere with 5% CO_2.

MTT assay. To detect cell survival subsequent to treatment, a standard cell proliferation assay was performed (MTT reduction assay) as in a previous study (18). Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and incubated with rat recombinant TNF-α (specific activity $2x10^7$ U/mg) for 24 h (TNF-α concentrations: 0, $1x10^4$, $1x10^5$, $2x10^5$ and $5x10^5$ U/L) (Sigma-Aldrich). MTT (20 μl; Amresco, Solon, OH, USA) was added to each well and the cells were incubated at 37˚C for 4 h. Subsequently, 150 μl dimethyl sulfoxide (Sigma-Aldrich) was added. The optical density (OD) was then measured at a wavelength of 570 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Vantaa, Finland). The inhibitory rate of tumor cell proliferation (%) was measured with the following formula: (OD value of the control group - OD value of the test group) x 100/OD value of the control group. Based on the IC_{50} of TNF-α in C6 cells, one concentration of TNF-α was selected for the experiments that followed.

Transmission electron microscopy (TEM). The morphology of apoptotic cells was measured using an H-800 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan). Subsequent to treatment, the C6 cells were fixed with glutaraldehyde (Sigma-Aldrich) for 1 h, washed twice with phosphate-buffered saline (PBS; Gibco-BRL) and then suspended with osmic acid (Sigma-Aldrich). Following a 1-h resting period, the cells were gradually dehydrated with acetone (Sigma-Aldrich) and embedded in resin (PRIMASET PT-30; Lonza Japan Ltd, Chiba, Japan). The cells were then placed onto slides and observed using TEM (JEM-2000EX; JEOL, Ltd, Tokyo, Japan).

Cell cycle analysis. Apoptosis was analyzed by flow cytometry. Subsequent to treatment, $1x10^6$ glioma C6 cells were prepared into a cell suspension by trypsinization (Sigma-Aldrich), and washed twice with ice-cold PBS. Cells were fixed with 70% ethanol (Sigma-Aldrich) at 4˚C overnight, then were treated with propidium iodide (Sigma-Aldrich) and RNase A (Nacalai Tesque, Inc., Kyoto, Japan) at 37˚C for 40 min. Subsequently, samples were analyzed with a FACSCalibur flow cytometry system (BD Biosciences, San Jose, CA, USA). The sub-G_1 population, representing apoptotic cells, was scored using the hypodiploid DNA content, which was quantified using ModFit LT software, version 3.2 (Verity Software House, Inc., Topsham, ME, USA); the percentages of DNA fragmentation reflecting apoptotic cells were determined by measuring the fraction of nuclei containing a hypodiploid DNA (sub-G1 peak), which based on >5,000 cells analyzed by flow cytometry.

Western blot analysis. For the detection of the p38MAPK (1:500; sc-166357; mouse anti-rat monoclonal antibodies), phosphorylated-p38MAPK (1:500; sc-166182; mouse anti-rat polyclonal antibodies), JNK (1:500; sc-7345; mouse anti-rat monoclonal antibodies) and phosphorylated-JNK (1:500; sc-6254; mouse anti-rat monoclonal antibodies) proteins (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), the whole-cell extracts were lysed in lysis buffer (Sigma-Aldrich) and then clarified by centrifugation at 12,000 x g for 10 min at 4˚C. The volume of protein was normalized by the Bradford method, as previously described (19). Equal amounts of total protein were loaded onto 10-15% SDS-PAGE gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for electrophoresis (Bio-Rad Laboratories, Inc.) and the separated proteins were subsequently electrotransferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% nonfat milk in PBS with Tween-20 (Sigma-Aldrich), and probed with various primary antibodies (as described above) overnight at 4˚C, then secondary antibodies (goat anti-mouse Immunoglobulin G-horseradish peroxidase; 1:2,000; sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h. The membranes were subsequently washed and the bands were examined using a ChemiDoc XRS system (Bio-Rad Laboratories, Inc.) with the enhanced chemiluminescence (ECL) western blot detection system (Amersham Life Science, Arlington Heights, IL, USA). The densities of the bands were determined by densitometry using Quantity One 4.5 software (Bio-Rad Laboratories, Inc.).

Inhibitor assay. To investigate whether MAPK influences TNF-α-induced apoptosis, C6 cells were also treated with TNF-α in the presence of a specific inhibitor of p38MAPK (SB202190) or JNK inhibitor (SP600125) (10 μmol/L; Calbiochem, San Diego, CA, USA) for 2 h. TNF-α was then added for the indicated experimental groups.

Gene transfection assay. Transient transfection was carried out using Lipofectin (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. During the logarithmic growth phase, C6 cells were transferred into a 6-well plate at a density of $2x10^8$cells/well and were incubated for 24 h. C6 cells were washed with Serum-free medium (0.8 ml; Gibco-BRL) prior to the addition of 2 μg pCMV5-p38MAPK (Promega Corp.) and 10 μl Lipofectin, which were dissolved in 100 μl serum-free medium, and then rested for 30 min at room temperature. Following 12-h incubation, 1 ml medium with 15% serum was added and then incubated for 24 h. The control was transfected with pCMV5 plasmid (Addgene, Inc., Cambridge, MA, USA) and non-transfected C6 cells. Subsequent to transfection, certain cells were fixed for TEM observation. The cell cycle distribution was analyzed by flow cytometry, and the expression of p38MAPK was detected by western blot analysis as described in the above methods.

ELISA. The rat soluble TNF-α (sTNF-α) ELISA kit (Invitrogen Life Technologies) was used to perform the ELISA in accordance with the manufacturer's instructions. Following p38MAPK transfection, the culture supernatants were collected. The coating TNF-α goat anti-rat polyclonal antibody (1:100; sc-1349; Santa Cruz Biotechnology, Inc.) was

added into an ELISA plate (Invitrogen Life Technologies) at 100 μl/well for 48 h at 4˚C. Subsequently, the plate was washed with PBS containing 0.05% Tween-20 three times. A total of 100 μl/well of the detected sample, negative control and standard sample were added at proportional dilutions with PBS (1:1), seperately. Following incubation at 37˚C for 1 h, the plate was washed three times. A total of 100 μl/well of the horseradish peroxidase-labeled donkey anti-goat monoclonal antibody (1:100; sc-2020; Santa Cruz Biotechnology, Inc.) was added, then following incubation at 37˚C for 1 h, the plate was washed three times with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich). A total of 100 μl/well 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS; Sigma-Aldrich) was added and 15 min later, the OD was detected by the ELISA reader (MR 5000; Dynatech Laboratories, Chantilly, VA, USA) at a wavelength of 410 nm. The standard curve was drawn and the concentrations of sTNF-α in the sample were calculated.

Flow cytometry for membrane TNF-α and membrane TNF receptor I (TNFRI) detection. Cells in each group were prepared into a cell suspension using trypsin and washed with PBS containing 1% BSA; the final concentration was $1x10^6$/ml. A total of 2 μl rabbit-anti-rat membrane TNF-α polyclonal antibody (1:1,000; 3707S; Cell Signaling Technology, Inc., Beverly, MA, USA) or TNFRI polyclonal antibody (1:1,000; 13377S; Cell Signaling Technology, Inc.) was added into 0.5 ml cell suspension. The cell suspension was shaken every 5 min for 30 min, at 37˚C. Subsequent to washing with PBS containing 1% BSA, goat-anti-rabbit monoclonal immunoglobulin G (1:2,000; 7074S; Cell Signaling Technology) labeled by fluorescein isothiocyanate was added and washed with PBS containing 1% BSA three times. Subsequent to the addition of 1 ml 4% paraformaldehyde (Sigma-Aldrich), the cells were observed by fluorescence microscopy (DP90; Olympus, Tokyo, Japan) and detected by flow cytometry using a FACS LSR II system (Becton Dickinson, San Jose, CA, USA).

Statistical analysis. All data are presented as the mean ± standard deviation of at least three independent experiments. Statistical differences between the means were analyzed by the independent-samples t-test. Rates were compared using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

TNF-α inhibits the proliferation of glioma C6 cells. The MTT assay was used to detect cell viability of glioma C6 cells. Subsequent to treatment with TNF-α, the proliferation of C6 cells was inhibited in a dose-dependent manner. The inhibitory rates of different concentrations of TNF-α on C6 cells are described in Table I. The IC_{50} of TNF-α in C6 cells was $2.53x10^5$ U/L, and this concentration was used for the subsequent experiments. Following treatment of glioma cells with SB202190 and TNF-α simultaneously, the inhibitory rate reduced to 9.849±0.675% (Fig. 1). However, following simultaneous treatment with SP600125 and TNF-α, the inhibitory rate was similar to that of TNF-α treatment alone (41.615±1.236% vs. 44.518±1.305%). These results suggest that TNF-α is antiproliferative in a dose-dependent manner in glioma cells, and that this effect of TNF-α can be partly blocked by the inhibition of p38MAPK with SB202190, but not by inhibition of JNK with SP600125.

Table I. Inhibitory rates of TNF-α on the proliferation of glioma cells.

TNF-α (U/l)	OD570 (mean ± SD)	Inhibitory rate (%)
0	0.995±0.004	0
$1x10^4$	0.962±0.018	3.316±0.603
$1x10^5$	0.645±0.031	35.180±1.276
$2x10^5$	0.552±0.030	44.518±1.305
$5x10^5$	0.378±0.009	62.012±2.229

TNF-α, tumor necrosis factor-α; OD, optical density; SD, standard deviation.

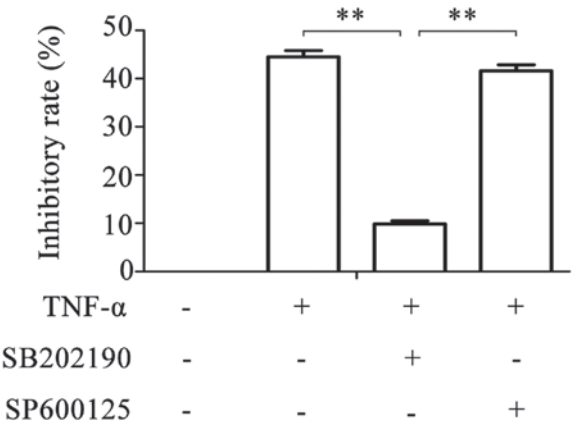

Figure 1. TNF-α-induced inhibition of glioma C6 cell proliferation. Glioma cells were treated with SB202190 and TNF-α simultaneously, and the inhibitory rates reduced to 9.849±0.675%. However, following the simultaneous treatment with SP600125 and TNF-α, the inhibitory rate was similar to that of TNF-α treatment alone (41.615±1.236% vs. 44.518±1.305%). **P<0.01, n=5. TNF-α, tumor necrosis factor-α.

TNF-α-induces apoptosis in rat C6 cells. To investigate the mechanisms associated with a TNF-α-induced reduction in cell proliferation, the effects of TNF-α treatment on cell cycle arrest and apoptosis were examined. Apoptosis was observed by TEM and quantified by flow cytometry. In the TNF-α and SP600125-treated groups, a number of the cells exhibited clear apoptotic characteristics, including pyknosis, chromatin condensation and formation of apoptotic bodies, when observed by TEM (Fig. 2A). Few apoptotic cells were observed in the control and SB202190-treated groups, whereas in the TNF-α- and SP600125-treated groups, the proportion of cells in the G_1 phase was increased and the proportions in the S and G_2 phases were reduced, and clear apoptotic peaks emerged (Fig 2B-E). The apoptotic rates in the TNF-α- and SP600125-treated groups were 37.5 and 34.1%, respectively (Fig. 2B and C). There was

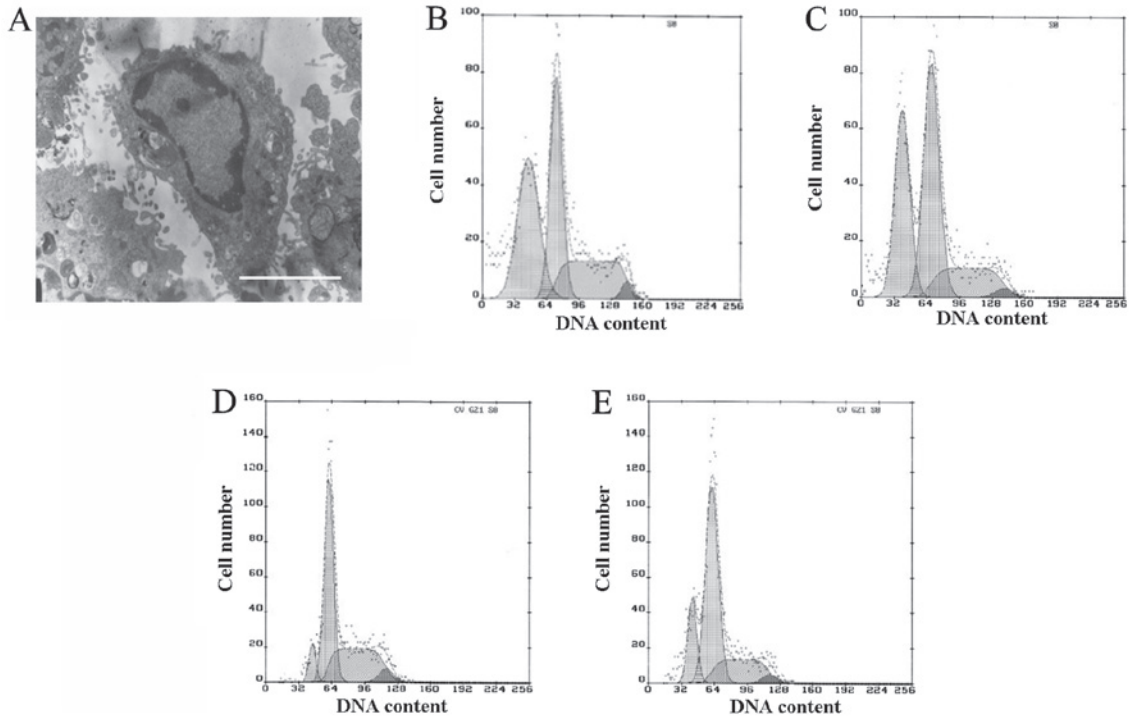

Figure 2. TNF-α-induced apoptosis. (A) Apoptotic glioma cells undergoing pyknosis and chromatin condensation subsequent to treatment of TNF-α observed by transmission electron microscopy (magnification, x6,500; Scale bar, 2 μm). Flow cytometry results from glioma C6 cells in the (B) TNF-α-treated, (C) SP600125, (D) TNF-α free and (E) SB202190 groups. The apoptotic rates were 37.5, 34.1, 7.0 and 16.9% in B-E, respectively. All experiments were performed in triplicate. TNF-α, tumor necrosis factor-α.

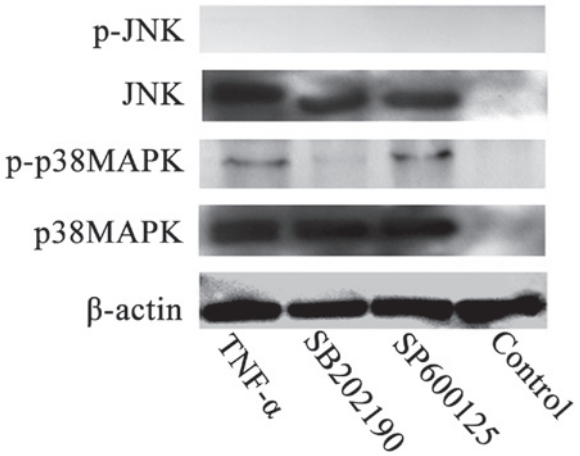

Figure 3. p38MAPK activation is involved in TNF-α-induced apoptosis. Western-blot analysis detected p-p38MAPK in the TNF-α- and SP600125-treated groups. No p-p38MAPK was detected in the SB202190-treated or control groups. p-JNK was not detected in any of the four groups. p38MAPK, p38 mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α; JNK, c-Jun N-terminal kinase; p-JNK, phosphorylated-JNK; p-p38MAPK, phosphorylated-p38MAPK.

a smaller apoptotic peak in the control and SB202190-treated groups, in which the apoptotic rates were 7 and 16.9%, respectively (Fig. 2D and E). The apoptotic rates of the TNF-α- and SP600125-treated groups were significantly different from that of the control or SB202190-treated group ($P<0.01$; data not shown). The results of the current study demonstrate that TNF-α-induced apoptosis may be partly blocked by the inhibition of p38MAPK, but not by inhibition of JNK.

p38MAPK activation is involved in TNF-α-induced apoptosis. To investigate the potential involvement of MAPK in TNF-α-induced apoptosis, the activation states of p38MAPK and JNK were investigated using western blot analysis with antibodies specific to the phosphorylated forms of these kinases. Subsequent to $2x10^5$ U/L TNF-α treatment, clear activation of phosphorylated-p38MAPK was observed (Fig. 3). In contrast, induction of JNK phosphorylation was not observed in any group. In addition, no phosphorylated-p38MAPK band was detected in the SB202190-treated or control group (Fig. 3). These data suggest that p38MAPK phosphorylation, but not JNK phosphorylation, may be involved in the mediation of TNF-α-induced apoptosis.

p38MAPK gene transfection induces apoptosis. To investigate the role of p38MAPK in TNF-α-induced apoptosis, transient transfection was carried out using Lipofectin. During the 24-h pCMV5-p38MAPK transfection, the asteroid-shaped cells became bipolar or round in shape, with reduced sizes and increased intracellular bubbles. After 24 h, a number of the dead cells floated in the medium. There were no distinct alterations in the non-transfected and pCMV5 groups. In addition, a number of cells demonstrated clear apoptotic alterations, including pyknosis, chromatin condensation and formation of apoptotic bodies (Fig. 4A). Cell cycle analysis identified a clear apoptotic peak in the pCMV5-p38MAPK group and the apoptotic index was 31.2% (Fig. 4B). Western blot analysis demonstrated that a distinct immunoreactive band at 38 kDa was present in the pCMV5-p38MAPK group. No band was present in the non-transfected or pCMV5 groups (Fig. 4C). Additionally, the sTNF-α concentration was analyzed subse-

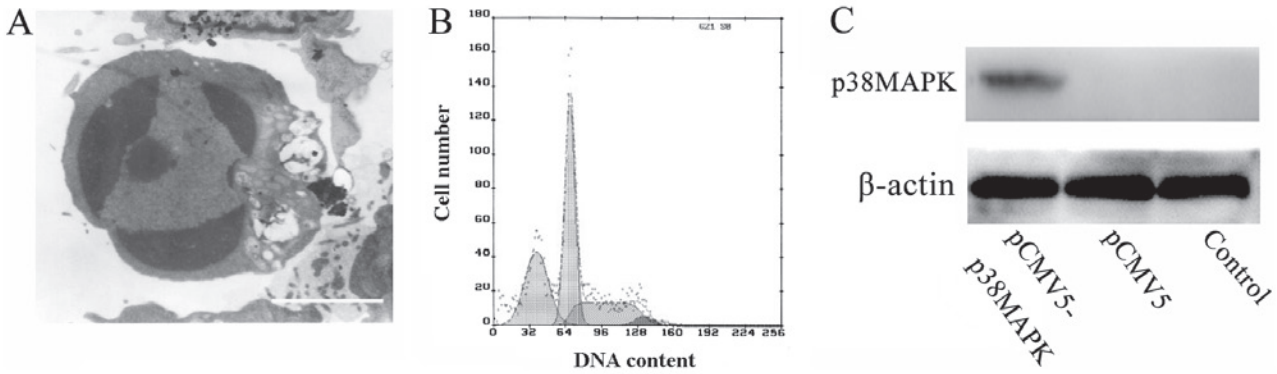

Figure 4. Apoptosis induced by p38MAPK gene transfection. (A) Apoptotic C6 cells subsequent to p38MAPK transfection observed using transmission electron microscopy (Scale bar, 2 μm). (B) Flow cytometry results from glioma C6 cells in the pCMV5-p38MAPK group, the apoptotic rate was 31.2%. (C) Western blot analysis detected that p38MAPK was present in the pCMV5-p38MAPK group, but not in the non-transfected (control) and pCMV5 groups. All experiments were performed in triplicate. p38MAPK, p38 mitogen-activated protein kinase.

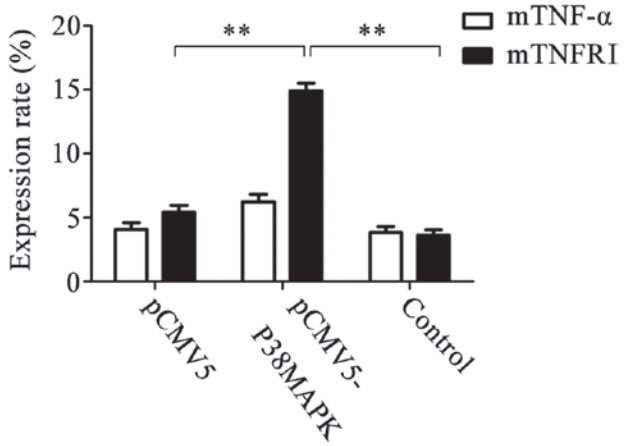

Figure 5. Expression of membrane TNF-α and membrane TNFRI subsequent to gene transfection. The results demonstrated that the expression rates of membrane TNF-α in the pCMV5 group, pCMV5-p38MAPK group and non-transfected group were 4.079±0.532, 6.218±0.601 and 3.845±0.482%, respectively. There were no significant differences between them. The expression rates of membrane TNFRI in the pCMV5 group, pCMV5-p38MAPK group and non-transfected group were 5.422±0.551, 14.906±0.627 and 3.633±0.404%, respectively. Among them, the expression in the pCMV5-p38MAPK group was significantly higher than that in the other two groups **P<0.01, n=5. TNF-α, tumor necrosis factor-α; TNFRI, TNF receptor I.

quent to gene transfection. The results demonstrated that the concentration of sTNF-α in the culture supernatant of the pCMV5-p38MAPK group increased and reached 43.4 pg/ml following treatment for 24 h, but there was no clear alteration in the pCMV5 and non-transfected groups (data not shown).

Expression of membrane TNF-α and TNFRI following gene transfection. The expression rates of membrane TNF-α in the pCMV5, pCMV5-p38MAPK and control (non-transfected) groups were 4.079±0.532, 6.218±0.601 and 3.845±0.482%, respectively, and were not significantly different. The expression rates of membrane TNFRI in the pCMV5, pCMV5-p38MAPK and control groups were 5.422±0.551, 14.906±0.627 and 3.633±0.404%, respectively. Out of these, the level in the pCMV5-p38MAPK group was significantly higher than that of the other two groups (P<0.01; Fig. 5).

Discussion

The current study demonstrated that TNF-α inhibits proliferation and induces apoptosis in glioma cells. Apoptosis, a biological process characterized by condensation of the nucleus and a distinctive pattern of chromosomal DNA fragmentation, is established to be essential for the development and maintenance of homeostasis during cell growth and the elimination of damaged cells in multicellular organisms (20). Disruption to apoptotic regulation is closely associated with the generation, development and prognosis of tumors. TNF-α regulates immune function and cytotoxicity in tumor cells by binding to its receptor, and the induction of apoptosis and cell cycle inhibition has been suggested as a potential mechanism for its anticancer effects. However, the sensitivity of different types of tumor cells to TNF-α varies, and little research has been undertaken regarding glioma cells. In the present study, the proliferation of C6 cells was inhibited by TNF-α through the induction of apoptosis. The mechanism that underlies TNF-α-induced apoptosis remains unclear. Yin *et al* (21) demonstrated that TNF-α may activate wild-type p53 protein by suppressing mutant p53, inducing p53-dependent apoptosis in glioma cells. However, studies have reported contradictory results that suggest that p38MAPK has an anti-apoptotic effect in TNF-α-induced apoptosis (16,17).

The findings of the current study demonstrated that p38MAPK activation was observed to partially mediate TNF-α-induced apoptosis of glioma cells. In 1994, Han *et al* (22) cloned p38MAPK from the murine liver cDNA library, then detected the expression of p38MAPK mRNA in murine macrophages (T and B cells). p38MAPK is activated by ultraviolet light, hyperosmolarity, arsenate, heat shock, H_2O_2, cytokines (such as IL-1 and TNF-α) and physiological stress (23). Following translocation from the nucleus to the cytoplasm, it initiates the activity of corresponding transcription factors. It has been reported that various transcription factors, including ATF2, MEF2C, CHOP10 and SAP1, are the physiological substrates of p38MAPK (24). p38MAPK acts in the pathophysiological processes of cell differentiation, development and regulation of apoptosis. In previous studies, p38MAPK activation was identified to induce apoptosis in non-tumor cells, such as nerve cells (7), fetal brown adipocytes (15) and tumor cells (25-27). However, a number of

studies have identified p38MAPK to be independent of apoptosis (28), or even to inhibit it (29,30).

The involvement of p38MAPK in TNF-α-induced apoptosis remains unclear and previous evidence is contradictory. In human cervix carcinoma cells, p38MAPK activation may be a key upstream signal of TNF-α-induced apoptosis and attenuation of the p38MAPK pathway by overexpression of DDB2 (a DNA repair protein) may be responsible for acquired TNF-α resistance (31). ASK1, a MAPKKK activated in cells treated with TNF-α, activates MKK3/MAPKK6 (or MKK6) and p38MAPK in turn, thus inducing apoptosis (32). However, another study demonstrated that ASK1 mediates anti-apoptotic signals (33). Insulin and insulin-like growth factor-I (IGF-I) protect HT29-D4 colon carcinoma cells from IFN-γ/TNF-α-induced apoptosis. The anti-apoptotic function of IGF-I is based on the enhancement of the survival pathways initiated by TNF-α and is mediated by p38MAPK, ERK and NF-κB, which act together to suppress proapoptotic signals (9). In L929 cells that overexpress SSI-1 and have been treated with TNF-α, the activation of p38MAPK was observed to be sustained, and the cells were resistant to TNF-α-induced apoptosis (34). In the current study, p38MAPK phosphorylation, but not JNK phosphorylation, was detected in the TNF-α-treated C6 cells. Subsequent to treatment with SB202190 and TNF-α, no positive p38MAPK signal was detected in the western blot analysis, and the apoptosis was higher than in the SB202190 and control groups. These data suggested that the p38MAPK, but not the JNK signaling pathway, is involved in TNF-α-induced apoptosis.

The current study demonstrated that p38MAPK transfection enhances apoptosis. To further investigate the role of p38MAPK in TNF-α-induced apoptosis, transient transfection was performed using Lipofectin. Subsequent to the transfection, p38MAPK was clearly expressed and confirmed to induce apoptosis. Activation of p38MAPK may contribute to the pathogenesis of apoptosis via the following mechanisms: (i) Upregulation of specific oncogenes, including c-Myc/s-Myc, c-Fos, c-Jun and bax (35); (ii) participating in Fas/FasL-mediated apoptosis (36); (iii) inducing p53 phosphorylation (37); and (iv) enhancing TNF-α expression (38). In endotoxin-activated glial cells, Bhat *et al* (39) demonstrated the importance of ERK and p38MAPK cascades in the transcriptional and post-transcriptional regulation of iNOS and TNF-α gene expression. Anti-TNF strategies targeting p38MAPK, NF-κB and TNF-α are being investigated as possible methods for the attenuation of renal ischemic injury (40). Soluble TNFRI, one of the two forms of TNFRI (soluble and membrane type), usually restricts the activity of TNF-α and is regarded as its antagonist. However, sTNF-α (the functional type of TNF-α) induces apoptosis when it binds to membrane TNFRI, which in turn binds to the TNF receptor-associated death domain (TRADD) molecules. TNFRI/TRADD binding results in the activation of a protein cascade (TRAF-2, ASK-1, MEK-4 and JNK) that ends with the activation of activator protein-1 (AP-1) (41). In the current study, the data demonstrated that p38MAPK transfection enhances apoptosis by the upregulation of sTNF-α and membrane TNFRI, which inferred that p38MAPK activation may contribute to the pathogenesis of apoptosis via these mechanisms.

In conclusion, the current study demonstrated that activation of p38MAPK, but not JNK, mediates TNF-α-induced apoptosis in glioma C6 cells. This suggests that the enhancement of p38MAPK activity can inhibit the progression of glioma cells. However, the MAPK signaling pathway is complex, thus it is necessary to further investigate the role of the upstream or downstream kinases in the p38MAPK cascade of apoptosis, in addition to other MAPKs, such as ERK1/2 or ERK5. *In vivo* experiments are also required to verify the results of the current study.

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