Caspase-dependent apoptotic death by gadolinium chloride (GdCl₃) via reactive oxygen species production and MAPK signaling in rat C6 glioma cells

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Abstract. Gadolinium (Gd) compounds serve as magnetic resonance imaging contrast agents and exert certain anticancer activities. Yet, the molecular signaling underlying the antitumor effect of Gd chloride (GdCl₃) on glioma remains unclear. In the present study, we aimed to ascertain the apoptotic mechanisms of GdCl₃ on rat glioma C6 cells. Our results demonstrated that GdCl₃ significantly reduced cell viability and shrunk cell morphology of C6 cells in a concentration-dependent manner. GdCl₃ led to apoptotic C6 cell death as detected by TUNEL staining. An increase in cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 occurred in GdCl₃-treated C6 cells as detected by immunoblotting analysis. The activities of caspase-3, caspase-8 and caspase-9 were increased, and the specific inhibitors of caspase-3/-8/-9 individually reversed cell viability, which caused apoptotic death in C6 cells prior to GdCl₂ exposure. GdCl₂ also caused an elevation in the cytoplasmic Ca²⁺ level and reactive oxygen species (ROS) production, as well as the loss of mitochondrial membrane potential ($\Delta \Psi m$) as shown by flow cytometric analysis in C6 cells. The results from the immunoblotting analysis demonstrated that there were upregulated protein levels of cytochrome c and Bax but a downregulated protein level of

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Dr Shih-Chang Tsai, Department of Biological Science and Technology, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. E-mail: sctsai@mail.cmu.edu.tw Bcl-2 in C6 cells after GdCl₃ treatment. Additionally, GdCl₃ decreased the protein levels of phosphorylated-extracellular signal-regulated kinases, phosphorylated-c-Jun N-terminal kinase and phosphorylated-p38 mitogen-activated protein kinases in C6 cells. In conclusion, ROS production and MAPKs signaling pathways contribute to GdCl₃-induced caspase cascade-mediated apoptosis in C6 cells. Our findings provide a better understanding of the molecular mechanisms underlying the role of GdCl₃ in rat glioma C6 cells.

Introduction

Glioma is characterized as a malignant brain tumor with a high recurrence rate, and surgery is utilized to remove the tumor (1,2). However, patients with glioma have a high mortality rate and an extremely poor prognosis (2). Currently, chemotherapy is the main method for prolonging survival or reducing symptoms (3,4). It is known that the existence of the blood-tumor barrier (BTB) in tumor tissue limits the efficacy and the delivery of therapeutic agents to brain tumor tissue (1,3). Therefore, the development of therapeutic agents to pass through the BTB is increasingly needed to effectively limit the rapid and invasive growth of malignant gliomas (5,6).

Lanthanide (Ln) ions have the chemical properties of both a high coordination number and a charge density, and they have been previously applied in agriculture and medicine (7-12). Gadolinium (Gd), a member of the Ln series, exerts a magneto-caloric effect (8). Gd compounds including Gd chloride (GdCl₃) have been applied as magnetic resonance imaging (MRI) contrast agents and may be promising anticancer drugs (8-12). GdCl₃ is widely used for in-activating tumor-associated macrophages (9,13). In addition, GdCl₃ was found to inhibit cell proliferation and induce apoptosis in human hepatoma HepG2 cells through a mitochondria-dependent pathway (11). Recently, we reported that GdCl₃ triggers apoptotic death in human osteosarcoma U-2 OS cells through the death receptor, mitochondria-dependent and ER stress pathways (8). The exact molecular mechanism of GdCl₃ that

Key words: gadolinium chloride, GdCl₃, apoptosis, reactive oxygen species production, MAPK pathway, caspase cascade

underlie the inhibition of the viability of rat C6 glioma cells remains undefined. Therefore, the present study aimed to investigate the effect of GdCl₃ on rat C6 glioma cells and the possible mechanism of GdCl₃-induced apoptosis.

Materials and methods

Chemicals and reagents. Fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA, and Ham's Nutrient Mixture F12 medium were obtained from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). GdCl₃, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and In Situ Cell Death Detection kit, Fluorescein were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Z-VAD-FMK (a pan-caspase inhibitor), Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), Z-LEHD-FMK (a caspase-9 inhibitor) and Caspase-3/-8/-9 Colorimetric Assay kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). All primary antibodies against caspase-3 (cat. no. GTX110543), caspase-8 (cat. no. GTX110723), caspase-9 (cat. no. GTX112888), cytochrome c (cat. no. GTX108585), Bax (cat. no. GTX109683), Bcl-2 (cat. no. GTX100064), phosphorylated-extracellular signal-regulated kinase (p-ERK) (cat no. GTX59568), ERK (cat no. GTX59618), phosphorylated-c-Jun N-terminal kinase (p-JNK) (cat no. GTX52326), JNK (cat no. GTX52360), phosphorylated-p38 (p-p38) (cat no. GTX24822), p38 (cat no. GTX110720), β-actin (cat. no. GTX109639), as well as anti-mouse (cat. no. GTX213111-01) and anti-rabbit (cat. no. GTX213110-01) immunoglobulin (Ig) G horseradish peroxidase (HRP)-linked secondary antibodies were purchased from GeneTex International Corporation (Hsinchu, Taiwan). Dihydrorhodamine 123, 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] and Fluo-3/AM were sourced from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. Rat glial cell tumor C6 cell line was obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and cultured in 75-cm² flasks with Ham's Nutrient Mixture F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human fetal glial SVG p12 cell line was purchased from BCRC and cultured in minimum essential Eagle's medium containing 10% FBS, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Cellviabilityassayandmorphologicalobservation.Cellviability was determined by a colorimetric MTT assay, as previously described (14). Briefly, C6 and SVG p12 cells (1x10⁴ cells/100 μ l) in a 96-well plate were incubated at 37°C with or without GdCl₃ at a final concentration of 50, 100, 150 and 200 μ M for 24 h. After incubation, the medium was removed, and the cells were mixed with 0.5 mg/ml MTT solution for 4 h. The insoluble formazan crystals were dissolved in 0.1 ml dimethyl sulfoxide (DMSO) for 30 min at 37°C. The absorbance was spectrophotometrically measured with an ELISA reader at a wavelength of 570 nm. The result was calculated as a ratio of sample absorbance to the control (vehicle) absorbance, considered as 100%. Additionally, the GdCl₃-treated C6 cells were measured for apoptotic characteristics and photographed via a phase-contrast microscope, as previously described (14).

DNA breaks for apoptosis. C6 cells $(1x10^5 \text{ cells/ml})$ were exposed to 50, 100, and 150 μ M of GdCl₃ for 24 h. The cells were subsequently collected and stained with the *In Situ* Cell Death Detection kit, Fluorescein (Sigma-Aldrich; Merck KGaA), following the manufacturer's instructions. The terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL)-positive cells were detected by flow cytometry (BD FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, San Jose, CA, USA), as previously described (14).

Comet assay and DAPI staining. C6 cells $(2x10^5 \text{ cells/well})$ were treated with 0, 50, 100, 150 and 200 μ M of GdCl₃ for 24 h. Comet assay was applied according to the vendor's instructions and a previous study (15). Additionally, chromatin undergoes a phase change from loose to condensed during apoptosis. DAPI dye was used to observe condensed chromatin, as previously described (16).

Immunoblotting analysis. C6 cells (5x10⁶ cells/75T flask) were incubated with the presence and absence of 0, 50, 100 and 200 μ M of GdCl₃ for 24 h. After cells were lysed, total protein (40 μ g) was separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer membrane; Merck Millipore, Darmstadt, Germany). As previously described (17,18), the membranes were probed with primary antibodies against caspase-3, caspase-8, caspase-9, cytochrome c, Bax, Bcl-2, p-ERK, ERK, p-JNK, JNK, p-p38, p38, (all 1:1,000 dilution) and β -actin (1:5,000 dilution). The membranes were then incubated with goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP linked antibodies (1:10,000 dilution) for 1 h at room temperature. All immunoblots were hybridized using an Enhanced Chemiluminescence system kit (Immobilon Western Chemiluminescent HRP substrate; Merck Millipore). The intensity of relative protein expression was normalized to β-actin signal and quantified with the NIH ImageJ program (version 1.47; National Institutes of Health, Bethesda, MD, USA).

Assays for caspase-3/-8/-9 activities. C6 cells ($5x10^6$ cells/75T flask) were exposed to 0, 50, 100, and 200 μ M of GdCl₃ for 24 h. The cell lysates were then harvested and incubated with a commercial lysis buffer according to the instructions of the manufacturer (Caspase-3,-8 and -9 Colorimetric Assay kits; R&D Systems).

Determinations of reactive oxygen species (ROS), the mitochondrial membrane potential ($\Delta\Psi m$) and intracellular Ca²⁺ levels via flow cytometry. C6 cells (2x10⁵ cells/well) were incubated with or without 50, 100, 150 and 200 μ M of GdCl₃ for 6 h. After treatment, the medium was replaced with 5 μ M dihydrorhodamine 123 (an ROS indicator), 4 nM DiOC6(3) (a $\Delta\Psi m$ fluorescent probe) and 3 μ g/ml Fluo-3/AM (cytoplasmic



Figure 1. Effects of GdCl₃ on the cell viability of rat glioma C6 and human normal fetal glial SVG p12 cells. (A) Viability of C6 cells treated with GdCl₃ at the indicated concentrations for 24 h via an MTT assay. Data are expressed as the mean \pm SD (n=3). *P<0.05 compared to the control. (B) Morphological changes of C6 cells treated with GdCl₃ at 50, 100, and 150 μ M of GdCl₃ for 24 h. Images were taken from one of three independent experiments; (magnification, x200). (C) SVG p12 cells were exposed to GdCl₃ (0, 50, 100, 150, and 200 μ M) for 24 h. Cell viability was determined via an MTT method. GdCl₃, gadolinium chloride; SD, standard deviation.

Ca²⁺ dye), respectively, and the plates were protected from light for 30 min at 37°C. The mean fluorescence intensity (MFI) was measured via flow cytometry (Muse Cell Analyzer; Merck Millipore) and expressed as % of the control (vehicle), as previously described (14,19).

Statistical analysis. All data are represented as the mean \pm standard deviation (SD) from at least three independent experiments. Statistical calculations of the data were carried out via one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

GdCl₃ inhibits viability of rat C6 glioma cells. The MTT assay was used to evaluate the impact of GdCl₃ on C6 cells. The cells were treated with GdCl₃ at the different concentrations $(0, 50, 100, 150, \text{ and } 200 \ \mu\text{M})$ for 24 h. The cell viability was significantly decreased with the treatment of >100 μ M GdCl₃ in a concentration-dependent manner (Fig. 1A). The half-maximal inhibitory concentration (IC₅₀) of GdCl₃ for C6 cells was $152.36\pm2.45 \mu$ M. GdCl₃ at the concentration of 150 μ M markedly caused apoptotic cell shrinkage and the morphological changes, but the untreated control was well spread in C6 cells (Fig. 1B). Our results suggested that GdCl₃ possessed a cytotoxic effect on C6 cells. However, no effect of viability (Fig. 1C) and morphological change (data not shown) was observed on normal SVG p12 cells after GdCl₃ treatment (0, 50, 100, 150, and 200 μ M). Therefore, GdCl₃ may exhibit lower toxicity in normal glial SVG p12 cells.

*GdCl*₃ *induces apoptosis in C6 cells*. To explore whether GdCl₃ induces C6 cell death through an apoptotic mechanism, a TUNEL assay was used to detect DNA breaks in apoptotic cells. The percentage of TUNEL-positive cells (cells undergoing apoptosis) was markedly increased in GdCl₃-treated C6 cells compared to that noted in the control cells, and the effect was concentration-dependent (Fig. 2A). We also measured DNA damage using comet assay. GdCl₃ triggered DNA damage through the production of a longer comet tail (Fig. 2B, upper panel) after DNA was stained with PI in C6 cells. Moreover, chromatin and apoptotic DNA condensation was observed in C6 cells after GdCl₃ exposure using DAPI staining (Fig. 2B, bottom panel). Our findings indicate that GdCl₃ provoked an apoptotic response in C6 cells.

GdCl3 elevates the activities of caspase-3, caspase-8 and caspase-9 in C6 cells. To characterize the mechanism of GdCl₃-induced apoptosis, we explored the expression levels of caspase-3, caspase-8 and caspase-9 using immunoblotting analysis. GdCl₂ increased cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 levels in C6 cells (Fig. 3A). We also examined the activities of caspase-3, caspase-8 and caspase-9 in GdCl₃-treated C6 cells. The activities of caspase-3 (Fig. 3B), caspase-8 (Fig. 3C) and caspase-9 (Fig. 3D) were elevated after GdCl₃ challenge, and the effects were concentration-dependent. Furthermore, the cells were pre-treated with a pan-caspase inhibitor (Z-VAD-FMK) (Fig. 4A), and selective inhibitors to caspase-3, caspase-8 and caspase-9 (Fig. 4B) to confirm the specificities of caspases. Data showed that specific inhibitors to caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) significantly prevented the GdCl₃-reduced cell viability (Fig. 4B). These data suggest



Figure 2. Effects of $GdCl_3$ on the apoptosis of rat glioma C6 cells. (A) Cells were treated with $GdCl_3$ at indicated concentrations for 24 h. Apoptotic cells were determined by TUNEL assay kit, as described in Materials and methods. Data are presented as the mean \pm SD (n=3). *P<0.05 compared to the control. (B) Fluorescence staining of apoptotic cells in $GdCl_3$ -treated cells were observed. PI was used to stain the nuclei via comet assay (upper panel), and the yellow arrows indicate the nuclei and DNA damage of apoptotic cells. DAPI dye was applied to probe the nuclei condensation (bottom panel); x400 magnification. Representative images are from one of three independent experiments. GdCl₃, gadolinium chloride; PI, propidium iodide.



Figure 3. Effects of GdCl₃ on caspase cascade protein expression and activities in rat glioma C6 cells. Cells were treated with 50, 100, and $150 \,\mu$ M of GdCl₃ for 24 h. The cell lysates were harvested to assess (A) the expression levels of caspase-3, caspase-8, and caspase-9 using immunoblotting analysis. β -actin was used as a control to ensure an equal loading amount. Representative chemiluminescent images were collected from one of independent duplicate experiments. The activities of (B) caspase-3, (C) caspase-8, and (D) caspase-9 were monitored, as described in Materials and methods. Data are presented as the mean \pm SD (n=3). *P<0.05 compared to the control. GdCl₃, gadolinium chloride; SD, standard deviation.

that the caspase cascade-mediated the signaling results in GdCl₃-induced apoptosis in C6 cells.

 $GdCl_3$ enhances the ROS production and loss of $\Delta\Psi m$ level in C6 cells. To detect the effect of GdCl₃ on ROS production in C6 cells, we measured intracellular ROS levels by dihydrorhodamine 123 fluorescent dye, and the stained cells were analyzed by flow cytometry. Intracellular ROS levels were concentration-dependently increased in C6 cells following GdCl₃ exposure (Fig. 5A). To further test the effect of GdCl₃ on $\Delta\Psi m$ levels, we used the specific fluorescent probe DiOC6(3) to detect the levels of $\Delta\Psi m$. GdCl₃ disrupted the $\Delta\Psi m$ level in C6 cells, and this impact was in a concentration-dependent manner (Fig. 5B). These results indicate that GdCl₃ triggered apoptosis via ROS production and mitochondrial dysfunction in C6 cells.

 $GdCl_3$ prompts cytoplasmic Ca^{2+} level in C6 cells. To further elucidate whether the Ca²⁺ level is involved in GdCl₃-induced apoptosis and the possible signaling in C6 cells, we detected intracellular Ca²⁺ levels by flow cytometric analysis. The cells were treated with 50, 100, and 150 μ M of GdCl₃ for 24 h. GdCl₃ significantly increased the intracellular Ca²⁺ level (Fig. 5C). These results revealed that cytoplasmic Ca²⁺ signaling and ER stress-mediated pathway may contribute to GdCl₃-induced apoptotic machinery in C6 cells.



Figure 4. Effects of caspase inhibitors to reverse GdCl₃-reduced viability of rat glioma C6 cells. Cells were treated with 150 μ M GdCl₃ for 24 h after being pre-incubated with or without (A) 15 μ M Z-VAD-FMK (a pan-caspase inhibitor) and (B) 15 μ M Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), and Z-LEHD-FMK (a caspase-9 inhibitor) for 2 h, respectively. Cell viability was determined by the MTT assay. Data are expressed as the mean \pm SD (n=3). *P<0.05 compared to the control. GdCl₃, gadolinium chloride; SD, standard deviation.

GdCl₃ alters the levels of apoptosis- and mitogen-activated protein kinase (MAPK)-related protein molecules in C6 cells. To understand the molecular mechanisms of the apoptotic pathway, we further explored the levels of apoptosis-related protein signals (cytochrome c, Bax and Bcl-2) using immunoblotting analysis. The levels of cytochrome c and Bax were increased. By contrast, the level of anti-apoptotic protein Bcl-2 was decreased in GdCl₃-treated cells (Fig. 5D). In addition, we also investigated the levels of MAPK-related pathway proteins (p-ERK, ERK, p-JNK, JNK, p-p38 MAPK and p38 MAPK) also using immunoblotting analysis. GdCl₃ downregulated the levels of the p-ERK, p-JNK and p-p38 MAPK pathway in C6 cells (Fig. 6). These data demonstrated that GdCl₃-induced apoptotic cell death may result from mitochondria-dependent and MAPK-mediated signaling in C6 cells.

Discussion

Malignant glioma is an invasive intracranial tumor that grows rapidly and has extremely high recurrence (1,20). At present, postoperative chemotherapy has become a powerful strategy for the treatment of adult glioma patients (1,2,20). However, treatment has been particularly inefficient for glioma patients administered chemotherapeutic agents due to the existence of the BTB. The permeability of the BTB and blood brain barrier (BBB) impedes the accumulation and uptake rate of potential drugs to brain tumor tissues (1,2,20). Liu *et al* (21) showed that the efficacy of killing brain tumor cells increased by 10-fold when the therapeutic agent was permeabilized into the area of the brain tumor tissue. Hopefully, a therapeutic agent can be found that can pass through the BTB and BBB, and with that the efficacy of chemotherapy in treating glioma would be markedly improved. Gd-based contrast agents (GBCAs) are applied as pharmaceuticals and have been approved for 30 years and are used daily in millions of patients worldwide (22). In recent years, numerous retrospective clinical studies have reported the unexpected long-term presence of Gd in the brain after receiving radiology practice (22,23). Previous studies have reported that the efficacy of chemotherapy in treating glioma would be markedly improved if the therapeutic agent can be found that can pass through the BTB and BBB (23). These results suggested that GdCl₃ can accumulate and be uptaken by tumors in the brain through favorable permeability of the BTB and BBB. In addition, Gd compounds possess diverse anticancer activities (8,11). Previous studies have shown that after treatment with a relatively low concentration of GdCl₃, cell cycle progression and cell growth were promoted, whereas human hepatoma HepG2 and osteosarcoma U-2 OS cells exposed to high concentrations of GdCl₃ exhibited apoptosis and suppressed cell proliferation (8,11). It is suggested that GdCl₃-induced apoptosis is specific to HepG2 cells (11), U-2 OS cells (8) and rat C6 glioma cells. In this study, we demonstrated that GdCl₃ caused anti-proliferative effects on rat C6 glioma cells in a concentration-dependent manner (Fig. 1A). Our data demonstrated that the half-maximal inhibitory concentration (IC₅₀) values for a 24-h treatment of GdCl₃ in U-2 OS and rat C6 glioma cells were 198.26±1.69 and $152.36\pm2.45 \,\mu\text{M}$, respectively. In addition, Shen et al (24) and Fu et al (25) demonstrated that Gd promoted cell proliferation in mouse embryo fibroblast NIH3T3 cells. Ferreira et al (26) indicated that no effect was noted in regards to the viability of Kupffer cells after GdCl₃ exposure. Our data also revealed that there was no viability impact (Fig. 1C) and morphological change (data not shown) on GdCl₃-treated normal SVG p12 cells, suggesting that GdCl₃ has an extremely low toxicity in normal glial cells.

Apoptosis (type I programmed cell death) is a vital mechanism in antitumor drugs and cancer therapies (27,28). It is a promising approach to induce apoptosis in glioma. Induction of tumor cell apoptosis is one of the best strategies for treating glioma and multiple types of cancers (27,28). Apoptotic death can be activated by three main pathways (17,29). The extrinsic pathway is triggered through binding of extrinsic signals to death receptors, which leads to activation of caspase-8 (29,30). The mitochondrial (intrinsic signaling) pathway is activated upon cellular stresses such as ROS production and $\Delta\Psi m$ disruption, and this results in activation of caspase-9. Both the extrinsic and intrinsic pathways can activate caspase-3 signaling and the major executioner caspase cascade (16,29,31). Therefore, induction of apoptotic pathways by a novel agent is a potentially powerful approach to fighting cancer cells (32,33).

Our results indicate that a high concentration of $GdCl_3$ caused the apoptosis of C6 cells (Fig. 1). This is the first study to report that $GdCl_3$ could be successfully applied to promote cell death in rat C6 glioma cells. In the present study, an increase of DNA damage and fragmentation of cells (an apoptotic characteristic) was demonstrated after exposure to $GdCl_3$ (Fig. 2). Previous studies have shown that Gd^{3+} caused distinct effects depending on the type of target cells (8,11). $GdCl_3$ was



Figure 5. Effects of GdCl₃ on ROS production, $\Delta\Psi$ m, and cytoplasmic Ca²⁺ level of rat glioma C6 cells. Cells were treated with 50, 100, and 150 μ M of GdCl₃ for 24 h. Cells were then mixed with (A) 5 μ M dihydrorhodamine 123 for detecting ROS levels, (B) 4 nM fluorochrome DiOC6(3) for detecting the disruption of $\Delta\Psi$ m, and (C) 3 μ g/ml Fluo-3/AM for detecting intracellular Ca²⁺ levels. MFI was measured by flow cytometry. Data are expressed as the mean \pm SD (n=3). *P<0.05 compared to the control. (D) Immunoblotting analysis against cytochrome *c*, Bax, and Bcl-2 signals after GdCl₃ challenge for 24 h. β -actin was used as a loading control. Representative chemiluminescent images were collected from one of independent duplicate experiments. ROS, reactive oxygen species; GdCl₃, gadolinium chloride; $\Delta\Psi$ m, mitochondrial membrane potential; MFI, mean fluorescence intensity; SD, standard deviation.



Figure 6. Effect of GdCl₃ on the levels of phosphorylation of MAPK signaling molecules in rat glioma C6 cells. Cells were exposed to 0, 50, 100, and 150 μ M of GdCl₃ for 24 h. Cell fractions were extracted and subjected to immunoblotting analysis using the specific antibodies (p-ERK, ERK, p-JNK, JNK, p-p38 MAPK, and p38 MAPK). β -Actin was used as a loading control. Representative chemiluminescent images were collected from one of independent duplicate experiments. GdCl₃, gadolinium chloride; p-ERK, phosphorylated-extracellular signal-regulated kinase; p-JNK, phosphorylated-c-Jun N-terminal kinase; p-p38, phosphorylated-p38.

found to exert a proliferation-promoting ability and to activate ERK and phosphoinositide 3-kinase (PI3K) signaling pathways in NIH 3T3 cells (24). Gd triggered cell apoptosis through the mitochondrial pathway in human osteosarcoma U-2 OS cells (8). Therefore, we sought to investigate the exact effect of GdCl₃ on cell proliferation and cell apoptosis in rat C6 glioma cells.

Apoptosis can be induced though activation of a cascade of caspases (29,31). Ye et al (11) showed that GdCl₃ triggered HepG2 cell apoptosis through death receptor-dependent and mitochondrial pathways. Our findings previously reported that GdCl₃-induced apoptosis may be mediated through the extrinsic pathway, the intrinsic pathway, and the ER stress pathway in human osteosarcoma U-2 OS cells (8). Caspase-3 (a 35-kDa protein), a critical executioner of apoptosis, is either partially or totally responsible for the proteolytic cleavage by nuclear enzyme poly (ADP-ribose) polymerase (PARP) (34). Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 fragments. Caspase-8 is a 55-kDa protein and is an inactive pro-enzyme. Activation of caspase-8 involves a two-step proteolysis: i) the cleavage of caspase-8 to generate a 43- and a 12-kDa fragment which is further processed to 10 kDa; and ii) p43 is then cleaved to yield p26 and the release of the active site containing p18 (35-37). Caspase-9 is an important family protein in the intrinsic apoptotic pathway (38). Upon apoptotic stimulation, cytochrome c released from mitochondria associates with the 47-kDa procaspase-9/Apaf-1. Apaf-1-mediated activation of caspase-9 involves intrinsic proteolytic processing resulting in cleavage on Asp315 and producing a p35 subunit. Another cleavage occurs on Asp330 producing a p37 subunit that can serve to amplify the apoptotic response (39-41). In this study, GdCl₃ increased the enzymatic activities of caspase-3, caspase-8, and caspase-9, as well as cleaved-caspase-3 (p19), cleaved-caspase-8 (p18), and cleaved-caspase-9 (p35) protein levels in vitro (Fig. 3). Our results suggested that GdCl₃-triggered apoptosis of C6 cells resulted from caspase-dependent signaling. Furthermore, GdCl₃ elevated the ROS production and decreased the levels

of $\Delta \Psi m$ (Fig. 5). The expression level of the anti-apoptotic protein Bcl-2 was decreased, while the expression levels of cytochrome c and pro-apoptotic protein Bax were increased after GdCl₃ exposure in C6 cells (Fig. 5D). Our results found that GdCl₃-triggered apoptosis of C6 cells was mitochondria-dependent.

The influx of Ca^{2+} is involved in several biological functions including cell proliferation, apoptosis and differentiation (42,43). Ca^{2+} rapidly flows into the cytoplasm and into the mitochondria, leading to cell apoptosis (29,31). Xia *et al* (44) demonstrated that Gd caused oxidative stress in rat cortical neurons. Feng *et al* (45) indicated that Gd triggered ER stress and unfolded protein responses in primary cultured rat cortical astrocytes through an increase in the influx of extracellular Ca^{2+} level. However, no report regarding GdCl₃-induced ER stress in rat C6 glioma cells has been conducted. The present study revealed that GdCl₃ caused the release of Ca^{2+} , which led to apoptosis in C6 cells (Fig. 5C). Thus, our results suggest that GdCl₃-induced cell death may be mediated via the apoptotic mechanism in C6 cells.

MAPKs are divided into three main subfamilies: ERK, JNK, and p38 MAPKs. Increasing evidence has shown that MAPKs play crucial roles and exhibit cell functions in cell survival, cell proliferation, cell cycle regulation, and apoptotic death (46,47). ERK is involved in cell survival, and JNK and p38 MAPK are thought to mainly promote cell apoptosis (48,49). Activation of ERK1/2 has been demonstrated to inhibit apoptosis in response to tumor necrosis factor (TNF), Fas ligand, radiation, stress, hypoxia, and chemotherapeutic agent stimulation (50). Subramanian et al (51) suggested that estrogen-induced increase in Ca2+ leads to ERK phosphorylation and consequently CREB phosphorylation, resulting in an increase in the anti-apoptotic Bcl-2 protein level. The inactivation of pro-apoptotic Bcl-2 family member BAD is mediated through phosphorylation on Ser112 by ERK activated p90 ribosomal S6 kinase (RSK). Inhibition of JNK2 activity can also suppress tumorigenesis by promoting apoptosis (52). Yu et al (53) demonstrated that one of the molecular mechanisms by which JNK suppresses apoptosis is through phosphorylation of BAD on Thr201, thereby inhibiting its pro-apoptotic activity. p38 plays a role in cell survival supported by increased levels of Bcl-2 and Bcl-xL in response to DNA damage and stress (54,55). Furthermore, the chemical inhibition of p38 has been strongly associated with increased chemosensitivity in tumor cells (56,57). Intriguingly, in this study, the expression of p-ERK, p-JNK, and p-p38 MAPKs were downregulated after GdCl₃ exposure (Fig. 6). MAPKs were crucial for GdCl₃-induced apoptosis in C6 cells. We suggest that the phosphorylation of MAPKs was involved in Bcl-2 modulation in GdCl₃-induced apoptosis of C6 cells. Wang et al (58) demonstrated that GdCl₃ inhibits PC3 cell migration by the inactivation of both ERK and p38 MAPK pathways. Our results are in accordance with that study (58), which indicated that GdCl₃ suppressed ERK and p38 MAPKs and triggered apoptosis in C6 glioma cells.

In conclusion, $GdCl_3$ provoked apoptosis in C6 cells through upregulation of cytochrome *c* and Bax, downregulation of Bcl-2, and activation of caspase-3, caspase-8, and caspase-9 signaling. $GdCl_3$ triggered C6 cell apoptosis via mitochondria-dependent pathway. $GdCl_3$ may be a promising therapy for human glioma and an adjunct to other chemotherapies. Our findings provide a new molecular mechanism underlying the action of GdCl₃ for the chemotherapy of glioma.

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Availability of data and materials

The datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YFT, SCT and JSY conceived and designed the experiments. YFC, CYH, CWH and CCL performed the experiments. CCL, SCT and JSY analyzed the data. YFT, SCT and JSY wrote and modified the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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