Gadolinium chloride elicits apoptosis in human osteosarcoma U-2 OS cells through extrinsic signaling, intrinsic pathway and endoplasmic reticulum stress

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Abstract. Gadolinium (Gd) compounds are important as magnetic resonance imaging (MRI) contrast agents, and are potential anticancer agents. However, no report has shown the effect of gadolinium chloride ($GdCl_3$) on osteosarcoma *in vitro*. The present study investigated the apoptotic mechanism of $GdCl_3$ on human osteosarcoma U-2 OS cells. Our results indicated that $GdCl_3$ significantly reduced cell viability of U-2 OS cells in a concentration-dependent manner. $GdCl_3$ led to apoptotic cell shrinkage and DNA fragmentation in U-2 OS cells as revealed by morphologic changes and TUNEL staining. Colorimetric assay analyses also showed that activities of caspase-3, caspase-8, caspase-9 and caspase-4 occurred in $GdCl_3$-treated U-2 OS cells. Pretreatment of cells with pan-caspase inhibitor (Z-VAD-FMK) and specific inhibitors of caspase-3/-8/-9 significantly reduced cell death caused by $GdCl_3$. The increase of cytoplasmic Ca^{2+} level, ROS production and the decrease of mitochondria membrane potential ($\Delta\Psi m$) were observed by flow cytometric analysis in U-2 OS cells after $GdCl_3$ exposure. Western blot analyses demonstrated that the levels of Fas, FasL, cytochrome *c*, Apaf-1, GADD153 and GRP78 were upregulated in $GdCl_3$-treated U-2 OS cells. In conclusion, death receptor, mitochondria-dependent and endoplasmic reticulum (ER) stress pathways contribute to GdCl_3-induced apoptosis in U-2 OS cells. $GdCl_3$ might have potential to be used in treatment of osteosarcoma patients.

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Introduction

Osteosarcoma is the most common type of malignant bone tumor in children and teenagers (1,2). Unfortunately, less than 50% of patients living beyond 10 years results from the response to the current preferred treatment of preoperative adjuvant chemotherapy followed by surgery (3). Furthermore, no reliable predictors are applied to guide the choice or the intensity of the chemotherapy (4). Currently, there is still a limited role in radiotherapy which should be reserved for inoperable situations. Doxorubicin, cisplatin, ifosfamide and high-dose methotrexate with leucovorin rescue are recognized as the most effective agents against osteosarcoma, but the ideal combination remains to be defined and needs to further well investigated (5). Targeting apoptosis (programmed cell death type I) is a promising approach in the fight against osteosarcoma (6). Therefore, the induction of cell apoptosis is one of best strategies for treating osteosarcoma and multiple cancers (7,8).

Apoptotic function is associated with several diseases, including cancer genesis, and diabetes, and this process is considered a critical element of cancer prevention and therapy (6,9). The three main pathways (death receptor signal, mitochondrial regulation and endoplasmic reticulum stress) contribute to apoptotic signaling (10-12). The death receptor signal is induced by the binding of extrinsic signals to surface receptors, resulting in activation of caspase-8 followed by the activation of caspase-3/-7 (10,11). The mitochondrial pathway is regulated by endoplasmic reticulum (ER) various damage stimuli, which increase reactive oxygen species (ROS) and subsequent damage DNA. These stresses disrupt mitochondrial membrane potential ($\Delta\Psi m$) to cause cells to undergo apoptosis cascade (11,13). Accumulation of unfolded/misfolded

proteins elicits ER-specific pathway aggregating in ER by excessive protein traffic (12). The hallmarks of ER stress are known to stimulate the protein level of growth arrest- and DNA damage-inducible gene 153 (GADD153), glucose-regulated protein 78 (GRP78), GRP94 and activating transcription factor 4 (ATF-4), which can induce intracellular Ca^{2+} level to activate calpain and caspase-12 and/or caspase-4 molecules (13,14). Therefore, induction of apoptosis by a novel target in these pathways is an attractive approach to fight the tumor cell system.

The rare earth metals of Lanthanides (Lns) exhibit a variety of physical or chemical properties and have been applied in agriculture and medicine for a long time (15,16). Gadolinium (Gd), a member of Lns family, has multi-biological effects on organisms (17), and its compounds have important applications in magnetic resonance imaging (MRI) as contrast medium and are potential anticancer agents (18,19). In clinic, chelates of Gd such as gadobenate dimeglumine (MultiHance) are used as contrast agent in MRI (20). Gadolinium texaphyrin complex (MGd) was evaluated in phase III clinical trials for treating brain metastases of non-small cell lung cancer (21). The Kupffer cell inhibitor gadolinium chloride (GdCl₃) has been demonstrated to inhibit human hepatoma HepG2 cell proliferation (22,23). It was also shown that GdCl₃ induces HepG2 cell apoptosis through mitochondria-dependent pathway (22). Hence, the present study investigated the effects of GdCl₃-induced apoptosis and the underlying mechanisms on human osteosarcoma U-2 OS cells. The experimental data indicated that GdCl₃ triggered U-2 OS cell apoptosis through death receptor-, mitochondria- and ER stress-dependent pathways.

Materials and methods

Chemicals and reagents. Gadolinium chloride (GdCl₃), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). McCoy's 5A (modified) medium, fetal bovine serum (FBS), trypsin-EDTA, penicillin/streptomycin, Fluo-3/AM, 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) and 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Pan-caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies (anti-Fas/CD95, anti-FasL, anti-cytochrome *c*, anti-Apaf-1, anti-GADD153 and anti-GRP78 and anti-β-actin) and second antibodies [goat anti-rabbit IgG-horseradish peroxidase (HRP) and goat anti-mouse IgG-HRP] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. Human osteogenic sarcoma U-2 OS cell line was obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and cultured in 75-cm² tissue culture flasks with McCoy's 5A (modified) medium with 10% FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO₂.

Cell viability and morphological observation. U-2 OS cells (1x10⁴ cells/well) in a 96-well plate were individually pretreated with or without 15 μM specific caspase inhibitors (Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK) for 2 h and then exposed to 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h. After challenge, cell viability was assessed by an MTT assay, as previously reported by Lu *et al* (24). The GdCl₃-treated cells were examined for apoptosis and photographed utilizing a phase-contrast microscope, as previously described (25).

Apoptosis by TUNEL and DAPI staining. U-2 OS cells (2x10⁵ cells/well) in 24-well plates were treated with 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h. Apoptotic DNA breaks were labeled with the In Situ Cell Death Detection kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the vendor's protocol, and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) positive cells were monitored using a BD FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, USA) and BD CellQuest Pro Software (BD Biosciences), as previously described (26). DAPI dye was used to countstain condensation nuclei (a characteristic of apoptosis) in U-2 OS cells with or without 200 μM GdCl₃ for 24 h, as detailed by previous studies (27,28).

Assays for caspase-8/-9/-3/-4 activities. U-2 OS cells (5x10⁶ cells per 75T flask) were exposed to 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h. The cell lysates were harvested and assessed subsequently in accordance with the manufacturer's instruction [caspase-8, caspase-9 and caspase-3 colorimetric assay kits (R&D System Inc.) and caspase-4 colorimetric assay kit (BioVision Incorporated, Milpitas, CA, USA)].

Determinations of intracellular Ca²⁺, ROS and ΔΨm levels by flow cytometry. U-2 OS cells (2x10⁵ cells/well) were incubated with or without 50, 100, 150 and 200 μM of GdCl₃ for 6 h. Cells were harvested and individually stained with 3 μg/ml Fluo-3/AM for cytoplasmic Ca²⁺, ROS indicator (5 μM H₂DCFDA) and ΔΨm probe [4 nM DiOC₆(3)] at 37˚C for 30 min. The mean fluorescence intensity (MFI) was quantified by BD CellQuest Pro software (BD Biosciences) before analysis by flow cytometry, as previously described (13,26).

Western blot analysis. U-2 OS cells (5x10⁶ cells/75T flask) were treated in the presence and absence of 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h. Cell lysates were collected, and protein was fractionated on SDS-polyacrylamide gel electrophoresis (PAGE) after being mixed with protein loading dye and boiled, as previously described (14,29). The membrane (iBlot Transfer Stack, PVDF regular; Thermo Fisher Scientific Inc.) was probed with antibodies against Fas, FasL, cytochrome *c*, Apaf-1, GADD153 and GRP78, respectively. All blots were normalized to β-actin signal, and each signal was quantified with NIH ImageJ program for Windows (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical calculations of the data for comparisons of two mean values were obtained using Student's t-test. Statistical significance was set at P<0.05. All data were expressed as mean ± standard deviation (SD) of three independent experiments.
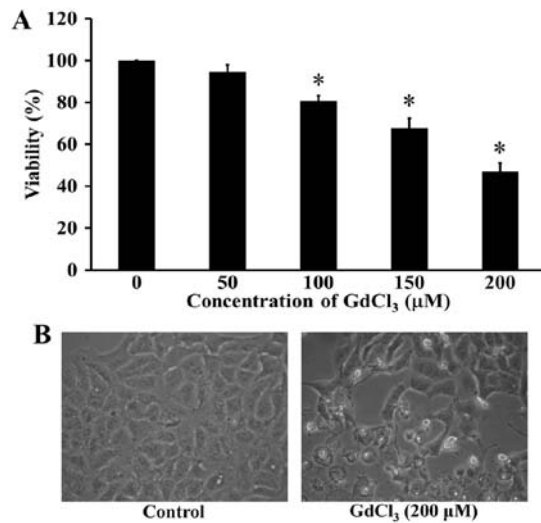
Figure 1. Effects of gadolinium chloride (GdCl$_3$) on cell viability in human osteosarcoma U-2 OS cells. Cells were treated with GdCl$_3$ (0, 50, 100, 150 and 200 μM) for 24 h. (A) Cell viability was determined by an MTT assay, and data are presented as the mean ± SD (n=3). *P<0.05 vs. untreated control. (B) Cells in response to 200 μM GdCl$_3$ showed morphological changes and were photographed from three independent experiments.

Figure 2. Effects of gadolinium chloride (GdCl$_3$) on apoptosis in U-2 OS cells. Cells were exposed to 200 μM GdCl$_3$ for 24 h and then harvested to determine by TUNEL assay kit as described in Materials and methods. (A) Immunofluorescence staining of apoptotic cells in GdCl$_3$-treated cells was observed, and 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. (B) TUNEL positive cells were quantified, and data are presented as the mean ± SD (n=3). *P<0.05 vs. untreated control.

Results

GdCl$_3$ reduces cell viability of human osteosarcoma U-2 OS cells. Cells were treated with GdCl$_3$ at the different concentrations from 0 to 200 μM for 24 h. The number of viable cells was assessed by the MTT method. The cell viability was markedly decreased in GdCl$_3$-treated U-2 OS cells in concentration-dependent manner (Fig. 1A). The inhibitory concentration 50% (IC$_{50}$) for U-2 OS cells was 198.26±1.69 μM. GdCl$_3$ at 200 μM led to the morphological changes and apoptotic cell shrinkage in U-2 OS cells. Also, GdCl$_3$-treated cells were detached from the surface, and some cell debris was observed, whereas the untreated control cells were well spread (Fig. 1B). These findings suggested that GdCl$_3$ possessed cytotoxic effect on human osteosarcoma U-2 OS cells.

GdCl$_3$ provokes U-2 OS cell apoptosis. To explore whether GdCl$_3$ induces U-2 OS cell death through an apoptotic mechanism, TUNEL assay was used for detecting DNA fragmentation in apoptotic cells. GdCl$_3$ markedly stimulated cell apoptosis (TUNEL positive cells) in comparison with control cells (Fig. 2A), and this effect was concentration-dependent (Fig. 2B). Furthermore, apoptotic DNA condensation by DAPI stain was shown in GdCl$_3$-treated U-2 OS cells (Fig. 2A). Our results indicated that GdCl$_3$ provoked apoptotic cell death in U-2 OS cells.

GdCl$_3$ stimulates the activities of caspase-3, caspase-8, and caspase-9 in U-2 OS cells. To clarify the mechanism of GdCl$_3$-induced apoptosis, we further individually investigated the activities of caspase-3, caspase-8, and caspase-9 in U-2 OS cells prior to GdCl$_3$ challenge. The activities of caspase-3 (Fig. 3A), caspase-8 (Fig. 3B) and caspase-9 (Fig. 3C) were increased after GdCl$_3$ exposure in a concentration-dependent manner. Pretreatment of cells with pan-caspase inhibitor (Z-VAD-FMK) (Fig. 4A), specific inhibitors to caspase-3,
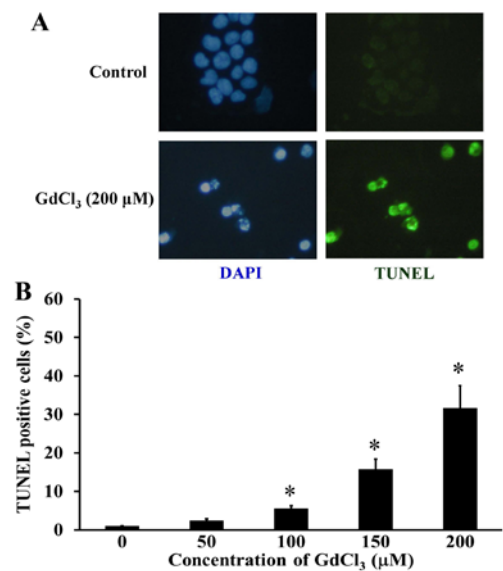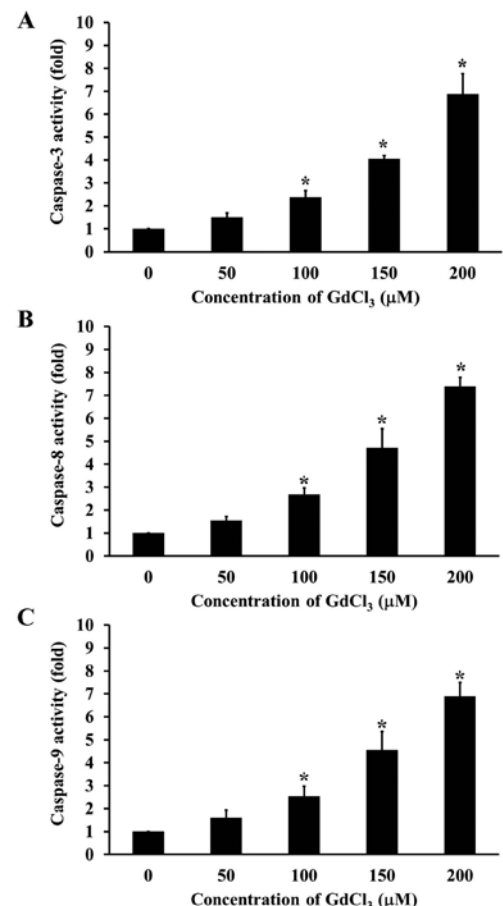

Figure 3. Effects of gadolinium chloride (GdCl$_3$) on caspase-3, caspase-8, and caspase-9 activities in U-2 OS cells. Cells were incubated with 0, 50, 100, 150 and 200 μM of GdCl$_3$ for 24 h. The cell lysates were harvested to assess (A) caspase-3, (B) caspase-8 and (C) caspase-9 activities as described in Materials and methods. Data are presented as the mean ± SD (n=3). *P<0.05 vs. untreated control.
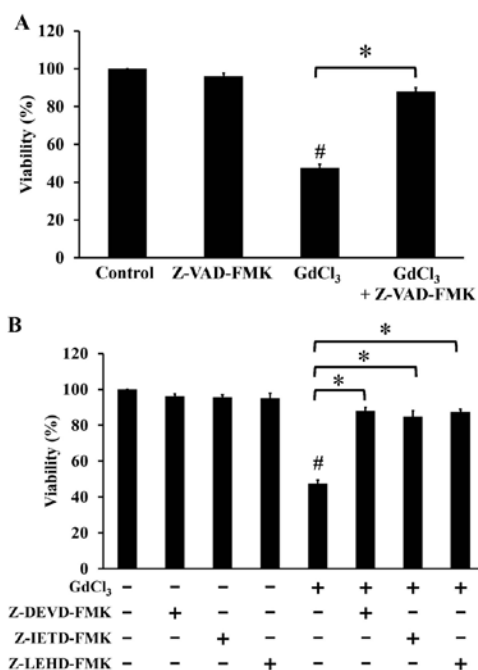
Figure 4. Effects of caspase inhibitors on U-2 OS cells after gadolinium chloride (GdCl₃) treatment. Cells were exposed to 200 μM GdCl₃ for 24 h after pretreatment with or without 15 μM of pan-caspase inhibitor (Z-VAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) for 2 h. Pretreatment with (A) Z-VAD-FMK and (B) inhibitors of caspase-3, caspase-8 and caspase-9, respectively, cell viability was carried out by an MTT assay, and data are presented as the mean ± SD (n=3). #P<0.05 vs. untreaed control; *P<0.05 vs. GdCl₃-treated only.

Figure 5. Effects of gadolinium chloride (GdCl₃) on cytoplasmic Ca²⁺ levels and caspase-4 activity in U-2 OS cells. Cells were exposed to 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h and then collected to employ (A) intracellular Ca²⁺ levels after stained with 3 μg/ml Fluo-3/AM by flow cytometry and (B) caspase-4 level as described in Materials and methods. Data are presented as the mean ± SD (n=3). *P<0.05 vs. untreated control.

caspase-8, and caspase-9 (Fig. 4B) significantly prevented the GdCl₃-induced cell apoptosis. Our results suggest that the caspase cascade-dependent pathway is important in GdCl₃-induced apoptotic death in U-2 OS cells.

GdCl₃ increases cytoplasmic Ca²⁺ level and stimulated the activity of caspase-4 in U-2 OS cells. To further elucidate the possible signaling of GdCl₃-induced apoptosis in U-2 OS cells, we determined intracellular Ca²⁺ levels and caspase-4 activity by flow cytometric analysis and caspase activity assay, respectively. Cells were treated with 50, 100, 150 and 200 μM of GdCl₃ for 24 h, and our data demonstrated that GdCl₃ significantly increased intracellular Ca²⁺ levels (Fig. 5A). In addition, caspase-4 activity was concentration-dependently increased in U-2 OS cells after GdCl₃ treatment (Fig. 5B). These results showed that GdCl₃ induced apoptotic response through cytoplasmic Ca²⁺ level and ER stress-mediated pathway.

GdCl₃ stimulates the ROS production and loss of ΔΨm level in U-2 OS cells. To examine the effect of GdCl₃ on ROS production and ΔΨm levels in U-2 OS cells, the specific fluorescence probes were individually used to detect the levels of ROS and ΔΨm. GdCl₃ concentration-dependently increased intracellular ROS level in U-2 OS cells (Fig. 6A). GdCl₃ disrupted ΔΨm level in U-2 OS cells in a concentration-dependent manner (Fig. 6B). These results suggested that GdCl₃ triggered apoptosis in U-2 OS cells by way of the ROS and mitochondria-dependent signaling.
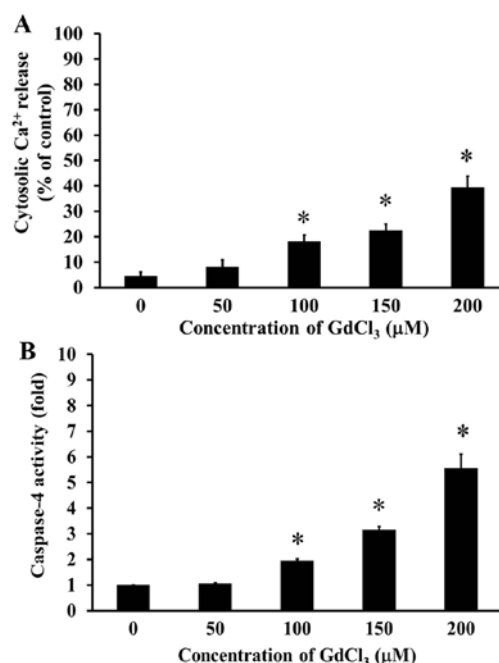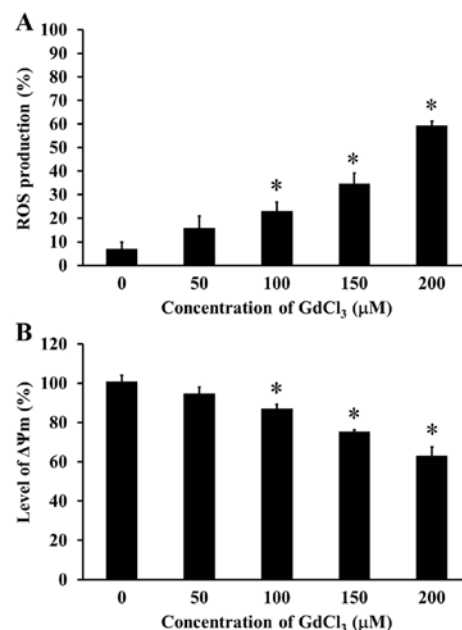

Figure 6. Effects of gadolinium chloride (GdCl₃) on ROS and ΔΨm in U-2 OS cells. Cells were incubated with 0, 50, 100, 150 and 200 μM of GdCl₃ for 24 h. (A) ROS level was assessed by staining with 5 μM H₂DCFDA, and (B) the disruption of ΔΨm was measured by using the fluorochrome 4 nM DiOC₆(3) by flow cytometry. Data are presented as the mean ± SD (n=3). *P<0.05 vs. untreated control.

GdCl₃ alters the levels of apoptosis-related proteins in U-2 OS cells. In more detail, in the molecular mechanisms of apoptotic pathway, we investigated these related protein levels by western blotting. The levels of Fas, FasL, cytochrome *c*, Apaf-1,
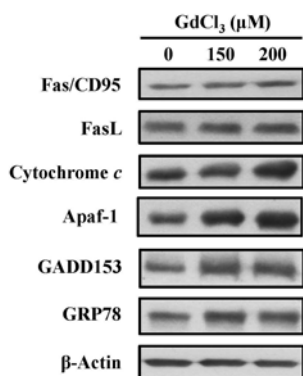
Figure 7. Effects of gadolinium chloride (GdCl$_3$) on the levels of apoptotic relative proteins in U-2 OS cells. Cells were exposed to 150 and 200 μM of GdCl$_3$ for 24 h, and whole-cell lysates were prepared and subjected to western blot analysis and to blot using the specific antibodies (Fas/CD95, FasL, cytochrome c, Apaf-1, GADD153 and GRP78). β-actin served as the loading control to ensure equal loading.
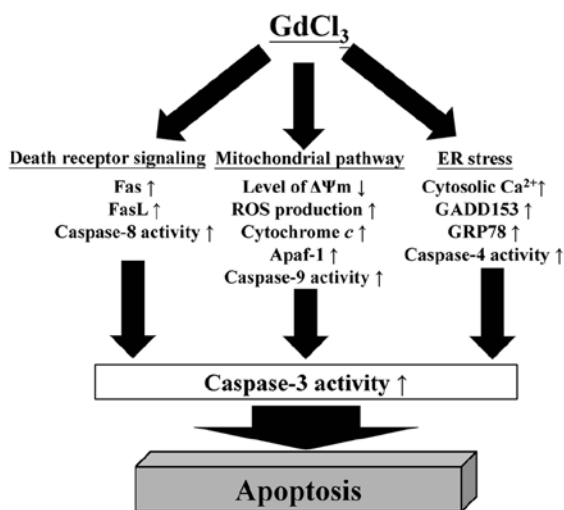

Figure 8. The proposed pathway indicates the three major apoptosis signaling (death receptor, mitochondria and ER stress pathways) for gadolinium chloride (GdCl$_3$)-induced apoptosis in human osteosarcoma U-2 OS cells.

GADD153 and GRP78 were increased in GdCl$_3$-treated cells (Fig. 7). These results suggested that GdCl$_3$-induced apoptotic action might be involved in death receptor-mediated signaling, mitochondria-dependent pathway and ER stress in U-2 OS cells.

Discussion

Lanthanide (Ln) compounds have been shown to possess contrary effects on anticancer activities such as promotion of cell cycle progression and cell growth by lower concentration treatment but induction of apoptosis and suppression of cell proliferation at higher dosing (16,18,19). Our results indicated that high concentration of GdCl$_3$ caused apoptotic U-2 OS cell death (Fig. 1). This is first to report that the MRI contrast agent GdCl$_3$ can be successfully applied to induce apoptosis of human osteosarcoma cells. Moreover, an increase of TUNEL positive cells (Fig. 2), activation of caspase-3, caspase-8, caspase-9 (Fig. 3) and caspase-4 (Fig. 5B) was found to suggest that GdCl$_3$ caused apoptosis of U-2 OS through multiple pathways.

It is well know that Gd^{3+}, like other Ln ions, caused different effects depending on the type of target cells (30,31). In NIH 3T3 cells, GdCl$_3$ at the concentrations of <100 μM exerts proliferation-promoting effect and activation of extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) signaling pathways (31). Gadolinium provokes cell apoptosis through mitochondrial pathway and oxidative stress in L-02 normal liver cells (32).

Ye et al (22) reported that GdCl$_3$ induces HepG2 cell apoptosis through mitochondrial and death receptor-dependent pathways. Similarly, our results also demonstrated that GdCl$_3$ induced apoptosis through death receptor and mitochondria-dependent pathways in U-2 OS cells. In the present study, we found that GdCl$_3$ increased the ROS production and decreased the levels of $\Delta\Psi$m (Fig. 6), and western blot analysis showed that GdCl$_3$ treatment resulted in an increase of Fas/CD95, FasL, cytochrome c and Apaf-1 protein expression in U-2 OS cells (Fig. 7). Thus, we suggest that GdCl$_3$-induced cell death may be mediated via extrinsic and intrinsic apoptotic pathway in U-2 OS cells.

Calcium (Ca^{2+}) regulates cell proliferation, apoptosis or differentiation and plays a pivotal role in carcinogenesis and progression (33). Xia et al (34) has indicated that gadolinium-induced oxidative stress causes ER stress in rat cortical neurons. Feng et al (35) also showed that gadolinium triggers unfolded protein responses (UPRs) in primary cultured rat cortical astrocytes through increasing an influx of extracellular Ca^{2+} level. However, there is no available report regarding GdCl$_3$-induced ER stress in U-2 OS cells. In the present study, we investigated the GdCl$_3$-induced ER stress in apoptotic mechanism of U-2 OS cells. Our findings demonstrated that the increased levels of GADD153 and GRP78 were followed by releasing Ca^{2+} from ER and activating caspase-4 activity (Fig. 5), finally leading to ER stress and cell apoptosis. Our results revealed that the activation of ER stress signaling contribute to GdCl$_3$-induced apoptosis in U-2 OS cells.

In conclusion, we found that GdCl$_3$ exhibits direct anticancer activity and triggers suppression of tumor cell proliferation in human osteosarcoma cells. GdCl$_3$ provoked U-2 OS cell apoptosis by the way of the death receptor, mitochondria-dependent and ER stress pathways. The proposed pathway of GdCl$_3$-induced apoptosis of human osteosarcoma U-2 OS cells is showing in Fig. 8. Taken together, our findings provide important possible molecular mechanism against osteosarcoma of GdCl$_3$, showing that GdClv may be a promising anti-osteosarcoma drug.

Acknowledgements

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