Gadolinium oxide nanoparticles enhance the cytotoxicity of chemotherapeutic drugs by blocking autophagic flux in cancer cells

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Abstract

During chemotherapy, drugs can damage cancer cells' DNA and cytomembrane structure, and then induce cell death. However, autophagy can increase the chemotherapy resistance of cancer cells, reducing the effect of chemotherapy. We found that gadolinium oxide nanoparticles (Gd₂O₃ NPs), which have great potential for use as a contrast agent in magnetic resonance imaging, could block the late stage of autophagic flux in a dose-dependent manner and then cause autophagosome accumulation in HeLa cells. When co-treated with 8 µg/mL Gd₂O₃ NPs and 5 µg/mL cisplatin, the number of dead cancer cells increased by about 20% compared with cisplatin alone. We observed the same phenomenon in cisplatin-resistant COC1/DDP cells. Thus, Gd₂O₃ NPs have significant potential for use in both cancer diagnosis and therapy.

Background

Cancer is a malignant invasion and uncontrolled growth of cells [1] and much energy and other resources are wasted at tumor sites. Chemotherapy is a common treatment for cancer; during chemotherapy, the chemical drugs can cause DNA damage and cell function failure [2], which can induce cell death [3].

In oncology, drug resistance is the dominant obstacle for therapy efficacy, and autophagy may help cancer cells to survive chemotherapy, to some extent, thereby leading to drug resistance [4]. In cells, material such as disabled organelles and proteins become coated by double-membranes in structures named autophagosomes [5]. Subsequently, the autophagosomes fuse with lysosomes, forming autolysosomes, in which the encapsulated contents are degraded into components for use in repair or cell structure formation. Cells perceive nanoscale materials to be foreign or aberrant, and these nanoscale materials can disturb the process of macroautophagy (defined in this article as autophagy) and cause lysosomal dysfunction [6]. Recently, our group reported that cadmium telluride quantum dots can block the late stage of autophagic flux in *Saccharomyces cerevisiae* and were toxic to the cells [7]. In addition, several other nanomaterials (such as fullerene C₆₀ nanocrystals and MnO nanocrystals) have been reported to have potential to treat cancer by disturbing the autophagy process [8,9]. Thus, using nanoparticles (NPs) as chemosensitizers to induce disruptions to the autophagic flux is a new method to treat cancer. The use of an autophagic inhibitor in combination with chemotherapeutic agents has already shown significant potential in cancer therapy [10,11].

Gadolinium oxide (Gd₂O₃) nanoscale materials were developed to be used as contrast agents for magnetic resonance imaging (MRI) due to their longitudinal relaxivity characteristics [12]. They have significant potential for improving image quality. For example, terbium-doped Gd₂O₃ NPs can be used as a fluorescence and MRI dual-modal contrast agent [13]. Due to the better T₁ contrast enhancement compared to Gd³⁺ chelates, Gd₂O₃ NPs are recognized as promising potential MRI T₁ contrast agents [14,15]. The ability of Gd₂O₃ NPs (which were used in this study) to enhance T₁ contrast was previously reported [16].

Faucher et al reported that Gd_2O_3 NPs could agglomerate in vesicles and be retained in glioblastoma multiforme cells [17]. Thus, the persistence of Gd_2O_3 NPs may influence the cellular degradation system. Autophagy is a major intracellular degradation process that involves delivering cytoplasmic constituents sequestered in double-membrane vesicles to lysosomes [18], and it is particularly important for cells under certain environmental stress conditions [19]. Li et al reported that Gd_2O_3 NPs (47 nm) induced autophagosome accumulation in THP-1 cells [20], but full details of the autophagic response to Gd_2O_3 NPs remain unknown.

We aimed to explore autophagic changes after cell exposure to Gd_2O_3 NPs by monitoring autophagic markers. In addition, to explore whether Gd_2O_3 NPs can treat cancer by disrupting cancer cells' autophagic function, we co-treated HeLa cells and cisplatin-resistant COC1/DDP cells with Gd_2O_3 NPs and cisplatin. If the Gd_2O_3 NPs used in our work can enhance the ability of chemical drugs to kill cancer cells by modulating autophagy, the Gd_2O_3 NPs would offer a new avenue for both cancer diagnosis and therapy.

Methods

Preparation of Gd_2O_3 NPs

The Gd_2O_3 NPs were provided by Prof. Jinhao Gao [16]. The collected product was purified by dialysis. The concentrations of gadolinium ions in the NPs were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Cell culture and treatment

HeLa and COC1/DDP cells were purchased from China Center for Type Culture Collection (Wuhan, China). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Antibiotics were purchased from Gibco (Grand Island, NY, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HeLa) or Roswell Park Memorial Institute (RPMI) 1640 medium (COC1/DDP) supplemented with 10% FBS and antibiotics (100 $\mu\text{g}/\text{mL}$ streptomycin and 100 U/mL penicillin) at 37 °C and 5% CO_2 .

For the treatment experiments, cells were grown at an appropriate density in 96- or 6-well plates for 24 h to reach 80% population. Thereafter, the cells were cultured in fresh medium supplemented with the treatments (add rapamycin (Rapa, 20 μM), chloroquine (CQ, 100 nM), Gd_2O_3 NPs (2, 4 or 8 $\mu\text{g}/\text{mL}$), cisplatin (5 or 10 $\mu\text{g}/\text{mL}$) alone or several of them together into the fresh medium).

Autophagy assay

Autophagosomes number changes were measured by monodansylcadaverine (MDC) staining, MDC is a selective fluorescent marker for autophagic vacuoles [21], to observe the autophagic process using

fluorescence microscopy, Rapa (an agent that can induce autophagy) and CQ (a lysosomal inhibitor) were used as positive controls. And also measured by analysis of microtubule-associated protein light chain 3 (LC3) immunoblot, ratio of LC3-II/GADPH was used to quantification of LC3-II. Autophagic flux status was measured by analysis of p62 immunoblot.

Immunofluorescence

After treatment of the cells in 6-well plates for 24 h, the supernatants were removed. The cells were then fixed with 4% paraformaldehyde at room temperature for 20 min. Subsequently, 1 mL 0.5% Triton X-100 was added to each well, and the cells were left for 20 min. Thereafter, the cells were probed with primary rabbit polyclonal anti-LC3 antibody (Danvers, MA, USA, Cell Signaling Technology) overnight at 4 °C, and the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Proteintech, Wuhan, China), was then added and the cells were incubated for 2 h at room temperature. Finally, after adding 1 mL 4',6-diamidino-2-phenylindole (DAPI, final concentration 0.1 µg/mL) to each well and leaving the cells for 6 min at 37 °C, the cells were observed by confocal microscopy (Olympus, Tokyo, Japan).

Protein extraction and immunoblot analysis

Cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer (P0013B; Beyotime, Shanghai, China) with 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. Thereafter, the cell lysates were heated at 95 °C for 20 min and centrifuged at 13,500× g for 10 min at 4 °C. The supernatant was collected and the protein concentration was determined with a Bicinchoninic Acid (BCA) Kit (P0012 BCA; Beyotime, Shanghai, China).

Samples were run on 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes. Each membrane was blocked with 5% skimmed milk and 0.1% Tween-20 overnight at 4°C and probed with LC3 or p62 or GAPDH primary antibody for 2 h. Regarding the secondary antibody, the incubation was performed with horseradish peroxidase-conjugated IgG for 2 h. Blots were developed using a chemiluminescent horseradish peroxidase substrate (Merck Millipore, Billerica, MA, USA). Each Western blot was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Cell viability assay

Cell viability was measured using a (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatment, medium supplemented with MTT solution was added to each well and the cultures were incubated for 4 h at 37 °C. Thereafter, the supernatants were removed and 100 µL dimethyl sulfoxide (DMSO) was added to the plates, which were then incubated for 10 min at 37 °C. The absorbance at 595 nm was detected using a microplate reader (Thermo, Grand Island, NY, USA).

Apoptosis assay

Apoptosis detection was performed with an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (C1062; Beyotime, Shanghai, China). Briefly, after 24 h treatment cells were stained by Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature, avoiding light, and analyzed using a BD FACS Aria flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Each experiment was performed in three replicates, and the values shown represent the means $\pm$ standard deviation. Significant differences between the treatments were determined by Student's *t*-test using SigmaPlot Version 12.5 (Systat Software, San Jose, CA, USA).

Results

Gd₂O₃ NPs cause autophagosome accumulation in HeLa cells

To investigate the effect of Gd₂O₃ NPs on autophagy, firstly, we used MDC staining, after treatment of HeLa cells with 4 and 8 μ g/mL Gd₂O₃ NPs, we observed increased numbers of MDC-labeled vesicles compared to in the control cells, and this effect was dose-dependent (Fig. 1A). This indicated that Gd₂O₃ NPs could induce the accumulation of autophagic vacuoles in HeLa cells.

In detail, immunofluorescence and immunoblotting analysis of LC3 were conducted. LC3 is the most widely used biomarker for autophagosomes [22], and the amount of LC3-II has a positive correlation with the autophagy level [23]. As shown in Fig. 1B, 4 and 8 μ g/mL Gd₂O₃ NPs induced significant accumulation of autophagosomes stained with LC3 antibody. In addition, Gd₂O₃ NP treatment resulted in an increase of LC3-II, and the amount of LC3-II increased in a dose-dependent manner (Fig. 1C and D). Taken together, these results indicate that Gd₂O₃ NPs induced accumulation of autophagosomes in a dose-dependent manner.

Autophagosome accumulation is caused by inhibition of late-stage autophagic flux rather than induction of autophagy

Accumulation of autophagosomes could be the result of induction of autophagy or inhibition of autophagosome clearance [24]. To determine what the case for Gd₂O₃ NPs was, we measured the turnover of LC3 in the presence or absence of 100 nM CQ and 20 μ M Rapa, respectively. Rapa caused LC3-II accumulation as expected and adding CQ further increased the Rapa-induced LC3-II accumulation. No increase in LC3-II accumulation was found in cells co-treated with 8 μ g/mL Gd₂O₃ NPs and CQ compared to cells treated with Gd₂O₃ NPs alone (Fig. 2A and B). This indicated that Gd₂O₃ NPs caused

autophagosome accumulation by inhibiting autophagosome clearance rather than by inducing autophagy.

To further confirm this conclusion, autophagic flux was monitored by measuring p62, a specific substrate that is preferentially degraded by autophagy [25]. As expected, as shown in Fig. 2C and D, 20 μ M Rapa caused p62 degradation while 100 nM CQ led to p62 accumulation. 4 and 8 μ g/mL Gd_2O_3 NPs treatment caused accumulation of p62, indicating possible impairment of autophagic degradation capacity. Taken together, these results indicate that Gd_2O_3 NPs induced autophagosome accumulation by inhibition of autophagosome clearance.

Gd_2O_3 NPs enhance cytotoxicity of cisplatin in HeLa cells

To find out whether Gd_2O_3 NPs can enhance the cytotoxicity of drugs, we firstly investigated the cytocompatibility of Gd_2O_3 NPs in HeLa cells using the MTT procedure. As shown in Fig. 3A, the cell viability decreased slightly with the concentration or incubation time, but it was above 85% even at a high concentration (64 μ g/mL), indicating the low cytotoxicity of Gd_2O_3 NPs in HeLa cells. Cisplatin at a low dose (5 μ g/mL) killed about 10% of the HeLa cells, while treatment for 24 h with a combination of 4 (or 8) μ g/mL Gd_2O_3 NPs and 5 μ g/mL cisplatin increased the HeLa cell death rate to about 20%, as assessed by MTT assay (Fig. 3B). The microscopy images support this conclusion, with more cells dying when co-treated with chemical drug and 8 μ g/mL Gd_2O_3 NPs compared with each agent alone (Fig. 3C).

Autophagic blocking contributed to enhanced cytotoxicity

Autophagy is a major method of cancer cells to combat chemical drugs. Thus, to find out whether the blocked autophagic flux caused by Gd_2O_3 NPs is responsible for the enhanced cytotoxicity, we assessed how low-dose Gd_2O_3 NPs affect the cell death caused by 5 μ g/mL cisplatin, a frequently used chemotherapeutic agent. Cell death induced by cisplatin is known as apoptosis [26]. For quantification of apoptotic cells, the HeLa cells were stained with Annexin V-FITC and PI after 24 h of treatment. The results showed that the combination of 8 μ g/mL Gd_2O_3 NPs and 5 μ g/mL cisplatin caused twice the apoptotic cell number compared to 5 μ g/mL cisplatin alone. Treatment with 8 μ g/mL Gd_2O_3 NPs alone did not cause a significant increase in the number of apoptotic cells (Fig. 4A).

To find out whether the blockage of the autophagic flux is responsible for the cell death, we replaced Gd_2O_3 NPs with 100 nM CQ in the co-treatment of HeLa cells with cisplatin. We then estimated the

number of apoptotic cells as we had done before. The number of apoptotic cells caused by the combination of 100 nM CQ and 5 $\mu\text{g/mL}$ cisplatin was increased by about 100% than the number caused by 5 $\mu\text{g/mL}$ cisplatin alone (Fig. 4B). These results indicated that Gd_2O_3 NPs can enhance cisplatin-mediated cell death.

To provide more proof, 20 μM Rapa was used to recover the autophagic flux impaired by Gd_2O_3 NPs. As shown in Fig. 5, not only did 20 μM Rapa decrease the level of p62 accumulation induced by 8 $\mu\text{g/mL}$ Gd_2O_3 NPs (Fig. 5C and D), indicating that Rapa can indeed recover autophagic flux impaired by Gd_2O_3 NPs, but Rapa also significantly reduced the chemosensitization caused by Gd_2O_3 NPs. With the addition of Rapa, about 20% more cells survived when co-treated with Gd_2O_3 NPs and cisplatin (Fig. 5A and B). These results indicated that inhibition of autophagic flux is required for chemosensitization induced by Gd_2O_3 NPs.

Gd_2O_3 NPs also enhance chemosensitization in cisplatin-resistant cancer cells

Some tumor cells develop resistance to chemotherapeutics via autophagy; thus, inhibiting autophagy could be a promising strategy for cancer therapy [11]. To assess whether the sensitization effect is HeLa-specific, we evaluated the chemosensitization effect of Gd_2O_3 NPs in cisplatin-resistant cancer cells, COC1/DDP, using an Annexin V/PI assay. Similar to the situation with HeLa cells, the combination of 8 $\mu\text{g/mL}$ Gd_2O_3 NPs and 10 $\mu\text{g/mL}$ cisplatin resulted in a lower cell viability (Figure S1), compared to treat with cisplatin alone, number of dead cells increased by about 100% (Figure 6). This result indicated that the chemosensitization effect of Gd_2O_3 NPs was also efficient in COC1/DDP cells.

Discussion

The most difficult factor that we confront when treating cancer is drug resistance among cancer cells [27], and autophagy is an important reason for drug resistance. Several nano-materials have been found to induce cancer cell death by causing autophagy failure [28]. Moreover, due to the enhanced permeability and retention (EPR) effect at tumor sites, NPs mainly become distributed at tumor sites [29], which means that NPs could improve the efficacy of chemotherapy. Nanomaterials such as C60 and MnO nanocrystal may kill cancer cells by inducing autophagy, and they have the potential for use in cancer therapy [8,9].

In this study, we found that Gd_2O_3 NPs had a strong ability to block autophagic flux and could result in cancer cell death when used with chemical drugs to treat cancer cells. We were convinced that the autophagic response induced by Gd_2O_3 NPs increased with the concentration of Gd_2O_3 NPs (Fig. 1), and the chemosensitization effect of Gd_2O_3 NPs also increased (Fig. 3B). The results demonstrated the interrelationship between the observed autophagic flux impairment and the chemosensitization effects of

Gd₂O₃ NPs. The autophagy failure occurred because of autophagosome accumulation caused by blockage of the late stage of autophagic flux rather than induction of autophagy.

The same chemosensitization effects occurred in both drug-resistant cancer cells and ordinary nonresistant cancer cells after exposure to Gd₂O₃ NPs. Gd₂O₃ nanomaterials are a great candidate for MRI. Thus, the Gd₂O₃ NPs used in our work have great potential to be used as a tool for both cancer diagnosis and treatment.

Declarations

Ethics approval and consent to participate

Not applicable. This manuscript does not report on or involve the use of any animal or human data or tissue.

Consent for publication

Not applicable. This manuscript does not contain data from any individual person.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Tianyu Zhang, Cheng Zhong and Zhixiong Xie contributed to the conception and design of this work. Both Tianyu Zhang and Cheng Zhong contributed to the cell culture and treatment, Tianyu Zhang contributed to the acquisition of all westernblot data, Cheng Zhong contributed to the acquisition of MDC, immunofluorescence and MTT data, both Tianyu Zhang and Cheng Zhong contributed to the acquisition of flow cytometry data. Tianyu Zhang and Zhixiong Xie contributed to the analysis and interpretation of

all the data. Tianyu Zhang contributed to drafting the article, Zhixiong Xie and Tianyu Zhang contributed to revising it critically for important intellectual content.

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Figures

Figure 1

Gd2O3 NPs cause autophagosome accumulation in a dose-dependent manner. Notes: After 24 h of treatment, (A) Monodasylcadaverine (MDC) staining showed that 4 µg/mL or 8 µg/mL Gd2O3 NPs caused autophagosome accumulation. (B) Immunofluorescence of LC3 and (C) immunoblotting of LC3-II showed that 4 µg/mL or 8 µg/mL Gd2O3 NPs upregulate LC3 expression in a dose-dependent manner. (D) Quantification of (C). The ratio of LC3-II/GADPH in each group was calculated. The results are expressed as mean ± standard deviation, n=3. *p<0.05, **p<0.01. Abbreviations: Rapa, rapamycin; CQ, chloroquine; Gd2O3, gadolinium oxide nanoparticles; NS, not significant.

Figure 2

Inhibition of late-stage autophagic flux causes autophagosome accumulation. Notes: After 12 h of treatment, (A) 20 µM Rapa increased the accumulation of LC3-II and the addition of 100 nM CQ increased the accumulation further; 8 µg/mL Gd2O3 NPs also increased the amount of LC3-II but the addition of 100 nM CQ did not further increase the accumulation of LC3-II. (B) Quantification of (A). The ratio of LC3-II/GADPH in each group was calculated. The results are expressed as mean ± standard deviation, n=3. *p<0.05. (C) 20 µM Rapa and 100 nM CQ caused the amount of p62 to be decreased and increased, respectively, and Gd2O3 NPs increased the amount of p62 in a dose-dependent manner. (D)

Quantification of (C). The ratio of p62/GADPH in each group was calculated. The results are expressed as mean $\pm$ standard deviation, n=3. *p<0.05 **p<0.01 ***p<0.001. Abbreviations: Rapa, rapamycin; CQ, chloroquine; Gd2O3, gadolinium oxide nanoparticles; NS, not significant.

Figure 3

Gd2O3 NPs are responsible for the enhanced cytotoxicity of cisplatin Notes: (A) Gd2O3 NPs have a very limited cytotoxic effect on HeLa cells, even at a relatively high dose: >90% of cells survived even after treatment for 48 h with 8 μg/mL Gd2O3 NPs. (B) 5 μg/mL cisplatin caused about 90% of the cells to die, while treatment of HeLa cells for 12 h with a combination of 4 or 8 μg/mL Gd2O3 NPs and 5 μg/mL cisplatin killed about 20% more HeLa cells. (C) Microscopy images showed the same effect described above. The results are expressed as mean $\pm$ standard deviation, n=3. ***p<0.001. Abbreviation: Gd2O3, gadolinium oxide nanoparticles. NS, not significant. Scale bars: 50 μm

Figure 4

Gd₂O₃ NPs and CQ showed the same pattern regarding the enhancement of cisplatin's cytotoxicity. Notes: (A) The combination of 8 µg/mL Gd₂O₃ NPs and 5 µg/mL cisplatin caused apoptotic cell death number increased by about 100% compared with 5 µg/mL cisplatin alone. Gd₂O₃ NPs alone had no effect on the number of apoptotic cells. (B) The combination of 100 nM CQ and 5 µg/mL cisplatin also caused apoptotic cell death number increased by about 100% compared with 5 µg/mL cisplatin alone. 100 nM CQ alone had no effect on the number of apoptotic cells. Abbreviations: Rapa, rapamycin; CQ, chloroquine; Gd₂O₃, gadolinium oxide nanoparticles.

Figure 5

Rapamycin partially recovered the blocked autophagic flux and weakened the cytotoxicity of Gd₂O₃ NPs and cisplatin. Notes: (A) Autophagy promotor rapamycin (20 μ M) weakened the cytotoxicity of the combination of 8 μ g/mL Gd₂O₃ NPs and 5 μ g/mL cisplatin in HeLa cells. The results are expressed as mean $\pm$ standard deviation, n=3. *p $\leq$ 0.05 ***p<0.001. (B) Co-treatment with 8 μ g/mL Gd₂O₃ NPs and 5 μ g/mL cisplatin with the addition of 20 μ M rapamycin decreased the apoptotic cell number. (C) 8 μ g/mL Gd₂O₃ NPs increase the accumulation of p62 while 20 μ M rapamycin partly decreased the accumulation. (D) Quantification of (C). The ratio of p62/GAPDH in each group was calculated. The results are expressed as mean $\pm$ standard deviation, n=3. *p $\leq$ 0.05. Abbreviations: Rapa, rapamycin; CQ, chloroquine; Gd₂O₃, gadolinium oxide nanoparticles.

Figure 6

Chemosensitization efficacy of the combination of Gd_2O_3 NPs and cisplatin in cisplatin-resistant COC1/DDP cells. Notes: The apoptotic COC1/DDP cell number caused by the combination of 8 $\mu\text{g/mL}$ Gd_2O_3 NPs and 10 $\mu\text{g/mL}$ cisplatin increased by about 100% compared to the number caused by 10 $\mu\text{g/mL}$ cisplatin alone. 8 $\mu\text{g/mL}$ Gd_2O_3 NPs alone did not cause an increase in the COC1/DDP apoptotic cell death number. Abbreviations: Gd_2O_3 , gadolinium oxide nanoparticles.

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