Gallic acid induces apoptosis and enhances the anticancer effects of cisplatin in human small cell lung cancer H446 cell line via the ROS-dependent mitochondrial apoptotic pathway

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Abstract. Small cell lung cancer (SCLC) is the most aggressive lung cancer subtype and accounts for more than 15% of all lung cancer cases. Cisplatin [cis-diamminedichloroplatinum (CDDP)]-based combination chemotherapy is the cornerstone for all stages of SCLC. However, acquired multidrug resistance (MDR) and intolerable toxicities lead to a high mortality rate in SCLC patients. Gallic acid [3,4,5-trihydroxybenzoic acid (GA)] is a natural botanic phenolic compound which can induce cell apoptosis in several types of cancers. In the present study, we aimed to explore the anticancer effects of GA on human SCLC H446 cells and its promotive effects on the anticancer activities of cisplatin. The viability of the H446 cells was analyzed by MTT assay. Morphological changes in the H446 cells were observed under an inverted microscope. Apoptosis induction was determined by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. The level of reactive oxygen species (ROS) was assessed by 2’7’-dichlorofluorescein diacetate (DCFH-DA), mitochondrial membrane potential (MMP) by JC-1, and western blotting was used to examine the expression of mitochondrial apoptosis-related proteins. The results showed that both GA and cisplatin changed the morphology, inhibited the growth and induced apoptosis in the H446 cells by inducing generation of ROS, disruption of MMP, downregulation of XIAP expression, and upregulation of Bax, Apaf-1, DIABLO and p53 expression. More importantly, GA combined with cisplatin exhibited synergistic effects on inducing of these pro-apoptotic mediators and modulating the activation of apoptosis-related molecules. However, inhibition of the generation of ROS by N-acetyl-l-cysteine (NAC), a specific ROS inhibitor, reversed the cell apoptosis induced by cisplatin combined with GA. In conclusion, the results from the present study revealed that GA exhibited an anticancer effect on human SCLC H446 cells and enhanced the antitumor activities of cisplatin via the ROS-dependent mitochondrial apoptotic pathway.

Introduction

Lung cancer accounts for 12% of all newly diagnosed cancer cases worldwide (1). Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer and it represents more than 15% of all lung cancer cases (2). Cisplatin [cis-diamminedichloroplatinum (CDDP)]-based combination chemotherapy has markedly improved the survival rate of SCLC patients (3). However, the median survival time of SCLC patients with limited and advanced stage are still less than 2 years, and the overall 5-year survival rate of SCLC patients remains 5-10% owing to acquired multidrug resistance (MDR) and intolerable toxicity (4). It has been found that apoptosis blockade is one of the most important mechanisms by which cancer cells escape from the cytotoxicity of cisplatin, leading to MDR (5). Therefore, it is of great significance for cancer treatments to explore the mechanisms of apoptotic resistance after cisplatin stimulation and to accelerate apoptosis in tumor cells in efficient ways.

Mitochondria are essential for regulation of the intrinsic apoptotic pathway and they are the main generation spots for reactive oxygen species (ROS) in most living cells (6). A low level of ROS generation exhibits critical physiological effects in normal cells, whereas excessive generation of ROS induces oxidative stress, which may further lead to loss of cell function and cell apoptosis(7).It has been shown that DNA damage caused by ROS can evoke p53 and result in mitochondrial-mediated cell apoptosis through downregulation of anti-apoptotic...
Bcl-2 proteins and upregulation of various pro-apoptotic proteins, such as Bax and apoptotic protease-activating factor-1 (Apaf-1) (8). In contrast, the mitochondrial membrane permeability is sensitive to redox stress and excessive ROS can upgrade mitochondrial membrane permeability (9), which ultimately leads to mitochondrial swelling, depolarization of mitochondrial membrane potential (MMP) and release of apoptosis-inducing proteins (10). In addition, many types of phytochemicals, including cisplatin, have been reported to kill cancer cells via the ROS-mediated mitochondrial apoptotic pathway (11).

Gallic acid [3,4,5-trihydroxybenzoic acid (GA)], a natural botanic phenolic compound, is abundantly found in green tea, grapes and red wine (12). GA possesses a wide range of pharmacological properties including anti-obesity, anti-inflammation and anticancer activities (13-15). More and more attention has been given to the anticancer effects of GA in recent years since it may induce cell apoptosis in various types of cancers, such as lung and cervical cancer, and oral squamous carcinoma (16-18). Evidence has also revealed that the accumulation of intracellular ROS caused by oxidative stress imbalance is an important predisposing factor for GA to exhibit its anticancer effects. For example, it was demonstrated that GA induced apoptosis in prostate cells and mouse lung fibroblasts via the ROS-dependent intrinsic apoptotic pathway and the ROS-dependent p53 activation pathway, respectively (15,19). However, the mechanisms by which GA induces apoptosis in cancer cells are not well illustrated and there is little evidence revealing its anticancer effects on human SCLC cells.

In the present study, based on these clues, we investigated the hypothesis that GA can exhibit an anticancer effect in the human SCLC H446 cell line by interfering with the generation of ROS and disrupting the function of the mitochondria. Further exploration also revealed that GA enhanced the anticancer effects of cisplatin via the ROS-dependent mitochondrial apoptotic pathway in H446 cells.

Materials and methods

**Materials.** The human SCLC H446 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). GA (Fig. 1) was purchased from Xi’an Grass Plant Technology Co. (Xi’an, China) with a purity above 98%. Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco-BRL (Grand Island, NY, USA). N-acetyl-l-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell apoptosis detection kit with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), MMP assay and intracellular ROS detection kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Primary antibodies against Bax, Apaf-1, DIABLO, XIAP, p53 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-rabbit IgG peroxidase conjugated secondary antibody, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) gels and polyvinylidene difluoride (PVDF) membrane were purchased from Amersham Biosciences (Piscataway, NJ, USA).

**Cell culture.** The human SCLC H446 cell line was cultivated in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37°C.

**Cell viability assay.** The effects of cisplatin, GA and co-administration of cisplatin and GA on cell viability were detected by MTT colorimetric assay. H446 cells in logarithmic growth phase were collected and seeded into 96-well plates at a density of 5x10^3 cells/well. After 12 h of incubation, the cells were treated with different concentrations of cisplatin and GA or their combination for 6-32 h. Then, 20 µl MTT was added to each well and cultured for another 4 h. Finally, the medium was removed and 150 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was recorded at 492 nm on an automated Bio-Rad 550 microplate reader.

**Morphological examination of the H446 cells.** The individual or/and combined effects of cisplatin and GA on morphological changes in the H446 cells were observed under an inverted microscope (10x10 magnification). Briefly, SCLC H446 cells were cultured in 6-well plates and treated with 5 µg/ml cisplatin and 3 µg/ml GA alone or in combination for 24 h. After that, images of the morphological features of the cells from the different groups were captured. Cytotoxicity of the different treatments on the H446 cells was evaluated according to the variations in cellular morphological changes and the amount of adherent cells.

**Analysis of cell apoptosis by flow cytometry.** The apoptosis of H446 cells was assessed by flow cytometry using Annexin V-FITC/PI staining according to the manufacturer's instructions. Firstly, H446 cells were treated with 5 µg/ml cisplatin and/or 3 µg/ml GA, in the presence or absence of 6 mM NAC for 2 h. After 24 h, both suspension and adherent cells were collected, washed twice with cold phosphate-buffered saline (PBS), and then stained with Annexin V and PI at room temperature for 15 min in the dark. Finally, the cells were analyzed by a flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA). As to the analysis, Annexin V-positive and PI-negative cells represent early apoptotic populations, Annexin V-positive and PI-positive cells represent late apoptotic or dead proportions.

**Measurement of ROS.** 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) is a highly sensitive probe which is usually used for detection of intracellular ROS. This non-fluorescent dye diffuses readily into cells and yields DCFH which cannot pass out of cells. In the presence of peroxidase, DCFH can generate the fluorescent compound dichlorofluorescein (DCF), which can be observed by fluorescence microscopy with an excitation wavelength of 488 nm and a 525-nm emission filter. In the present study, H446 cells were divided into four groups: the
normal group, 5 µg/ml cisplatin group, 3 µg/ml GA group and a combination of the two drugs group. Cells from each group were incubated with 10 µM DCFH-DA for 30 min after 24 h of drug treatment and finally covered with 1 ml serum-free DMEM before observation. Green fluorescence was detected to evaluate the concentration of intracellular ROS. 

Measurement of mitochondrial membrane permeability (MMP). MMP was measured using a JC-1 assay kit. The JC-1 dye can enter the mitochondrial matrix in normal cells, form JC-1 aggregates and emit red fluorescence (excitation by 540 nm), while JC-1 exists as a monomeric form and exhibits green fluorescence (excitation by 490 nm), when MMP is decreased. In the present study, the cells were seeded into 6-well plates and stimulated with 5 µg/ml cisplatin, 3 µg/ml GA and 5 µg/ml cisplatin combined with 3 µg/ml GA for 24 h. After that, cells in each well were incubated in the dark at 37°C with 1 ml culture medium and 1 ml 5 µM JC-1 for 30 min. At last, fluorescence was detected by an inverse fluorescence microscope (DMI6000B; Leica, Germany). The relative ratio of green to red fluorescence indicated the depolarization of MMP.

Western blotting. Cells from the different groups were collected and lysed in RIPA lysis buffer containing protease inhibitor on ice. Then, cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and cell supernatants were collected. Protein concentration was confirmed by a BCA protein assay kit (Beyotime, China). Proteins (20 µg) from each group were separated on 12% SDS-polyacrylamide gels and subsequently transferred onto a PVDF membrane (Amersham, Braunschweig, Germany). The membranes were blocked in 5% skimmed milk dissolved in Tris-buffered saline and Tween-20 (TBST) (20 mM Tris, pH 7.5) for 2 h and then incubated with appropriate primary antibodies at 4°C overnight. After three times washing in TBST, the membranes were incubated with the secondary antibody conjugated with anti-rabbit IgG peroxidase for 2 h at room temperature. Bands were monitored with chemiluminescence using an ECL detection system (Amersham Pharmacia Biotech).

Statistical analysis. All experiments were repeated three times. Numeric data are expressed as mean ± SD. Statistical differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s test after these data were confirmed to have a normal distribution. A p-value of <0.05 was considered to indicate a statistically significant result.

Results

Cell viability of the H446 cells. The effects of cisplatin, GA and cisplatin combined with GA on cell viability were evalu-
ated by MTT assay. The results revealed that both cisplatin and GA effectively suppressed the cell viability in a dose-dependent manner, and the survival rate of the H446 cells was reduced to 65 and 75% when treated with 5 µg/ml cisplatin and 3 µg/ml GA for 24 h, respectively (Fig. 2A and B). Moreover, the viability of the cells decreased in a time-dependent manner and was reduced to 40% at 24 h when stimulated with a combination of 5 µg/ml cisplatin and 3 µg/ml GA (p<0.05) (Fig. 2C and D). In addition, the growth inhibitory effects of their combination became more and more dramatic with decreasing P-values with increasing time compared with that of the single cisplatin-treated group (Fig. 2D).

**Cell morphological changes in the H446 cells.** When apoptosis occurs, cells present characteristic structural changes which can be observed through inverted microscopy. As shown in Fig. 3, almost all cells were regular in shape and confluent, rarely sloughing off in the normal control group, while many H446 cells became smaller, round and blunt in size and some floated on the medium when treated with cisplatin and GA. More importantly, the above changes became more extreme in cells exposed to cisplatin combined with GA. Moreover, the number of H446 cells adhering onto the plates decreased according to the following order: normal group > GA group > cisplatin group > cisplatin combined with GA group.

**Apoptosis of the H446 cells.** To further explore the mechanisms underlying the inhibitory effects of the two agents on H446 cells, the percentage of apoptotic cells in each group was detected by flow cytometry. As shown in Fig. 4, the percentage of apoptotic cells increased in both the cisplatin and GA groups compared with that of the control (30.47±1.56 and 12.43±2.89 vs. 1.53±1.25%) (p<0.05). Of particular note, joint application of cisplatin and GA induced more apoptosis compared with that in the cisplatin treatment alone group (40.97±1.92 vs. 30.47±1.56%) (p<0.05).

**Intracellular ROS levels in the H446 cells.** Based on the results above, we hypothesized that apoptosis induced by cisplatin, GA and combined treatment of the two agents was related to the abnormal intracellular ROS level. We then examined the generation of ROS using the DCFH-DA probe. As shown in Fig. 5, there was rare ROS detected in the normal cells, while a 24-h treatment with both cisplatin and GA obviously increased the accumulation of ROS. Moreover, the amount of intracellular ROS was maximally elevated when H446 cells were treated with CDDP plus GA.

**Loss of MMP in H446 cells.** The depolarization of MMP reflects mitochondrial dysfunction which is an early marker of mitochondrial-mediated cell apoptosis. In the present study,
we evaluated the changes in MMP by a fluorescence microscope. As shown in Fig. 6, the red light was most obvious in the normal group and it was markedly decreased 24 h after cells were treated with cisplatin and GA. Strikingly, the red light reached the lowest point when cells were exposed to a combination of the two agents. In contrast, there was barely any green light detected in the normal cells, while cisplatin and GA treatment resulted in the generation of green light. Moreover, opposite to that of red light, the brightness of green peaked when cells were treated with cisplatin and GA in combination.

Expression of mitochondrial apoptosis-related proteins in the H446 cells. In order to elucidate the molecular mechanisms involved in the cell apoptosis induced by GA in H446 cells, we measured the expression of several apoptosis-related proteins by western blotting. As shown in Fig. 7, we found that the expression levels of Bax, Apaf-1, DIABLO and p53 were markedly increased by GA accompanied with decreased expression of XIAP in a time-dependent manner (p<0.05).

To further explore whether the changes in expression of Bax, Apaf-1, DIABLO, p53 and XIAP were associated with enhanced apoptosis-inducing effects by the combined treatment of cisplatin and GA, we examined the expression levels of these proteins in the H446 cells treated with cisplatin and/or GA for 24 h. As shown in Fig. 8, combined treatment with cisplatin and GA significantly increased the levels of Bax, Apaf-1, DIABLO and p53, whereas it resulted in a decrease in XIAP levels relative to the treatment with either drug alone (p<0.05).

Pretreatment with NAC prevents apoptosis induced by cisplatin and GA. To confirm whether elevated intracellular ROS levels are related to the apoptosis induced by cisplatin...
combined with GA. The H446 cells were pretreated with 6 mM NAC for 2 h, followed by treatment of cisplatin plus GA for 24 h. The proportion of apoptotic cells was determined by flow cytometric analysis. As shown in Fig. 9, the percentage of apoptotic cells following the combination treatment was reduced from 43.90±2.19% in the non-NAC treatment group to 32.57±0.69% following NAC treatment; the difference was statistically significant (p<0.05).

Discussion

Lung cancer is one of the leading causes of cancer-related mortality worldwide (1). In addition, SCLC is the most deadliest subtype with fast growth and early widespread dissemination as its characteristic hallmark (20). Although combined chemotherapy with cisplatin and etoposide has been widely adopted to increase the remission rate of SCLC (21), almost all patients suffer from recurrent cancer with MDR and eventually succumb to this disease. Recently, identification of novel effective chemotherapeutic agents has become a research focus in the study of SCLC treatments.

Polyphenols are abundant natural sources of potential cancer chemotherapeutic agents, which can reduce the toxicities of typical anticancer drugs and help to overcome drug resistance of standard anticancer treatments (22). As one type of polyphenol acids, gallic acid (GA) is attracting increased attention due to its high bioavailability and non-toxicity (12). In the present study, we demonstrated for the first time that GA inhibited the growth of SCLC H446 cells and enhanced the apoptosis-inducing effects of cisplatin through an increase in intracellular ROS, reduction in MMP, an increase in BAX, Apaf-1, DIABLO and p53 expression and reduction in XIAP expression. These results further indicate that GA may be an effective supplementary drug in the clinical treatment of SCLC which would help to reduce cisplatin-induced MDR and toxicities.
Apoptosis, or programmed cell death, is a highly regulated process and is characterized by a series of morphological changes. Continuous studies have revealed that the activation of apoptosis plays an important role in inhibiting the development and progression of cancers (23,24). It was firstly confirmed, in the present study, that GA altered cell morphology, inhibited the growth and induced apoptosis in H446 cells. Moreover, combined treatment of GA and cisplatin exhibited a better effect on cell morphological changes than that of single treatment of cisplatin. These findings indicate that cell apoptosis may be the mechanism by which cisplatin and GA exhibit their anticancer effects in H446 cells.

As one of the by-products of normal cellular oxidative processes, ROS is an important mediator of intracellular signaling. Accumulating evidence suggests that a high level of ROS induces oxidation of cellular macromolecules, disruption of MMP and finally cell apoptosis (7,25). Ma et al believes there is a positive feedback loop in ROS-mediated mitochondrial membrane dysfunction which amplifies the death signals in liver cancer cells (26). Accordingly, we investigated the possibility that ROS may play a role in cisplatin and GA-mediated apoptosis in H446 cells. We found that intracellular ROS increased while MMP was reduced when H446 cells were exposed to cisplatin, GA and the combination of the two agents for 24 h. In addition, compared with individual treatment, the combined treatment of cisplatin and GA obviously increased the accumulation of ROS and the loss of MMP in H446 cells. Moreover, we found that cisplatin and GA-induced apoptosis was greatly reduced by pretreatment with the ROS scavenger NAC. These data indicated that ROS acted as an upstream signaling molecule to initiate mitochondrial-mediated apoptosis when H446 cells were treated with cisplatin and GA.

Mitochondrial-mediated cell apoptosis mainly depends on the abnormal activation and expression of the Bcl-2 family, which consists of anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, and pro-apoptotic factors, such as Bak and Bax.
Figure 8. Evaluation of apoptotic regulators in H446 cells by cisplatin and gallic acid (GA). (A) Cells were treated with cisplatin (Pt) or GA alone or in combination (Pt+GA) for 24 h, and then 20 µg protein was subjected to 12% SDS-PAGE to measure the expression of Bax, Apaf-1, DIABLO, p53 and XIAP in H446 cells. Representative bar diagrams show quantitative results for the relative levels of (B) Bax, (C) Apaf-1, (D) DIABLO, (E) p53 and (F) XIAP proteins. Data are mean ± SD, n=3. *p<0.05 vs. the normal group (N); **p<0.05 vs. the cisplatin group.

Figure 9. Induction of ROS-dependent apoptosis by cisplatin combined with gallic acid (GA) in H446 cells. (A) Apoptosis assay by flow cytometry using PI and Annexin V-FITC double staining. H446 cells were treated with or without 6 mM NAC for 2 h before challenge with 5 µg/ml cisplatin combined with 3 µg/ml GA for 24 h. (B) In the graph, each column represents the mean ± SD percentage of PI-positive and Annexin V-positive cells of three independent experiments. *p<0.05 vs. the normal group (N); **p<0.05 vs. cisplatin combined with GA group (Pt + GA).
Overexpressed Bax may translocate and integrate into the mito-
chondrial membrane resulting in altered protein interaction
of mitochondrial membranes, which may further lead to opening
of the permeability transition pore (PTP), disruption of MMP
and finally the release of secondary mitochondrial-derived
activator of caspase (SMAC, also called DIABLO) and cyto-
chrome c (27,28). DIABLO facilitates caspase processing and
activation by antagonizing an endogenous inhibitor of
caspase called XIAP. Also, cytochrome c initiates a caspase
cascade by enhancing the formation of apoptosomes through
accelerating the expression of Apaf-1 (29,30). Moreover, as a
critical tumor suppressor, p53 decreases cell proliferation and
induces caspase-mediated apoptosis via upregulation of
its downstream target Bax (31). Based on this knowledge,
we investigated the expression of the above apoptosis-related
proteins, and the results showed that both cisplatin and GA
markedly increased the expression of Bax, DIABLO, Apaf-1
and p53 while decreased XIAP expression. More importantly,
GA markedly enhanced the effects of cisplatin on expression of
these proteins. These findings further confirmed that cisplatin
and GA-induced apoptosis of H446 cells occur through the
mitochondrial-mediated pathway.

In conclusion, we demonstrated for the first time that GA
suppressed the proliferation and induced the apoptosis of
H446 cells and enhanced the anticancer effects of cisplatin on
these cells through the ROS-mediated mitochondrial apoptotic
pathway. However, studies are still needed to fully evaluate the
anticancer effects of GA in combination with cisplatin in vivo
and to further assess whether GA could be adopted as a novel
auxiliary therapeutic in cancer treatment.

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