Radiosensitizing effect of oleanolic acid on tumor cells through the inhibition of GSH synthesis in vitro

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Abstract. Oleanolic acid (OA) is a natural pentacyclic triterpenoid that has been used in traditional medicine as an anticancer and anti-inflammatory agent. The aim of our study was to investigate whether or not OA increases the radiosensitivity of tumor cells, and the relative mechanism was also investigated. Clonogenic assay was used to observe the radiosensitivity of C6 and A549 cells following different treatments. The alteration of intracellular DNA damage was determined using a micronucleus (MN) assay. In order to identify the mechanism of OA-mediated radiosensitization of tumor cells, the levels of glutathione (GSH) in irradiated cells following various pretreatments were determined using glutathione reductase/5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay. Under the same condition, the activities of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthase (GSS), both key enzymes for GSH synthesis, were detected using appropriate methods. In order to confirm the radiosensitizing effect of OA on cancer cells by attenuating GSH, N-acetylcysteine (NAC) was added to cells in culture for 12 h before irradiation. The results showed that the combined treatment of radiation with OA significantly decreased the clonogenic growth of tumor cells and enhanced the numbers of intracellular MN compared to irradiation alone. Furthermore, it was found that the synthesis of cellular GSH was inhibited concomitantly with the downregulation of γ-GCS activity. Therefore, the utilization of OA as a radiosensitizing agent for irradiation-inducing cell death offers a potential therapeutic approach to treat cancer.

Introduction

Radiotherapy is an effective tool for malignant tumor treatments. It is particularly beneficial for rapidly growing tumors due to their high susceptibility to radiation (1,2). Ionizing radiation can produce high amounts of reactive oxygen species (ROS) and free radicals, which further induce cellular DNA single-strand breaks (SSBs) and double-strand breaks (DSBs), eventually resulting in the death of cancer cells (3,4). However, local failure after irradiation remains a challenge due to intrinsic and acquired resistance of tumor cells to radiation treatment (5).

Cellular antioxidants are important for the protection of cells against ROS and free radicals generated through endogenous or exogenous oxidative stress (6-8). Glutathione (GSH), the tripeptide thiol L-γ-glutamyl-L-cysteinyl-glycine, is one of the most abundant antioxidants in the cells with concentrations in the low millimolar range. It plays a key role in maintaining intracellular redox equilibrium and in augmenting cellular defenses to harmful factors (9,10). In general, GSH is synthesized from its constituent amino acids, glutamic acid, cysteine and glycine, via two sequential ATP-consuming steps, which are catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthase (GSS). The synthesis of the di-peptide γ-glutamylcysteine by γ-GCS is the rate-limiting step in de novo GSH synthesis (11,12). The level of GSH is reported to be increased in various types of tumor cells, for example in bone marrow, breast, glioma, colon, larynx and lung cancers (13-18). Owing to its protection, an increased level of GSH is often associated with a refractory response to ionizing radiation via GSH scavenging ROS and free radicals. Conversely, depletion of intracellular GSH favorably increases the radiosensitivity of cancer cells (19,20).

Oleanolic acid (3b-hydroxy-olea-12-en-28-oic acid; OA) belongs to the triterpenoid family (Fig. 1), which is widely distributed in the plant kingdom (21). The pentacyclic triterpenoid with a wide variety of functional groups is commonly used to treat various diseases. OA demonstrates anti-inflammatory activity (22), protection against hepatotoxicity (23,24), and recovery of the hematopoietic system after irradiation (25). Meanwhile, OA was found to induce differentiation of malignant cells, to inhibit proliferation of carcinoma cells and to promote apoptosis (26,27). It has been well established that plant extracts and their derivatives have high potential capability as new anticancer therapeutic agents, either alone or in combination with conventional chemotherapeutic agents for cancers that exhibit resistance to standard drugs (28,29). However, whether OA increases the radiotherapeutic effect on cancer cells or not remains unclear. Therefore, in the present study, we investigated the influence of the monomer OA on the
radiosensitivity of cancer cells and the alteration in intracellular GSH level. Our results demonstrated that OA enhances the radiosensitivity of C6 and A549 cells through inhibition of intracellular GSH synthesis.

Materials and methods

Cell culture and treatment. The rat glioma C6 and human lung cancer A549 cell lines (Cell Bank, Chinese Academy of Sciences) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in an incubator containing a humid atmosphere of 95% air and 5% CO2 and propagated according to the protocol supplied by the American Type Culture Collection. OA was purchased from NanJing-Zelang (China) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a stock concentration of 250 μg/ml and stored at -20°C. All cells were treated with OA at different concentrations for 24 h prior to exposure to irradiation. In addition, 5 mM N-acetylcysteine (NAC) (Sigma-Aldrich), an antioxidant and GSH precursor, was added to the cultured cells 12 h prior to irradiation in order to further confirm the mechanism of OA modulation of the radiosensitivity of tumor cells by influencing the GSH level.

Cell viability assay. The influence of OA on cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. C6 and A549 cells were seeded in 96-well plates at a density of 5×103 cells/well. They were then treated with different concentrations of OA for 24 h. Furthermore, the medium was replaced with fresh medium allowing cells to be continuously grown up to 72 h. MTT dye was added to a final concentration of 0.5 mg/ml, and cells were subsequently incubated for an additional 4 h at 37°C. The medium containing residual MTT dye was carefully aspirated from each of the wells, and 200 μl DMSO was added to each well to dissolve the reduced formazan dye. The fraction of viable cells was calculated by comparing the optical absorbance of the culture exposed to OA treatment with that of the untreated control.

Irradiation. Irradiation was emitted using a 6-MV X-ray linear accelerator (Varian Inc., Palo Alto, CA, USA) at a dose rate of 250 cGy/min.

Clonogenic assay. The radiosensitivity of tumor cells was determined using a clonogenic assay. Both tumor cell lines were seeded and cultured overnight at an appropriate density in T25 flasks, and subsequently different drugs were added into the medium for 24 h. After being pretreated with control, OA, or OA and NAC, cells were subjected to 0, 1, 2, 3, 5, or 7 Gy X-ray irradiation. The medium was then replaced with fresh medium allowing cells to continuously grow for colony formation for 9 to 12 days. Cell colonies were fixed by absolute methanol and stained with Giemsa for counting. Clonogenic survival fraction (SF) was calculated as the number of colonies/the number of seeded cells x plating efficiency. Plating efficiency was defined as the number of colonies/the number of seeded cells of the untreated control. Survival curve was fitted with the single target multi-model of the equation: S = 1-1(1-e^{-D/D0})^N.

Micronucleus assay. The number of micronuclei (MN) were measured with the cytokinesis-block technique as a biological end point for the response of mimetic hypoxia to irradiation. Briefly, the cells were exposed to 0.83 μg/ml cytochalasin B (Sigma-Aldrich) for 19-20 h followed by 75 mM KCl hypotonic treatment for 1-3 min and then fixed in situ with methanol:acetic acid (9:1 v/v) for 30 min. Air-dried cells were stained with 5% Giemsa (Sigma-Aldrich) for 10 min. Micronuclei were scored in binucleated cells, and the formation of binucleated cells was measured as the percentage of the total number of cells scored. For each sample, at least 1,000 binucleated cells were counted. The MN yield, Y_MN, was the ratio of the number of micronuclei to the number of binucleated cells scored.

Intracellular GSH assay. After triplicate samples of 10⁶ cells were treated with different reagents, the intracellular levels of GSH were measured with the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay kit (Beyotime Biotechnology, Jiangsu, China) under the methods recommended by the manufacturer. Briefly, GSH was determined by using a reaction mixture, containing 50 μl of cell lysates, 50 μl of 2.4 mM DTNB and 50 μl of 10.64 mU/μl glutathione reductase in the assay buffer (pH 7.5) containing 153 mM sodium phosphate and 8.4 mM EDTA. After a 5-min incubation at 25°C, the reaction was started by the addition of 50 μl NADPH solution (0.16 mg/ml) in the assay buffer. The standard sample and checking sample cuvettes were placed into a dual-beam spectrophotometer, and the increases in absorbance at 412 nm were followed as a function of time.

Intracellular γ-GCS activity assay. Cells (10⁶) were homogenized in 50 mM potassium phosphate (pH 7.5) containing TES/SB buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, 2 mM serine) for the γ-GCS assay. Homogenates were centrifuged at 12,000 rpm (15 min, 4°C), and the supernatants were maintained on ice for determination of enzyme activity. The protein concentration of cell supernatants was measured using the Bio-Rad DC Protein Assay kit (Bio-Rad, Herts, UK), and the enzyme activity was reported as U/mg protein, where a unit of activity was the amount of enzyme required to convert one micromole of substrate to product per min at 25°C. The γ-GCS assay was an adaptation of a previously described method, in which γ-GCS in...
cell extracts synthesized γ-glutamylcysteine which was then reacted with 2,3-naphthalenedicarboxaldehyde (NDA) to form a highly fluorescent product that was able to be measured fluorimetrically at 520 nm (30).

Intracellular GSS activity assay. Tumor cells were plated in 60-mm culture dishes at a density of 10^6 cells/dish and then divided into different groups with various methods of pretreatment. The intracellular GSS activities were measured using GSS assay kit (Lanxu Biotech Co. Ltd., China). Briefly, the cells were subjected to repeated freeze-thaw cycles to lyse the cells for release of intracellular components. Cell lysates were centrifuged at 3,000 rpm (20 min, 4°C), and the supernatants were maintained on ice for determination of enzyme activity. All procedures were performed according to the protocol of the kit. After the reaction was terminated, the absorbance was measured at 450 nm on an enzyme-linked immunosorbent assay (ELISA) reader. The activity of GSS in the sample was then determined by comparing the OD of the samples to the standard curve.

Statistical analysis. Data are reported as the means ± SEM of 3 separate experiments. Statistical analysis was measured by the independent sample t-test and analysis of variance. P<0.05 was considered to indicate a statistically significant result.

Results

Selection of the experimental concentration of OA. In order to select the experimental concentration of OA for this study, a 24 h dose-response study was conducted by exposing the cells lines to different concentrations of OA. The results of the MTT assay showed that the half inhibition concentration (IC_{50}) of OA for C6 cells was 35 µg/ml and for the A549 cells this value was 27 µg/ml, respectively (Fig. 2). 10% IC_{50}, 20% IC_{50} and 30% IC_{50} of drug concentrations were used to determine the influence of OA on the radiosensitivities of both tumor cell lines.

Influence of OA on the radiosensitivities of the tumor cells. No statistically significant changes were noted in the formation of C6 and A549 clonogenic cells following treatment with different concentrations of OA. Based on the single target multi-model, the fitting curves of the irradiated C6 and A549 cells had a gradually declining tendency with increasing doses. The SFs of irradiated cells, after OA pretreatment for 24 h, were further decreased when compared to those of the irradiated cells without OA treatment (Fig. 3). By calculating the D_0 value and the sensitive enhancement ratio (SER), the D_0 and SERs of irradiated cells were reduced in a OA concentration-dependent manner. When the concentration of OA reached 10% IC_{50}, 20% IC_{50} and 30% IC_{50}, the SERs of C6 and A549 cell were 1.16, 1.81, 2.23 and 1.13, 1.26, 1.55, respectively. In contrast, the treatment of NAC partially reduced the sensitizing effect of OA at 30% IC_{50} on the irradiated cells. Therefore, OA obviously enhanced the radiosensitivity of both tumor cell lines.

Changes in the intracellular micronucleus rate by OA pretreatment. The results from the MN assay showed there was no obvious influence of the various concentrations of OA on the formation of the numbered MN in both cell lines unexposed to X-rays. Subsequently, the numbers of intracellular MN were significantly increased with concomitant irradiation doses. In both irradiated cell lines pretreatment with various concentrations of OA, further enhanced the numbers of intracellular MN. Meanwhile, it was found that, compared with the irradiated cells without OA treatment, the irradiated cells treated with 20% IC_{50} and 30% IC_{50} of OA displayed a statistically significant increase in cellular MN formation. Additionally, it was obvious that the capability of 30% IC_{50} OA-induced MN generation in irradiated cells was reduced after NAC was added into the culture medium. These results indicate that OA prompts the generation of MN in irradiated tumor cells (Fig. 4).

Effect of OA on the GSH level. To further study the mechanism of the influence of OA on the radiosensitivity of tumor
cells, intracellular GSH levels were measured after pretreatment with different concentrations of OA for 24 h. As shown in Fig. 5, when compared with the cells in the absence of OA, significant decreases in the GSH levels of C6 cells in the presence of OA were noted. Furthermore, the same phenomenon was noted in A549 cells, where intracellular GSH levels showed a gradually declining tendency concomitant with increases in OA concentrations. In contrast, supplementation of NAC significantly restored the inhibition of GSH synthesis by OA. The results revealed that OA effectively inhibits the synthesis of GSH in tumor cells.

Inhibition of γ-GCS activity by OA. Since γ-GCS is the rate-limiting enzyme in the synthesis of intracellular GSH, its activity was further measured. As showed in Fig. 6, the activity of γ-GCS in C6 cells was inhibited by the treatment with 20% IC\textsubscript{50} and 30% IC\textsubscript{50} of OA, while no statistical reduction in the activity of γ-GCS was noted following 10% IC\textsubscript{50} of OA treatment. Unlike C6 cells, all concentrations of OA treatment inhibited the enzyme activity in A549 cells. The results indicated that NAC could not reverse the OA-inhibited activity of γ-GCS in both tumor cell lines. It was shown that OA has the capacity of inhibiting the activity of γ-GCS in tumor cells.

Influence of OA on the activity of GSS. GSS is another synthetic enzyme of GSH. Thus, the activity of GSS in both cell lines following OA treatment was determined. As shown in Fig. 7, we found that OA pretreatment failed to affect the intracellular activity of GSS in the C6 cells. A similar phenomenon was also observed in A549 cells when OA concentration was 10% IC\textsubscript{50} or 20% IC\textsubscript{50}, even 30% IC\textsubscript{50} of OA could statistically enhance the activity of GSS. Meanwhile, compared to the cells treated with 30% IC\textsubscript{50} of OA, both C6 and A549 cells treated with 30% IC\textsubscript{50} of OA and NAC did not show an obvious alteration in GSS activity. The results thus revealed that OA does not decrease the activity of GSS in tumor cells.

Discussion

Ionizing radiation continues to be a frontline therapy for local control of glioma and lung cancer where surgery is either not possible or undesirable (31,32). However, regarding the
Figure 4. Alterations in the micronucleus rate of irradiated tumor cells by oleanolic acid (OA) pretreatment. Micronucleus (MN) induction in (A) C6 and (B) A549 cells irradiated with different doses of X-rays prior to OA treatment. The data are from three independent experiments. **P<0.01, compared to the Y_MN of the same cell line without OA treatment and irradiation (IR). #P<0.05 and ##P<0.01, compared to the Y_MN of the same irradiated cell line without OA treatment. (C and D) N-acetylcysteine (NAC) statistically reduces the enhancement of the ratio of MN by pretreatment with 30% IC_{50} OA; C6 and A549 cells are shown in C and D, respectively. *P<0.05 and **P<0.01, compared to the Y_MN of the same irradiated cell line without NAC treatment.

Figure 5. Inhibition of oleanolic acid (OA) on the synthesis of intracellular glutathione (GSH). Data are from 3 independent experiments. **P<0.01, compared to the cells in the absence of OA treatment. ***P<0.01, compared to the cells without N-acetylcysteine (NAC) treatment.

Figure 6. Inhibition of oleanolic acid (OA) on the activity of γ-glutamyl-cysteine synthetase (γ-GCS). Data are from 3 independent experiments. *P<0.01, compared to the cells in the absence of OA treatment.
radiotherapeutics of lung cancer, radiation pneumonia is one serious complication after patient exposure to high-dose irradiation (33). Thus, it is favorable to decrease the incidence of radiation pneumonia by enhancement of the radiosensitivity of cancer cells with pretreatment of a radiosensitizing agent. Regarding glioma, an appropriate radiosensitizing drug needs to have the capability for permeating the blood-brain barrier for achieving effective plasma concentrations in the region of the tumor. There are numerous natural compounds from the extracts of plants, which have the potential to increase the radiosensitivity of tumor cells (34-36). In the present study, we investigated the radiosensitizing effect of OA on glioma and lung cancer cell lines. The natural OA extract in the form of free acid has the ability of infiltrating the blood-brain barrier (37). The rationale for combined treatment of non-toxic concentrations of OA with radiation is to observe whether it leads to a greater extent of tumor cell death. We thus selected three different concentrations of OA, which had no obvious influence on cell viability, to carry out the radiosensitizing experiment. Different doses of radiation combined with OA greatly inhibited the cell growth and achieved an additive effect. According to the calculation of D_50 and SERs, the sensitivity of tumor cells to radiation was significantly enhanced by OA treatment. In a previous study, the radiosensitizing effect of ursoic acid, an isomer of OA, was demonstrated in diverse cell types and in vivo (38). Additionally, experimental data indicate that betulinic acid, another pentacyclic triterpenoid, may be a useful agent for tumors that are resistant to irradiation such as head and neck cancer and melanoma (39,40). The results from our research also support that OA has radiosensitizing capacities in tumor cells as confirmed by the effects of combined treatment of other pentacyclic triterpenoids and irradiation.

The protective effect of GSH is important for the resistance of cancer cells against radiotherapeutics. Many compounds of natural origin are capable of regulating intracellular GSH levels. For instance, gelomulide K, a natural diterpene extract, induces a decrease in cellular GSH in cancer cells (41). It was also observed that ferulic acid potentiates the efficacy of radiosensitization in human cervical carcinoma cells by the attenuation of intracellular GSH (42). Similarly, we further observed the alteration of intracellular GSH levels in tumor cells after treatment with different concentrations of OA. The results showed that OA obviously reduces GSH levels in a concentration-dependent manner. Because γ-GCS is the key rate-limiting enzyme synthesizing intracellular GSH, the intracellular GSH level was decreased by inhibition of γ-GCS (12,43). Our study demonstrated that the activity of γ-GCS in tumor cells could be downregulated following OA treatment. Although GSS is another synthetic enzyme of GSH, which is rarely considered in the field of radiation biology, we still observed its activity for the sake of clarifying the target of the radiosensitizing effect of OA. The experimental results found that OA does not evidently inhibit the activity of GSS. On the contrary, its activity was upregulated with 20% IC_{50} of OA. Consequently, the synthesis of GSH in tumor cells was decreased via the downregulation of the activity of γ-GCS by OA, but not by the inhibition of GSS.

Data indicate that MN are the result of small chromosome fragments that are not incorporated into the daughter nuclei during cell division, which arise from exposure to nonrepaired or misrepaired DSBs by various clastogenic agents (44,45). It has been suggested that DNA damage by ROS induction leads to formation of (SSBs) and DSBs (46,47). Meanwhile, the number of radiation-induced MN was strongly correlated with radiation damage. The MN assay is an appropriate biological tool for evaluating the radiosensitivity of cells in vitro due to the good reliability and reproducibility of the assay (48). In the present study, treatment of OA alone did not statistically enhance the ratio of MN in tumor cells. Our results clearly showed that the ratio of MN was conspicuously increased when tumor cells were exposed to X-rays. Subsequently, it was found that the combination of OA at different concentrations and radiation led to higher ratios of MN compared to radiation alone. According to previous reports, the depletion of intracellular GSH results in the generation of MN. In order to verify this theory, NAC, an antioxidant and GSH precursor, was further used in our study. The outcome demonstrated that the level of intracellular GSH was enhanced after NAC was added to the culture medium, but no significant changes in γ-GCS and GSS activity were noted. The phenomenon was contributed to the enhancement of this enzymatic reaction substrate, but not the increasing activity of the enzymes by themselves (49). Moreover, concomitant with the enhancement of reduced GSH levels, the high ratios of MN with OA at 30% IC_{50} concentration and irradiation treatment was decreased by NAC. Based on the high level of intracellular GSH, the following phenomenon was found. Compared to the cells without NAC pretreatment, both tumor cell lines treated with NAC showed a weaker damage after exposure to irradiation.

Importantly, previous data also indicate that OA attenuates hepatotoxicity and nephrotoxicity of various toxic agents by increasing the level of GSH, further protecting normal tissue and cells (50,51). Conversely, our results and other studies indicate that high levels of GSH in tumor cells are markedly suppressed by pretreatment of OA and its derivatives (52,53). Regardless this difference, we presume that the following factors possibly contributed to the controversial findings. The protective effect of OA and its derivatives are via the stimula-
tion of the synthesis of GSH in normal tissues damaged by the depletion of GSH, while in contrast the effect of OA is through inhibition of GSH in malignant tumors with high levels of GSH. Since extremely high or low levels of GSH may disorder the redox equilibrium of the intracellular microenvironment (54), OA sustains the balance via regulation of GSH.

Taken together, our experimental results suggest for the first time that OA sensitizes rat glioma C6 cells and human lung cancer A549 cells to radiation in vitro. The mechanism of this sensitization may involve the inhibition of reduced GSH synthesis via the downregulation of γ-GCS activity, which in turn may explain the reduction in clonogenic survival and the increase in cellular MN. The effect of the natural medicine OA on regulating the radiation response may provide novel benefit for the treatment of tumors.

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References