Ursolic acid sensitizes radioresistant NSCLC cells expressing HIF-1α through reducing endogenous GSH and inhibiting HIF-1α

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Abstract. In previous studies, the present authors demonstrated that effective sensitization of ionizing radiation-induced death of tumor cells, including non-small cell lung cancer (NSCLC) cells, could be produced by oleanolic acid (OA), a pentacyclic triterpenoid present in plants. In the present study, it was investigated whether ursolic acid (UA), an isomer of OA, had also the capacity of sensitizing radioresistant NSCLC cells. The radioresistant cell line H1299/M-hypoxia inducible factor-1α (HIF-1α) was established by transfection with a recombinant plasmid expressing mutant HIF-1α (M-HIF-1α). Compared with parental H1299 and H1299 cells transfected with empty plasmid, H1299/M-HIF-1α cells had lower radiosensitivity. Following the use of UA to treat NSCLC cells, elevation of the radiosensitivity of cells was observed by MTT assay. The irradiated H1299/M-HIF-1α cells were more sensitive to UA pretreatment than the irradiated cells with empty plasmid and control. The alteration of DNA damage in the irradiated cells was further measured using micronucleus (MN) assay. The combination of UA treatment with radiation could induce the increase of cellular MN frequencies, in agreement with the change in the tendency observed in the cell viability assay. It was further shown that the endogenous glutathione (GSH) contents were markedly attenuated in the differently irradiated NSCLC cells with UA (80 µmol/l) pretreatment through glutathione reductase/5,5′-dithiobis-(2-nitro-enzoic acid) (DTNB) recycling assay. The results revealed that UA treatment alone could effectively decrease the GSH content in H1299/M-HIF-1α cells. In addition, the inhibition of HIF-1α expression in radioresistant cells was confirmed by western blotting. It was then concluded that UA could upregulate the radiosensitivity of NSCLC cells, and in particular reduce the refractory response of cells expressing HIF-1α to ionizing radiation. The primary mechanism is associated with reduction of endogenous GSH and inhibition of high expression of intracellular HIF-1α. UA should therefore be deeply studied as a potential radiosensitizing reagent for NSCLC radiotherapy.

Introduction

Radiotherapy is an established treatment modality for non-small cell lung cancer (NSCLC), which can provide an effective cure for a proportion of patients (1). Although NSCLC is a moderately radiation-responsive tumor, local control is still not achieved in numerous patients, primarily due to intrinsic and acquired resistance of tumor cells to ionizing radiation (2,3). There are difficulties in delivering sufficiently high radiotherapy doses to the tumor due to potential toxicity development in the normal tissue (4). Therefore, it is particularly beneficial for radiotherapy of NSCLC to increase the radiosensitivity of resistant NSCLC cells by using natural or synthetic compounds.

Local failure of radiotherapy is associated with a series of factors, where the radiosensitivity of irradiated cells depends on a complex interplay of nuclear and cytoplasmic signaling cascades (5-8). Ionizing radiation induces DNA double-strand breakages (DSBs) and production of free radicals (FRs) and reactive oxygen species (ROS), which can cause DSBs of DNA and lipid oxidation of the cellular membrane; these events are widely acknowledged as the principal determinants of radiation-induced cell death (9,10). Intracellular antioxidants such as the tripeptide thiol L-γ-glutamyl-L-cysteinyl-glycine (glutathione, GSH) play a key role in the protection of cells against the oxidative stress induced by FR and ROS (11). GSH, which is present in all mammalian cells, is the most important intracellular thiol-based scavenger of FR and ROS (12-14). The level of GSH is increased in various tumor cells, including NSCLC cells (15,16). Agents that decrease the cellular content of GSH could effectively inhibit the DNA damage repair to increase the response of tumor cells to ionizing radiation (17,18).

Numerous compounds extracted from natural sources have been observed to elevate the radiosensitivity of tumor cells (19,20). Ursolic acid (3-beta-hydroxy-urs-12-en-28-oic acid, UA) is one of the pentacyclic triterpenoids that exist widely...
in the plant kingdom (Fig. 1) (21). UA is a compound of interest in oncology research due to its cytotoxicity, its anti-invasive and anti-migration activities, and its ability to induce cell differentiation (22,23). UA also interferes with damage repair of cancer cells and induces apoptosis in cancer cells by regulating different signaling pathways, including inhibition of Wnt/β-catenin and activation of the c-Jun N-terminal kinase and the phosphoinositide 3-kinase/Akt/nuclear factor (NF)-κB signaling pathways (24-26). Previous studies have demonstrated sensitization caused by UA towards chemotherapy and radiotherapy (27-29). With regard to the treatment of NSCLC, chemosensitization to low doses of UA was found in the ASTC-a-1 cell line (human lung adenocarcinoma cells) through suppression of NF-κB (27). However, it remains unclear whether the radiosensitivity of NSCLC cells, particularly that of cells with refractory response to radiation, could be increased by UA. In present study, a radioresistant NSCLC cell line was established by transfecting a mutant HIF-1α (M-HIF-1α) plasmid, and the radiosensitivity of the parental and radioresistant NSCLC cells was investigated upon UA pretreatment. In order to preliminarily analyze the mechanism, the alteration of intracellular GSH level was also analyzed.

**Materials and methods**

**Cell culture and treatment.** The human lung cancer H1299 cell line was kindly provided by Professor Qinghua Shi (College of Biological Science, University of Science and Technology of China, Hefei, China). Cells were maintained in Dulbecco’s modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma-Medical, 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 (Manassas, VA, USA). UA was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China), dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a stock concentration of 5 mmol/l and stored at -20˚C.

**Plasmids and transfection assays.** The pcDNA3.0 vector with enhanced green fluorescence protein (EGFP) was kindly provided by Professor Qinghua Shi. HIF-1α complementary DNA (cDNA) for with three mutant motifs, including the prolines at the 402 and 564 sites in the oxygen-dependent degradation domain (ODDD) of HIF-1α, and the aspartic acid at the 803 site (Sigma-Medical, St. Louis, MO, USA) were cloned into the pcDNA3.0 vector to construct the pcDNA3.0-EGFP-HIF-1α recombinant plasmid. The pcDNA3.0-EGFP empty vector was used as control. H1299 cells (5x10⁵) were transfected with 4 µg plasmid DNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The neomycin-resistant clones were selected in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 500 µg/ml G418 (Promega Corporation, Madison, WI, USA), and transferred into a 24-well culture plate with cloning discs (Sigma-Aldrich). The selected clones were expanded in medium containing 200 µg/ml G418, and identified by detecting the messenger RNA and protein expression of M-HIF-1α.

**Cell viability assay.** The influence of UA on cell growth was determined using the MTT (Sigma-Aldrich) assay. The parental H1299 cells and H1299 cells expressing the M-HIF-1α fragment were seeded in 96-well plates at a density of 5x10³ cells/well, and then treated with various concentrations of UA for 24 h. Next, the medium was replaced with fresh medium to allow cells to continuously grow for 72 h. MTT dye was then added to a final concentration of 50 mg/ml, and the cells were subsequently incubated for additional 4 h at 37˚C. The medium containing residual MTT dye was carefully aspirated from each of the wells, and 200 µl dimethyl sulfoxide was added to each well to dissolve the reduced formazan dye. The survival rates of viable cells were calculated by comparing the optical absorbance of the culture exposed to UA 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.

**Micronucleus (MN) assay.** MN frequencies were tested with the cytokinesis-block technique as a biological end point for the response of mimetic hypoxia to irradiation (30). 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 for 10 min. MN 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, ≥1,000 binucleated cells were counted. The MN yield was calculated as the ratio of the number of MN to the number of binucleated cells scored.

**Western blot analysis of HIF-1α expression.** Cells subjected to different treatments were scraped off from culture flasks and lysed in lysis buffer containing 10% glycerol, 10 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5 mM dithiothreitol and 1X complete protease inhibitor cocktail (Sigma-Aldrich).
The Bradford method was used to detect concentrations of protein in diverse samples. Protein concentration was measured using an automatic multifunctional microplate reader. Proteins (50 µg) were separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes, which were then blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 1 h, and then incubated with mouse anti-HIF-1α antibody (catalog no. ab82832; Abcam, Cambridge, MA, USA) at a 1:500 dilution overnight at 4°C, followed by goat anti-mouse immunoglobulin G (catalog no. ab8226; Abcam) for 1 h at room temperature. Signals were detected with enhanced chemiluminescence (ECL Plus; GE Healthcare Life Sciences, Chalfont, UK). An antibody against the microtubule protein tubulin (anti-tubulin; Abcam) at a 1:1,000 dilution was used as an internal control to observe the changes in the HIF-1α bands.

**Intracellular GSH assay.** Following the treatment of triplicate samples of $10^6$ cells with different reagents, the intracellular GSH content was measured with a glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay kit obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China), following the protocol recommended by the manufacturer. Briefly, GSH was determined 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 (153 mM sodium phosphate and 8.4 mM ethylenediaminetetraacetic acid, pH 7.5). After 5 min incubation at 25˚C, the reaction was started by the addition of 50 µl of reduced nicotinamide adenine dinucleotide phosphate (NADPH) solution (0.16 mg/ml) in the assay buffer. The standard and the sample cuvettes were placed into a dual-beam spectrophotometer, and the increases in absorbance at 412 nm were followed as a function of time.

Measurement of intracellular ROS. Cell suspension from the different treatments was incubated with 10 µM of 2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min, and then washed three times with phosphate-buffered saline for removing excess DCFH probe. Upon counting the viable cells, the fluorescence intensities of the cells were observed under an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan), at excitation and emission wavelengths of 488 and 525 nm, respectively.

Statistical analysis. Data are reported as the mean ± standard error of the mean of three separate experiments unless stated otherwise. Statistical significance was measured by independent samples t-test and analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of radioresistant NSCLC cells. The radiosensitivity of H1299 cells was regulated by transfecting recombinant pcDNA3.0-EGFP-HIF-1α plasmid. As shown in Fig. 2A and B, under aerobic conditions, overexpression of HIF-1α was detected in H1299 cells transfected with the H1299/M-HIF-1α recombinant plasmid, named H1299/M-HIF-1α cells. Both the parental H1299 cells and the H1299 cells transfected with empty vector (H1299/E cells) exhibited loss of HIF-1α expression. Upon exposure to 2 Gy irradiation, cellular viability was significantly upregulated in H1299/M-HIF-1α cells compared with that in H1299 and H1299/E cells (Fig. 2C). By contrast, no obvious difference was observed between H1299 cells and H1299/E cells. It was therefore demonstrated that H1299/M-HIF-1α cells had lower radiosensitivity than H1299 cells and H1299/E cells.
Figure 6. Alteration of the intracellular reactive oxygen species levels by ursolic acid treatment. (A) Blank control for H1299/M-HIF-1α cells without DCFH-DA treatment. (B) Control for H1299/M-HIF-1α cells with DCFH-DA treatment. (C) H1299/M-HIF-1α cells with 50 µmol/l DCFH-DA treatment. (D) H1299/M-HIF-1α cells with 80 µmol/l DCFH-DA treatment. (E) Blank control for H1299/E cells without DCFH-DA treatment. (F) Control for H1299/E cells with DCFH-DA treatment. (G) H1299/E cells with 50 µmol/l DCFH-DA treatment. (H) H1299/E cells with 80 µmol/l DCFH-DA treatment. (I) Blank control for H1299 cells without DCFH-DA treatment. (J) Control for H1299 cells with DCFH-DA treatment. (K) H1299 cells with 50 µmol/l DCFH-DA treatment. (L) H1299 cells with 80 µmol/l DCFH-DA treatment. Fluorescence microscope images were captured at x20 magnification. DCFH-DA, 2',7'-dichloro-dihydro-fluorescein diacetate; M, mutant; HIF-1α, hypoxia inducible factor-1α; E, empty.

Figure 7. Alteration of intracellular reactive oxygen species levels by the combination of UA with irradiation treatment. (A) Control for H1299/M-HIF-1α cells with DCFH-DA treatment. (B) H1299/M-HIF-1α cells exposed to irradiation at 2 Gy. (C) H1299/M-HIF-1α cells with the combination of 50 µmol/l UA with radiation treatment. (D) H1299/M-HIF-1α cells subjected to the combination of 80 µmol/l UA and radiation treatment. (E) Control for H1299/E cells with DCFH-DA treatment. (F) H1299/E cells exposed to irradiation at 2 Gy. (G) H1299/E cells exposed to the combination of 50 µmol/l UA with radiation treatment. (H) H1299/E cells subjected to the combination of 80 µmol/l UA and radiation treatment. (I) Control for H1299 cells with DCFH-DA treatment. (J) H1299 cells exposed to irradiation at 2 Gy. (K) H1299 cells upon combination of 50 µmol/l UA with radiation treatment. (L) H1299 cells subjected to the combination of 80 µmol/l UA with radiation treatment. Fluorescence microscope images were captured at x20 magnification. DCFH-DA, 2',7'-dichloro-dihydro-fluorescein diacetate; M, mutant; HIF-1α, hypoxia inducible factor-1α; E, empty; UA, ursolic acid.
of MN frequencies, however, was not observed in H1299 or H1299/E cells subjected to the combination treatment of UA at 50 µmol/l concentration and radiation. It was thus obvious that UA effectively promoted the formation of MN in irradiated NSCLC cells, particularly in irradiated H1299/M-HIF-1α cells.

Changes in intracellular GSH content by UA treatment. Due to the strong radioprotection of endogenous GSH, the intracellular GSH content was analyzed in NSCLC cells upon non-exposure or exposure to irradiation following UA pretreatment. The results revealed that UA could remarkably decrease the endogenous GSH content in H1299/M-HIF-1α cells not exposed to radiation but not in H1299 or H1299/E cells, as shown in Fig. 5A. Additionally, following NSCLC cells exposure to irradiation, the level of cellular GSH in H1299/M-HIF-1α cells was higher than that in H1299 cell and H1299/E cells, as shown in Fig. 5B. Furthermore, UA at high concentration (80 µmol/l) could effectively attenuate the intracellular GSH content of H1299 and H1299/E cells. The combination treatment of UA with radiation could decrease the GSH intracellular contents in H1299/M-HIF-1α cells, both at 50 and 80 µmol/l concentration of UA.

Alteration of intracellular ROS levels by UA treatment. Under an inverted fluorescence microscope, there was fluorescence in H1299/E and H1299/M-HIF-1α cells without DCFH-DA probe treatment, due to the presence of EGFP in the transfected plasmid (Fig. 6A and E). The fluorescence intensities in the three groups of cells evaluated were weakly increased following DCFH-DA treatment. UA at different concentrations could enhance the levels of intracellular ROS and FR in these cells, particularly in H1299/M-HIF-1α cells (Fig. 6). The results shown in Fig. 7 further demonstrate that the combination of UA with radiation treatment significantly enhanced the generation of ROS and FR. It was revealed that UA with or without irradiation could promote an increase in cellular ROS and FR, particularly in H1299/M-HIF-1α cells.

Discussion

In our previous study, it was observed that oleanolic acid (OA), an isomer of UA, could effectively increase the radiosensitivity of aerobic and hypoxic A549 cells, a NSCLC cell line, by inhibition of intracellular GSH synthesis and HIF-1α expression (31,32). The radiosensitizing efficiency of UA on NSCLC cells, particularly on radioresistant cells, was also observed in the present study, due to the higher anti-tumor activity of UA compared with that of OA (33). In the current study, radioresistant cell line was firstly established by transfection with an M-HIF-1α plasmid. It is known that HIF-1α is closely associated with the radiosensitivity of tumor cells,
including NSCLC cells (34,35). Numerous studies have shown that, following inhibition of the master transcription factor triggered in response to hypoxia, the radiotherapeutic effect on NSCLC may be significantly increased (36,37). Under aerobic conditions, HIF-1α is hydroxylated by prolyl hydroxylases at the proline residues 402 and 564 in the ODDD (38). Subsequently, it is targeted for proteasome-mediated degradation via a protein ubiquitin ligase complex containing the product of von Hippel-Lindau tumor suppressor (39). Another blockage pathway of HIF-1α activity is hydroxylation of asparagine 803 by factor inhibiting HIF-1 within the CTAD, followed by interruption of the binding of HIF-1α to the p300/CREB-binding protein coactivator, thus preventing the transactivation capabilities of HIF-1 (40). In the present study, the cDNA of HIF-1α within the pcDNA3.0-EGFP-HIF-1α recombinant plasmid contained three mutant sites, namely proline residues 402 and 564, and asparagine 803, which enable HIF-1α to evade hydroxylation by maintaining HIF-1α expression and transcriptional activity under aerobic conditions. Thus, H1299/M-HIF-1α cells exhibited high level of HIF-1α under aerobic conditions, concomitant with a refractory response to radiation (41). This in vitro model is convenient to observe alteration of the sensitivity of resistant cells to radiation, as HIF-1α need not be induced by physical or chemical hypoxia.

As a natural antitumor drug, the activity of UA has been reported in numerous studies (22,23,42). Previous data reported the sensitization of cancer cells to ionizing radiation-induced apoptosis by UA, including human prostate cancer DU145 cells, mouse colon cancer CT26 cells and mouse melanoma B16F10 cells (28). The present study demonstrated that UA exerted a similar radiosensitizing effect on NSCLC cells. Notably, a stronger sensitizing effect of UA was observed on radiosensitive H1299 cells with high level of HIF-1α expression compared with that observed in H1299 cells without HIF-1α expression. Similarity, sensitization to ionizing radiation of radiosensitive tumor cells was previously observed to be effected by other natural or synthetic compounds (43,44). For instance, Biddlestone-Thorpe et al (43) reported that glioma cells with a mutant p53 gene that induced tolerance to radiation were markedly more sensitive to radiosensitization induced by KY-60019 (an ataxia telangiectasia mutated kinase inhibitor) than genetically matched wild-type glioma cells. The mechanism could be associated with changes in the expression of various genes and modification of intracellular homeostasis.

There are numerous studies demonstrating that the numbers and frequencies of intracellular MN represent DNA damage induced by radiation; thus MN is considered as an appropriate biological tool to evaluate in vitro radiosensitivity due to its high reliability and reproducibility (45-47). Based on the results of the present cell viability assay, intracellular MN frequencies were detected as a biomarker of DNA damage caused by ionizing radiation. The results revealed that the intracellular MN formation was in agreement with the change in tendency observed in the cell viability assay. Both 50 and 80 µmol/l of UA could increase the MN frequencies in binucleated H1299/M-HIF-1α cells following irradiation. By contrast, elevation of MN formation in irradiated H1299 cells and H1299/E cells was only observed with a UA concentration of 80 µmol/l. These findings indicate the marked sensitization caused by UA on radiosensitive NSCLC cells.

According to previous studies, the depletion of GSH content strongly resulted in the production of cellular MN and the death of irradiated NSCLC cells by increasing the levels of cellular ROS and FR (31,32,48). Thus, the present study measured the intracellular GSH content and the levels of ROS and FR through either UA treatment alone or combination treatment with UA and irradiation. The results indicated that treatment of UA alone decreased the GSH content and increased the levels of ROS and FR in H1299/M-HIF-1α cells, but not in H1299 cells or H1299/E cells, whereas the combination of UA at high concentration (80 µmol/l) and irradiation could diminish the GSH content and increase the levels of ROS and FR in H1299 cells and H1299/E cells. For H1299/M-HIF-1α cells, either exposed to irradiation or not, there was a reduction in cellular GSH content, concomitant with an enhancement in ROS and FR, following treatment with UA at various concentrations. To explore the mechanism behind these observations, the expression of HIF-1α in H1299/M-HIF-1α cells was detected upon UA treatment. It was observed that increasing HIF-1α expression was inhibited by UA, which is in agreement with previously reported data (49,50). According to the study by Guo et al (51), the inhibition of HIF-1α could decrease the cellular GSH content and increase the generation of ROS by regulating the level of NADPH, which led to a more oxidizing environment for the cells. In present study, the GSH content of H1299/M-HIF-1α cells could further be reduced due to UA-mediated suppression of HIF-1α expression. Therefore, the increased sensitization of H1299/M-HIF-1α cells by UA was associated with reduced HIF-1α expression.

In summary, the present results demonstrated that UA significantly exerted a radiosensitizing effect on NSCLC cells, particularly on radiosensitive cells overexpressing HIF-1α. The primary mechanism is explained as follows: i) Radiosensitivity of NSCLC cells without HIF-1α expression was upregulated through UA, which decreased the intracellular GSH content; and ii) two pathways, including the attenuation of GSH and the suppression of HIF-1α by UA, enhanced the sensitization towards ionizing radiation-induced cell death of NSCLC cells overexpressing HIF-1α. UA should therefore be deeply studied as a potential radiosensitizing reagent for NSCLC radiotherapy.

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