Original Article

Human lung adenocarcinoma cells with an EGFR mutation are sensitive to non-autophagic cell death induced by zinc oxide and aluminium-doped zinc oxide nanoparticles

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ABSTRACT — Lung cancer, mostly non-small cell lung cancer (NSCLC), is the leading cause of cancer deaths; however, efficient treatments for NSCLC remain insufficient. The objective of this study was to investigate the effects of an epidermal growth factor receptor (EGFR) mutation on autophagic cell death in human lung adenocarcinoma cells by 20-nm zinc oxide nanoparticles (ZnONP20) and aluminum-doped ZnONPs (Al-ZnONP20). Two types of human lung adenocarcinoma cells were used throughout the study: wild-type EGFR A549 cells and EGFR-mutated CL1-5 cells. We observed that a significant reduction in cell viability resulting from ZnONP20 and Al-ZnONP20 occurred in A549 and CL1-5 cells after 18 and 24 hr of exposure. A colony formation analysis showed that A549 cells re-grew after exposure to 20 µg/mL Al-ZnONP20. Levels of light chain 3 (LC3) II conversion were activated by ZnONP20 and Al-ZnONP20 in A549 cells, whereas LC3 II was inhibited by ZnONP20 and Al-ZnONP20 in CL1-5 cells. In conclusion, we have shown that human lung adenocarcinoma cells with an EGFR mutation are sensitive to ZnONP20 and Al-ZnONP20, which may have resulted in non-autophagic cell death. ZnONP20 and Al-ZnONP20 may have the potential for personalized therapeutics in NSCLC with an EGFR mutation.

Key words: Cell death, Epidermal growth factor receptor, Lung cancer, Nanoparticle, Zinc oxide

INTRODUCTION

Lung cancer, mostly non-small cell lung cancer (NSCLC), is the leading cause of cancer deaths; however, efficient treatments for NSCLC remain insufficient. Frontline therapies using standard platinum-based doublet combination regimens have reached a plateau, with 1-year survival rates of only 35%~40% and a median overall survival of 12 months recently being barely achieved (Ciuleanu *et al.*, 2009). Although molecular targeted therapy, particularly epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), seem to

have achieved promising results, a prolongation of overall survival compared to conventional chemotherapy as the first-line therapy for those with EGFR-mutation-positive tumors has never been demonstrated (Shepherd *et al.*, 2005; Mok *et al.*, 2009). This suggests that patients still need to rely on chemotherapy as part of their standard treatment. Therefore, novel therapeutic regimens need to be developed and evaluated in order to complement current treatments.

Nanotechnology has rapidly developed in importance over time, especially in biomedical applications. For example, nanomaterials were proven to exhibit

promising properties that allow them to improve tumor responses to chemo- and radiotherapy in cancer therapy (Beik *et al.*, 2016; Ma and Yang, 2016). Recently, zinc oxide nanoparticles (ZnONPs) were considered to have the potential for cancer treatment (Zhang *et al.*, 2013). Hackenberg and colleagues observed that ZnONPs followed by UVA-1 irradiation were able to induce cell death of human head and neck squamous cell carcinoma cells (Hackenberg *et al.*, 2014). Additionally, previous reports demonstrated that ZnONPs caused cytotoxicity in cell-specific and proliferation-dependent manners (Hanley *et al.*, 2008; Premanathan *et al.*, 2011; Ostrovsky *et al.*, 2009). Therefore, it is important to investigate the efficiency of treating different types of cancer cells with ZnONPs.

Previous findings suggested that autophagy may play a protective role in the early stage of cancer (Sui *et al.*, 2013; Yang *et al.*, 2011). Autophagy is also reported to induce lethal mechanisms in tumor cells (Morselli *et al.*, 2009). Chemotherapy- and metabolic stress-induced activation of autophagy may result in the survival of tumor cells, leading to resistance in cancer cells. On the contrary, autophagy inhibition may represent a major therapeutic target for chemosensitization (Yang *et al.*, 2011). Therefore, oncogenesis and tumor survival are influenced by the molecular machinery that controls autophagy.

Genetic variations leading to overexpression or mutations of the EGFR play essential roles in the occurrence and progression of lung cancers such as NSCLC (Li *et al.*, 2016a). EGFR-TKIs are recognized as a standard NSCLC therapy and have great clinical benefits (Farhat and Houhou, 2013). However, primary or acquired drug resistance remains a big challenge to the success of TKI therapy in NSCLC. Therefore, ZnONPs for NSCLC have a great potential due to their unique characteristics. The objectives of this study were: (1) to investigate the effects of ZnONPs and aluminum-doped ZnONPs (Al-ZnONPs) on the cell viability and proliferation in wild-type EGFR A549 cells and EGFR-mutated CL1-5 cells; and (2) to observe the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3) in A549 and CL1-5 cells after ZnONP and Al-ZnONP exposure.

MATERIALS AND METHODS

Particles and reagent sources

The physical properties of ZnONP20 and Al-ZnONP20, obtained from Nanostructured & Amorphous Materials (Houston, TX, USA), used in this study were previously reported (Pan *et al.*, 2014). All of the other reagents were obtained from Sigma (St. Louis, MO, USA), unless the

source is explicitly stated.

Particle characterization

Inspect™ FE-SEM (JEOL 2100, Jeol, Tokyo, Japan) was used to characterize the morphology of the particles. Preparation of the FE- scanning electron microscopic (SEM) samples was previously described (Chuang *et al.*, 2011). Briefly, nanoparticles adhered to 12-mm carbon sticky tabs, which were fixed on 13-mm aluminum SEM stubs. Nanoparticles were coated with platinum (Pt) to an average thickness of 10 nm using a sputter-coater and then imaged. FE-SEM was operated at an accelerating voltage of 15 kV and a 2.5-nm spot size. An elemental analysis was conducted using the EDX Genesis Microanalysis System. Specific surface areas of nanoparticles were calculated by the Brunauer-Emmett-Teller (BET) method at a relative pressure (P/P₀) of 0.99 using a specific surface area and pore size distribution analyzer (ASAP 2020, Micromeritics Instrument, Norcross, GA, USA).

Cell culture and treatment

Human lung adenocarcinoma A549 cells (with the wild-type EGFR) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human lung adenocarcinoma CL1-5 cells (with an EGFR mutation) were originally established from a single cell clone isolated from lung adenocarcinoma tissues of a patient with a poorly differentiated adenocarcinoma (Lee-Huang *et al.*, 2000), which was kindly provided by Dr. C-T. Yeh (Shuang Ho Hospital, New Taipei City, Taiwan). A549 and CL1-5 cells are commonly used for EGFR studies on lung cancer. (Lee *et al.*, 2011) These cells were seeded in surface-treated, 96-well transwells at a density of 10⁵ cells/mL and incubated for 24 hr (BD Biosciences, Oxford, UK). Cells were cultured in RPMI medium containing 10% fetal bovine serum (FBS), penicillin, and streptomycin and were incubated at 37°C, 95% humidity, and 5% CO₂. ZnONP20 and Al-ZnONP20 solutions were freshly prepared in 10% FBS-containing cell medium. To determine the cell viability, cells were incubated with 0, 20, 50, 150, and 300 µg/mL ZnONP20 or Al-ZnONP20 at 37°C for 1, 6, 18, and 24 hr in a humidified atmosphere with 5% CO₂. For the colony formation assay, cells were incubated with 0, 20, 50, and 150 µg/mL ZnONP20 or Al-ZnONP20. For the Western blot analysis, cells were incubated with 0, 20, 50, and 150 µg/mL ZnONP20 or Al-ZnONP20. Each experiment was conducted at least in triplicate.

Cell viability

A sulforhodamine B (SRB) colorimetric assay was used

to investigate cell viability according a previous method (Vichai and Kirtikara, 2006). Briefly, cells were fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min. The protein-bound dye was dissolved in 10 mM of a Tris base solution after removing excess dye. The OD was measured at 510 nm using a microplate reader (Bio-Rad Model 680 microplate reader, Hertfordshire, UK). Cell viability is presented as a percentage (%) after adjusting for the control.

Colony formation assay

The protocol of the colony formation assay was described previously (Franken *et al.*, 2006). Briefly, 500 cells were resuspended in RPMI medium and seeded in six-well surface-coated plates following a 24-hr incubation. Cells were exposed to 0, 20, 50, and 150 $\mu\text{g/mL}$ of particles for 5 days. Next, cells were washed and cultured for a further 9 days. Finally, cells were washed and fixed with 3.7% paraformaldehyde following staining with 0.05% crystal violet. Plates were stained with 0.5 mg/mL p-iodonitrotetrazolium violet. Colonies with a diameter of > 1 mm were counted. Triplicate samples were used in the experiment. Images were then analyzed for the colony formation rate (%) using ImageJ software (vers. 1.49, Bethesda, MD, USA) (Fertsch-Gapp *et al.*, 2011).

Western blot analysis

Western blot analyses were conducted as described previously (Chen *et al.*, 2004). Briefly, cells were cultured in 6-cm dishes for nanoparticle exposure. Proteins isolated from lysed cells were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Darmstadt, Germany). Primary antibodies for LC3 (1:1000) and β -catenin (1:1000) were obtained from Cell Signaling (Danvers, MA, USA). Anti-rabbit (1:2000) horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Chemicon International (Billerica, MA, USA). Blots were then blocked with 5% skim milk in PBST and were probed with primary antibodies overnight at 4°C. An HRP-labeled secondary antibody was then incubated for 60 min at room temperature and washed with TBST. Enhanced chemiluminescence (ECL) Western blotting reagents were used, followed by imaging using the BioSpectrum Imaging System (UVP, Upland, CA, USA). Quantitative data were obtained using ImageJ software (Fertsch-Gapp *et al.*, 2011).

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (S.D.). A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for comparisons among multiple values. Statistical analyses were performed using GraphPad vers. 5 for Windows. The level of significance was set to $p < 0.05$.

RESULTS

Characterization of ZnONP20 and Al-ZnONP20

ZnONP20 (with an average diameter of 20 nm) and Al-ZnONP20 (with an average diameter of 20–40 nm) were characterised using FE-SEM coupled with EDX (Fig. 1). The purity was $\text{ZnO} = 99.5$ wt% for ZnONP20 and $\text{ZnO:Al}_2\text{O}_3 = 98:2$ wt% for Al-ZnONP20, which are values provided by the supplier. ZnONP20 presented a hexagonal structure, whereas an irregular morphology of Al-ZnONP20 was observed. Both ZnONP20 and Al-ZnONP20 were Zn dominant, but Al-ZnONP20 contained a minor amount of Al. Surface areas of ZnONP20 and Al-ZnONP20 were 22.3 and 3.6 m^2/g , respectively.

Cell viability and proliferation

To investigate the particle toxicity caused by ZnONP20 and Al-ZnONP20 in A549 and CL1-5 cells, cell viability and colony formation were determined. First, we observed that a significant reduction in cell viability resulted from ZnONP20 at 300 $\mu\text{g/mL}$ for 18 and 24 hr of exposure in A549 cells ($p < 0.05$), whereas cell viability of CL1-5 cells significantly decreased with 20, 50, 150, and 300 $\mu\text{g/mL}$ after 18 and 24 hr of exposure ($p < 0.05$) (Fig. 2). The cell viability of A549 cells decreased with Al-ZnONP20 at 150 and 300 $\mu\text{g/mL}$ for 18 and 24 hr of exposure ($p < 0.05$), whereas Al-ZnONP20 significantly reduced the cell viability of CL-15 cells when exposed to 150 and 300 $\mu\text{g/mL}$ for 6 hr and 20, 50, 150 and 300 $\mu\text{g/mL}$ for 18 and 24 hr ($p < 0.05$). The results suggest that A549 cells were more resistant to ZnONP20 than were CL1-5 cells, and Al-ZnONP20 caused higher cell death than ZnONP20.

Next, the effects on cell proliferation by ZnONP20 and Al-ZnONP20 in A549 and CL1-5 cells were determined using a colony formation assay. As shown in Fig. 3, ZnONP20 significantly affected the growth rates of A549 and CL1-5 cells at 20, 50, and 150 $\mu\text{g/mL}$. Notably, the growth rate of Al-ZnONP20-treated A549 cells was faster at 20 $\mu\text{g/mL}$ than that of CL1-5 cells. The colony formation rate of A549 cells was inhibited by ZnONP20, but not by Al-ZnONP20 at 20 $\mu\text{g/mL}$. The colony formation rate of CL1-5 cells was inhibited by both ZnONP20 and

Fig. 1. FE-SEM and EDX analyses of 20-nm zinc oxide nanoparticles (ZnONP20) and aluminum-doped (Al)-ZnONP20. ZnONP20 presented a hexagonal structure, whereas an irregular morphology of Al-ZnONP20 was observed. Both nanoparticles were Zn dominant.

Al-ZnONP20.

LC3 expression

Expressions of LC3 II (after controlling for LC3 I) after exposure to ZnONP20 and Al-ZnONP20 in A549 and CL1-5 cells were determined. As shown in Fig. 4, expression of LC3 II increased after exposure to ZnONP20 in A549 cells, particularly at 50 $\mu\text{g/mL}$ ($p < 0.05$), whereas LC3 II was steadily activated by Al-ZnONP20 and reached a significant increase at 150 $\mu\text{g/mL}$ ($p < 0.05$). Although the expression of LC3 II slightly increased by exposure to ZnONP20 and Al-ZnONP20 in CL1-5 cells, the increase in LC3 II was not significant. LC3 conversion caused by ZnONP20 and Al-ZnONP20 was found in A549 cells, but not observed in CL1-5 cells, suggesting that distinct effects of LC3 activation by exposure to ZnONP20 and Al-ZnONP20 occurred in A549 and CL1-5 cells.

DISCUSSION

Autophagy is considered to be an important pathway

in lung disease pathogenesis and therapeutics. ZnONPs are considered to have potential for cancer therapy. In the present study, we observed that ZnONP20 and Al-ZnONP20 may have potential for treating lung adenocarcinoma cells with an EGFR mutation. Three major findings are reported in the present study: (1) the cell viability and proliferation of CL1-5 cells with an EGFR mutation were sensitive to ZnONP20 and Al-ZnONP20; (2) cell viability of A549 cells with the wild-type EGFR was resistant to ZnONP20 and Al-ZnONP20; and (3) autophagy was activated by ZnONP20 and Al-ZnONP20 in A549 cells with the wild-type EGFR, whereas autophagy was inhibited by ZnONP20 and Al-ZnONP20 in CL1-5 cells with an EGFR mutation.

To investigate the effects of ZnONPs on autophagic cell death in lung adenocarcinoma cells with or without an EGFR mutation, the 20-nm-diameter engineered ZnONPs (ZnONP20) and Al-ZnONP (Al-ZnONP20) were used throughout the study. These nanoparticles present differences in morphology as observed in this study. Their physicochemical properties, including size, elements, electronic properties, zinc concentration, and pH, were

Inhibition of cell survival by ZnONPs and Al-ZnONPs in cells with an EGFR mutation

Fig. 2. Effects of 20-nm zinc oxide nanoparticles (ZnONP20) and aluminum-doped (Al)-ZnONP20 on cell viability of A549 (with the wild-type epidermal growth factor receptor (EGFR)) and CL1-5 (with an EGFR mutation) cells. Cell viability was determined in response to ZnONP20 and Al-ZnONP20 at 0, 20, 50, 150, and 300 µg/mL for 1, 6, 18, and 24 hr. A549 cells were more resistant to ZnONP20 than were CL1-5 cells, and Al-ZnONP20 caused higher cell death than ZnONP20. * $p < 0.05$.

Fig. 3. Effects of 20-nm zinc oxide nanoparticles (ZnONP20) and aluminum-doped (Al)-ZnONP20 on cell proliferation of A549 (with the wild-type epidermal growth factor receptor (EGFR)) and CL1-5 (with an EGFR mutation) cells. Colony formation was analyzed in response to ZnONP20 and Al-ZnONP20 at 20 µg/mL. The colony formation rate of CL1-5 cells was significantly inhibited by both ZnONP20 and Al-ZnONP20.

Fig. 4. Effects of 20-nm zinc oxide nanoparticles (ZnONP20) and aluminum-doped (Al)-ZnONP20 on light chain 3 (LC3) expression of A549 (with the wild-type epidermal growth factor receptor (EGFR)) and CL1-5 (with an EGFR mutation) cells. LC3 II expression (adjusted by LC3 I) was determined after exposure of A549 and CL1-5 cells to ZnONP20 and Al-ZnONP20 at 0, 20, 50, and 150 μ g/mL for 24 hr. LC3 conversion caused by ZnONP20 and Al-ZnONP20 significantly increased in A549 cells, but was inhibited in CL1-5 cells.* $p < 0.05$.

reported previously (Pan *et al.*, 2014). To reduce particle aggregation/agglomeration when suspended in solution for toxicity testing, serum-containing cell medium was used throughout the study. Serum proteins act as an excellent dispersant for nanoparticles in biological solutions (Cho *et al.*, 2012; Chuang *et al.*, 2014), but the dispersive ability was only maintained for a short time, after which aggregation/agglomeration recurred (Pan *et al.*, 2014). Physicochemical characteristics of nanoparticles play important roles in regulating nanoparticle toxicity (Buerki-Thurnherr *et al.*, 2013; Pan *et al.*, 2014). Solubility, for example, is recognized as an important determinant of regulating ZnONP toxicity. In the present study, Al-ZnONP20 was used to slow Zn ion leaching during exposure to cells, which was served as control nanoparticles. Notably, the long-term difference of nanotoxicity caused by their physicochemistry between ZnONP20 and Al-ZnONP20 might not be significant due to plateau levels of Zn ion in biological systems. Therefore, the solubility of the Zn-based may influence the bioreactivity *in vitro*, which should be considered in future works.

The ability to avoid cell death by cancer cells is a

hallmark of cancer, leading to cancer development and progression. Therefore, inhibition of cell viability and re-growth is an essential indicator for the treatment efficiency in lung cancer. Lung cancer is a malignant tumor, characterized by uncontrolled cell growth in lung tissues (CDC, 2015). Clinically, mutations in the EGFR were observed in 15%~20% of lung adenocarcinomas (Siegelin and Borczuk, 2014). In the present study, A549 cells with a wild-type EGFR and CL1-5 cells with an EGFR mutation were exposed to ZnONP20 and Al-ZnONP20. We observed that both ZnONP20 and Al-ZnONP20 were efficient at reducing cell viability and inhibiting cell re-growth in CL1-5 cells. Also, ZnONP20 and Al-ZnONP20 were able to reduce cell viability, but A549 cells exhibited re-growth after exposure to Al-ZnONP20. Consistent with previous reports, ZnONPs are able to decrease cell viability (Kumar *et al.*, 2015; Wang *et al.*, 2014; Meyer *et al.*, 2011), suggesting the potential for cancer therapy. Additionally, Hackenberg and colleagues found that ZnONPs were able to induce the photocatalytic death of head and neck squamous cell carcinoma cells (Hackenberg *et al.*, 2014). Therefore, based on our find-

ings, both ZnONP20 and Al-ZnONP20 may have the potential for treating NSCLC with an EGFR mutation.

Regulation of autophagy is associated with the survival of tumor cells and therapeutic efficiency. Recently, autophagic cell death was reported to be associated with ZnONP exposure (Hackenberg *et al.*, 2014; Johnson *et al.*, 2015; Yu *et al.*, 2013). However, in the present study, activation of autophagy caused by ZnONP20 and Al-ZnONP20 was only observed in A549 cells, but not in CL1-5 cells. LC3 was indicated to increase in expression of autophagy after exposure to metal-based nanoparticles, including As, Cd, Cr, Hg, and Fe (Chatterjee *et al.*, 2014). Together, we suggest that cell-specific autophagy was associated with exposure to ZnONP20 and Al-ZnONP20. Previous studies showed activation of LC3 expression in CL1-5 cells for drug screening (Li *et al.*, 2016b; Chen *et al.*, 2015), suggesting that autophagy functions well in CL1-5 cells. Based on our observations, we hypothesized that autophagy inhibition occurred due to ZnONP20 and Al-ZnONP20 in CL1-5 cells. The difference between cell death in A549 and CL1-5 cells may have resulted from the EGFR mutation status. In other words, the EGFR mutation in CL1-5 cells makes them sensitive to ZnONP20 and Al-ZnONP20 by reducing cell viability and inhibiting cell re-growth via a non-autophagic pathway. The results imply that ZnONP20 and Al-ZnONP20 may have the potential to treat NSCLC with an EGFR mutation. However, confirmation experiments are required to investigate the up- and downstream pathways in autophagy.

In conclusion, we showed that human lung adenocarcinoma cells with an EGFR mutation are sensitive to ZnONP20 and Al-ZnONP20, which may have resulted in non-autophagic cell death. On the other hand, lung adenocarcinoma cells with the wild-type EGFR were resistant to ZnONP20 and Al-ZnONP20, and may have been protected by an autophagy pathway. To investigate the role of autophagy in response to ZnONP20 and Al-ZnONP20, *in vivo* experiments should be conducted in further works. Our results demonstrate that ZnONP20 and Al-ZnONP20 may have the potential for personalized therapeutics in NSCLC with an EGFR mutation based on the safety dose in human. Further research on other types of lung cancer cells and *in vivo* experiments are required.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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