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Water-soluble germanium nanoparticles cause necrotic cell death and the damage can be attenuated by blocking the transduction of necrotic signaling pathway

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ABSTRACT

Water-soluble germanium nanoparticles (wsGeNPs) with allyamine-conjugated surfaces were fabricated and emit blue fluorescence under ultraviolet light. The wsGeNP was physically and chemically stable at various experimental conditions. Cytotoxicity of the fabricated wsGeNP was examined. MTT assay demonstrated that wsGeNP possessed high toxicity to cells and clonogenic survival assay further indicated that this effect was not resulted from retarding cell growth. Flow cytometric analysis indicated that wsGeNP did not alter the cell cycle profile but the sub-G1 fraction was absent from treated cells. Results from DNA fragmentation and propidium iodide exclusion assays also suggested that apoptotic cell death did not occur in cells treated with wsGeNP. Addition of a necrosis inhibitor, necrostatin-1, attenuated cell damage and indicated that wsGeNP caused necrotic cell death. Cell signaling leads to necrotic death was investigated. Intracellular calcium and reactive oxygen species (ROS) levels were increased upon wsGeNP treatment. These effects can be abrogated by BAPTA-AM and N-acetyl cysteine respectively, resulting in a reduction in cell damage. In addition, wsGeNP caused a decrease in mitochondrial membrane potential (MMP) which could be recovered by cyclosporine A. The cellular signaling events revealed that wsGeNP increase the cellular calcium level which enhances the production of ROS and leads to a reduction of MMP, consequentially results in necrotic cell death.

1. Introduction

Germanium (Ge) is a metalloid with semiconductor property and is dispensable for human health. Inorganic Ge compound, such as GeO_2, is generally non-toxic although renal and neural damages are reported after long-term, high-dose consumption. However, organic Ge compound, such as Ge-132, is regarded as an elixir in several countries since Ge-132 has been shown to inhibit cancer development (Kumano et al., 1985), induce erythropoietic efficiency (Dozono et al., 1996), exert antimicrobial activity (Aso et al., 1989) or modulate immunopotency (Fukazawa et al., 1994). We have shown that GeO_2 blocks cell cycle progression at G2 phase and causes radiosensitizing effect despite the chemical itself is very low in cytotoxicity (Chiu et al., 2002). High concentration of GeO_2 is required to generate the radiosensitizing effect. However, GeO_2 has a low solubility which hinders its biological application. In addition, GeO_2 cannot be delivered to a specific target to exert its radiosensitizing effect. Alternatively, nano-sized germanium particles may be utilized for this purpose.

Quantum dots (QDs) are generally defined as nanometer-sized crystals fabricated from materials with semiconductor properties. Due to their unique physical properties, QDs are currently utilized in various photoelectronic and biomedical researches. Materials with direct bandgap, such as CdSe, CdSe/ZnS, InP and PbSe, have photonic property under defined particle size (Alivisatos, 2004; Michalet et al., 2005). Semiconductor materials (Group IV) with indirect bandgap, i.e., Si(0) or Ge(0) are rarely characterized since they do not emit photons or fluoresce effectively at infrared region (Warner and Tilley, 2006; Zhou et al., 2003). However, germanium nanoparticles (GeNP) with sizes lower than the relatively large excitation Bohr radius (R_b = 11.5 nm) exert direct bandgap and produce radiative recombination. Hence GeNP fluoresces at the visible region. Due to quantum confinement and narrow size distribution, GeNPs were also defined as quantum dots(Kauzlarich et al., 2004; Warner and Tilley, 2006).

With a semiconductor property, Ge or Ge compounds are widely used in industries. It has been applied to fiber-optic systems, infrared optics, polymerization catalyst, various electronic devices and solar cells (Bailey et al., 2002; Rieke, 2007; Thiele, 2001;

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Washio, 2003). With the development of nanotechnology, nano-sized particles of Ge or Ge compounds have been fabricated. Owing to the distinct physical and optical properties, GeNP can potentially be used in a variety of fields (Chiu et al., 2006; Singh et al., 2005; Xie et al., 2009).

Fabrications of GeNP have been described using physical and chemical approaches. Physically, GeNP can be constructed with reactive laser ablation (Riabinina et al., 2006) or pulse laser with ion implantation (Ngiam et al., 1994). Chemically, several methods employing high temperatures and high reducing environment have been reported (Chiu and Kauzlarich, 2006; Fok et al., 2004; Lu et al., 2005). The drastic reaction conditions complicated the concoction procedures. Furthermore, it is difficult to control the size of the fabricated particles or modify the particle surfaces. The vapor condensation method (Warner and Tilley, 2006) for the manufacturing of GeNP is easy to follow and without the high temperature or reducing environment. Nonetheless, the prepared particles aggregated in aquatic phase and difficult to use in biological research (Lin et al., 2009). Recently, the method of synthesizing water-soluble GeNP (wsGeNP) has been reported and it potentially avoided the defects of previous methods (Lambert et al., 2007). GeNP produced by vapor condensation method has less toxicity to cells (Lin et al., 2009). The toxicity of GeNP fabricated through other methods has not been investigated.

With the synthesis of wsGeNP, the surface chemistry can be modified. The modifications may be useful for potential biological applications, such as cell type specific targeting. Since we have demonstrated that GeO_2 and GeNP are radiosensitizers in Chinese hamster ovary (CHO) K1 cells, we examined whether wsGeNP had the same property. We therefore prepared wsGeNP for cellular studies. The cytotoxicity of the wsGeNP was examined. Different from that of GeO_2 and GeNP, the wsGeNP damages cells at low concentration. The toxicological mechanism was studied and chemicals that attenuate the toxicity were signified.

2. Materials and methods

2.1. Cell culture and chemicals

CHO K1 cells were cultured as monolayers at 37 °C in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum, 0.22% sodium bicarbonate, 100 U/ml ampicillin and 100 μg/ml streptomycin, in 5% CO_2/95% air and 100% humidity. Reagents for cell culture were purchased from GIBCO (Invitrogen). BAPTA-AM [1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester] was obtained from BIOMOL. Necrostatin-1 [5-(indol-3-ylmethyl)-(2-thio-3-methyl)hydantoin] was obtained from Merck. Cyclosporin A and Fluo-3/AM (1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid pentaacetoxymethyl ester) were acquired from Kelowna. $DiOC_6$ [3,3′-dihexloxa-carbocyanine iodide] was a product of Calbiochem. MTT [3-(4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide)] was purchased from USB Crop. ATPLite 300 assay kit was a product of Blossom. Other chemicals were purchased from Sigma unless specified.

2.2. Fabrication of wsGeNP

GeNP was synthesized in reverse micelles by reducing the solution-phase $GeCl_4$ (Warner and Tilley, 2006). All reactions were processed under a nitrogen atmosphere to slow down the oxidation of germanium. The reverse micelles solution was prepared by stirring 100 μl of $GeCl_4$ (1.0 M, anhydride) and 1.5 g of tetraoctylammonium bromide (TOAB) in 100 ml of anhydrous toluene for 30 min. GeNP were formed by rapidly adding 2 ml of the reducing agent (1 M lithium triethylborohydride [$Li(C_2H_5)_3BH$] in tetrahydrofuran) to the reverse micelles solution, which turned from clear to a translucent yellow color. The solution was left to react for a further 2 h then quenched with 20 ml of anhydrous methanol. In order to add amino groups onto the GeNP surface, 40 μl of a platinum catalyst (0.05 M H_2PtCl_6 in isopropyl alcohol) and 10 ml of allyamine were mixed with the GeNP solution and stirred for 30 min. After surface capping, the mixture was removed from the nitrogen environment and dried in a rotary evaporator, leaving behind the TOAB (white powder) and the GeNP. The allyamine capped GeNP was solubilized with 50 ml of distilled water and the TOAB was removed by filtration through a 0.22 μm filter. The product was designated as wsGeNP.

2.3. Fourier transfer infra red (FTIR) spectrophotometric analysis

Allyamine or allyamine-coated GeNP was mixed with oven-dried FTIR grade KBr to a final concentration of 1% (w/w). The mixture was grounded to a fine powder then flattened into thin films under 10 tons in a hydraulic press for FTIR measurements. FTIR spectra were obtained with a Nicolet Avatar 320 FTIR spectrometer (Nicolet. Instrument Co., Madison, WI, USA). Thirty two scans were collected at a spectral resolution of 1 cm^{-1}.

2.4. MTT assay

The cytotoxicity of the synthesized wsGeNP was evaluated using MTT assay. Briefly, CHO K1 cells were seeded in 96-well plates at a density of 3.5×10^3 cells/well and treated with various concentrations of wsGeNP at 37 °C for 24 h. Cells were then incubated with MTT (final concentration 0.4 mg/ml) for 4 h before harvesting. Cells were washed with phosphate-buffered saline (PBS) and 200 μl DMSO was added to each well. The absorbance of formazan was recorded at 565 nm with a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Clonogenic survival assay

Two hundred and fifty cells were seeded in 60 mm dish before treating with various concentrations of wsGeNP for 24 h. The wsGeNP was removed and cells were cultured in fresh medium for 7 days, then fixed and stained with 1% (w/v) crystal violet dissolved in 30% ethanol. Colonies containing at least 50 cells were counted. The survival fraction of each treatment was determined by dividing the number of colonies in the treated sample by that of the non-treated control sample.

2.6. Cell cycle analysis

Cells were treated with various concentrations of wsGeNP for 24 h. Treated cells were removed by trypsinization and collected by centrifugation at 1500 rpm for 10 min. The cell pellets were resuspended in 70% ethanol and stored at 4 °C overnight. Cells were then centrifuged and resuspended in 1 ml PBS containing RNase A (100 μg/ml). After 30 min at room temperature, the cells were spun down and the pellets stained with 1 ml of propidium iodide (PI, 20 μg/ml in PBS) for 30 min. Flow cytometric analysis was then carried out on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. DNA fragmentation assay

For DNA ladder analysis, cells were treated with 5 μM wsGeNP or irradiated with 25 J/m^2 UV. After further incubating at 37 °C for 24 h, cells were washed twice with PBS and the low-molecular-weighted DNA fragments were extracted with TTE buffer (0.2% Triton X-100, 10 mM Tris, 15 mM EDTA, pH 7.6) for 15 min at room temperature. After centrifuging at 12,000 rpm for 15 min, the supernatants were transferred to new tubes before RNAase A (100 μg/ml) was added and incubated at 37 °C for 1 h. DNA was extracted with 1 volume of phenol/chloroform/isoamyl alcohol and precipitated in 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropyl alcohol. After standing at −70 °C for 15 min, the DNA was spun down at 12,000 rpm for 20 min, and washed with 70% alcohol. The DNA pellet was dissolved in TE buffer and analyzed electrophoretically on a 2% agarose gel.

2.8. Caspase-3 activity assay

Cells were lysed in 1% Triton X-100, 1% NP-40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM PMSF and incubated on ice for 10 min. After centrifugation at 13,000 rpm and 4 °C for 30 min, the supernatants were transferred to new tubes. Protein concentrations were determined using a protein assay kit (Bio-Rad). 50 μg of proteins were incubated at 37 °C in 100 μl reaction buffer (10 mM HEPES, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT) containing 50 μM Ac-DEVD-AFC for 1 h. The AFC fluorescence was measured at excitation wavelength of 405 nm and emission wavelength of 505 nm with a microplate reader (Wallac 1420 Multilabel Counter, Perkin Elmer).

2.9. PI exclusion assay

The integrity of plasma membrane was assessed by determining the ability of cells to exclude PI. Cells were trypsinized, collected by centrifugation, washed once with PBS then suspended in PBS containing 10 μg/ml PI. The cells were stood at room temperature in the dark for 15 min. The levels of PI incorporation were determined by flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The proportion of cells stained with PI was expressed as percentage of PI uptake.

2.10. Determination of intracellular Ca^{2+}, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP)

Intracellular Ca^{2+}, ROS and MMP were measured by Fluo-3-AM, H_2DCF-DA and $DiOC_6$, respectively. Cells were treated with various concentrations of wsGeNP in the