Oxidative stress and apoptosis induced by hydroxyapatite nanoparticles in C6 cells

Jing Xu, Pengjuan Xu, Zhigui Li, Jie Huang, Zhuo Yang

1Department of Physiology, College of Medicine, The Key Laboratory of Bioactive Materials, Ministry of Education, Nankai University, Tianjin 300071, China
2Department of Mechanical Engineering, University College of London, London WC1E 7JE, United Kingdom

Received 13 July 2011; revised 22 August 2011; accepted 12 September 2011
Published online 31 December 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.33270

Abstract: Hydroxyapatite (HA) nanoparticles have been reported to exhibit anti-tumor effects on various human cancers, but the effects of HA on glioma cells remain unclear. The aim of this study was to explore whether HA can inhibit the proliferation and induce the apoptosis of C6 cells. Use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that HA induced C6 cell death in a concentration-dependent and time-dependent manner. Results from Hoechst 33342 staining and flow cytometry assay showed that HA induced C6 cell apoptosis significantly. Meanwhile, the flow cytometric assay gave clear indication that HA induced intracellular accumulation of reactive oxygen species (ROS). The measurement of superoxide dismutase (SOD) generation showed that HA decreased the total SOD of cellular levels. Interestingly, pretreatment of N-(mercaptopropionyl)-glycine (N-MPG), known as a type of ROS scavenger formulations, could somehow inhibit C6 cell apoptosis induced by HA. These results may provide potential anti-glioma treatment in the future.

Key Words: hydroxyapatite, C6 cells, apoptosis, ROS, SOD


INTRODUCTION

Hydroxyapatite [HA, Ca_{10}(PO_{4})_{6}-(OH)_{2}], owing to its excellent biocompatibility and bioactivity, has been widely used as reconstructive and prosthetic material for the repair and regeneration of osseous tissue. It is suggested that HA particles would accumulate at prosthetic interface and influence the cell behaviors such as morphology, proliferation, and cytokines production. HA nanoparticles are chosen as the particulate carrier due to its excellent biocompatibility and ease of labeling with lanthanides, especially in the therapy of malignant tumors. Luo et al. have showed that HA nanoparticles, more than simple drug carriers, may play an active role in enhancing the docetaxel-induced apoptosis of prostate cancer cells. HA nanoparticles can be used as vectors in the in vivo delivery of plasmid-based siRNAs into cancers to inhibit cancer growth. In addition, many studies have showed that HA nanoparticles exert direct anti-tumor activities both in vivo and in vitro, which have been shown to inhibit the proliferation of various tumors, such as hepatoma, gastric cancer, and osteosarcoma.

Malignant gliomas remain essentially lethal cancers despite maximal therapy because of resistance to all conventional therapies. Gliomas commonly develop mechanisms through which they resist cell death either by disruption of apoptotic processes or by activation of survival signals. Therefore, effective treatment of malignant gliomas has been targeted to inhibit tumor cell proliferation and induce tumor cell apoptosis. Although many studies have showed the anti-tumor ability of HA nanoparticles among various tumor cells, there is little information about the effects of HA on glioma cells. As we known this is the first study that assesses the toxicity of HA on gliomas in vitro.

HA nanoparticles have potency on changing mitochondrial membrane potential and triggering apoptosis in mitochondria pathways in gastric cancer. Reactive oxygen species (ROS), which are produced primarily at the level of the mitochondrial respiratory chain, have been implicated in the regulation of apoptosis. These years diverse chemotherapeutic agents have been reported to induce apoptosis in cancer cell lines through increasing ROS generation, including glioma cells. In the present study, the role of ROS in HA-treated glioma cells was investigated.

Originally generated from rats exposed to N-nitrosomethylurea, the rat C6 glioma cell line shares a number of similar characteristics with human glioblastoma. So it is widely used as the model of malignant glioma in vitro. The purpose of this study was to assess whether HA can inhibit the proliferation and induce the apoptosis of C6 cells, thereby providing an interesting view of the potential application of HA in future clinical application as an anti-cancer material.
MATERIALS AND METHODS

Materials

Dulbecco’s modified eagle media (DMEM) cell culture medium was purchased from GIBCO Invitrogen. The fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-(2-mercaptobenzonitrile)-glycine (N-MPG), and hoechst 33342 dye were purchased from Sigma Chemical Co., St Louis, MO. The ROS testing kit was purchased from Genmed Sciences Inc., USA. The Annexin V-fluorescein isothiocyanate (FITC) propidium iodide (PI) apoptosis detection kit was from Bipec Biopharma Corporation, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY). The superoxide dismutase (SOD) assay kit was purchased from the Nanking Jiancheng bio-engineering research institute (Nanking, China).

Dispersion and characterization of the HA particles

HA with a Ca/P ratio of 1.67 was synthesized by a precipitation reaction between calcium hydroxide (Ca(OH)₂) and orthophosphoric acid (H₃PO₄). H₃PO₄ solution (0.3M) was added drop-wise to 0.5M Ca(OH)₂ solution under continuous stirring at room temperature, while the PH was kept above 10.5 by the addition of ammonia solution. Stirring was maintained for a further 16 h after completion of the precipitate addition. The precipitate obtained was aged for a week. The sizes of particles were tested using a JEOL 200CX transmission electron microscopy (TEM) with an accelerating voltage of 200 keV.

Cell culture and preparation of HA

The rat C6 glioma cell line was obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. The cells were cultured with the DMEM medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. Cultures were propagated at 37°C in a humidified atmosphere of 5% CO₂.

The suspension of HA particles was prepared using DMEM media and dispersed for 10 min by using an ultrasonic agitation (Branson Inc., Danbury, CT) to prevent aggregation. Then different concentrations of HA were prepared from the suspension.

Cell viability assay

Cytotoxicity of HA was evaluated by using the MTT assay, which was based on the reduction of the dye MTT to formazan crystals, an insoluble intracellular blue product, by cellular dehydrogenases. Briefly, cells were treated at 1 × 10⁶ cells/mL with final concentrations of 10, 100, 250, and 500 μg/mL HA. After 24, 36, and 48 h incubation, 20 μL MTT was added to each well and further incubated for 4 h at 37°C. The medium was then removed carefully and 150 μL DMSO was added in and mixed with the cells thoroughly until formazan crystals were dissolved completely. This mixture was measured in an ELISA reader (Elx 800, Bio-TEK, USA) with a wave length of 570 nm. The concentrations of the HA used in assays of apoptosis, ROS, and SOD were based on the results of the MTT test. The cell viability was expressed as a percentage of the viability of the control culture.

Cell viability(%) = (the viability of HA – treated group/ the control group) × 100.

Nuclear staining analysis by hoechst 33342

To further address the death pattern, HA-treated C6 cells were stained with hoechst 33342 dye. The dye Hoechst 33342 dye is sensitive to chromatin and is used to assess the changes in the nuclear morphology. Briefly, the cultured cells were grown on the cover slip of a 35-mm chamber. After treated with HA for 48 h, the C6 cells were washed with phosphate buffer saline (PBS), and stained with hoechst 33342 dye (5 μg/mL) for 20 min at the room temperature in the dark. After washing twice more with PBS, the hoechst-stained nuclei were visualized by using the laser scanning confocal microscope (FV1000S-IX81; Olympus, Tokyo, Japan). All quantitative analyses of the fluorescence images were performed with the FV1000 Viewer (Ver.1.6) software (Olympus). Ten random frames were imaged using confocal microscopy for each concentration, and the number of total cells and apoptotic cells were obtained by counting. The percentage of apoptotic cells was calculated as follows:

%apoptotic cells = (the number of apoptotic cells/ the number of all cells) × 100

Detection of apoptotic cells with flow cytometry

Apoptosis was assessed by annexin V-FITC and PI staining followed by analysis with flow cytometry (Beckman-Coulter, USA). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit. The cultured cells were exposed to HA with the concentrations of 10, 100, 250, and 500 μg/mL. Eventually, the cells were resuspended in a 400 μL 1 × binding buffer solution with a concentration of 1 × 10⁶ cells/mL, and the cells were stained with 5 μL annexin V-FITC and 5 μL PI for 15 min at room temperature in the dark. Then the cell suspension was ready for the analysis by the flow cytometry.

Measurement of reactive oxygen species

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA). The cells were incubated in a 6-well plate for 24 h for stabilization, then the medium was replaced with medium containing different concentrations (10, 100, 250, and 500 μg/mL) of HA for 12 h. After exposure, the cells were washed with phosphate-buffered saline (PBS), then they were resuspended at a concentration of 1 × 10⁶ cells/mL, and were stained by the staining solution for 20 min. The cells were detected and analyzed by flow cytometry.

Total SOD assay

The generation of total SOD (T-SOD) for the cells was evaluated by assay kit. The C6 cells were incubated in a 6-well plate for 24 h for stabilization, then the medium was replaced with the medium containing the different
concentrations (0, 10, 100, 250, and 500 μg/mL) of HA for 48 h. The levels of T-SOD were measured using a UV–visible spectrophotometer (V-530UV/UlSINIR Spectrophotometer, Jasco, Japan) at 550 nm according to the manufacturer’s instructions. The percentage of T-SOD activity was obtained as follows:

\[
\% T - \text{SOD activity} = \left( \frac{\text{the activity of HA - treated group}}{\text{the control group}} \right) \times 100
\]

The effect of N-(2-mercaptopyrrolyl)-glycine

To assess the effect of ROS on cell apoptosis, cells were pre-treated with N-MPG (300 μmol/L),26 known as a kind of ROS scavenger.27 Then the cells were exposed to 250 μg/mL HA, the cell apoptosis was measured by hoechst 33342 staining and flow cytometry.

Statistical analysis

The results were expressed as mean ± SEM. The statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison post test using the SPSS (11.5) software. The significant difference was taken as \( p < 0.05 \).

RESULTS

Characterization of HA

The sizes of HA particles were distributed from 50 to 80 nm as shown in the image of TEM (Fig. 1).

HA inhibited the viability of C6 cells

The viability of C6 cells was determined by MTT assay. C6 cells were treated with medium containing different concentrations (0, 10, 100, 250, and 500 μg/mL) of HA, and viability was determined at 24, 36, and 48 h after treatment. The viability in control group was defined as 100%, while the cell viability in HA-treated group was expressed as a percentage of the viability of the control group. As shown in Figure 2(A), the cell viability was decreased when the concentration and time period increased. After 24-h incubation, the cell viability was decreased. There were no significant differences in the viability of the cells incubated with the concentrations (10 and 100 μg/mL) of HA, but significant changes were showed in the concentrations of 250 and 500 μg/mL. The viability of C6 cells was also significantly inhibited by HA of 10 and 100 μg/mL with time increasing. When cells were exposed to 500 μg/mL HA for 48 h, the cell viability decreased greatly to (49.10 ± 5.19)% compared with that of control group. ANOVA analysis and Dunnett’s test revealed that C6 cells were inhibited both in a concentration-dependent and time-dependent manner by HA. As shown in Figure 2(B), the viability of C6 cells

![Figure 2(A)](image)

**FIGURE 2.** (A) Effects of HA nanoparticles on C6 cell viability determined by MTT assay. C6 cells were treated with different concentrations of HA (0, 10, 100, 250, and 500 μg/mL) for 24, 36, and 48 h. (B) Effect of N-MPG on the viability of C6 cells incubated with the HA nanoparticles. Cells were pre-treated with N-MPG for 30 min, then the HA was added to the final concentration of 250 μg/mL and followed by incubation for 48 h. Cells cultured without HA served as the control. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. (Statistics: one-way ANOVA with Dunnett’s post hoc test. * \( p < 0.05 \) compared with the control group, ** \( p < 0.01 \) compared with the control group, *** \( p < 0.01 \) compared with 250 μg/mL HA group).
FIGURE 3. Hoechst 33342 staining of C6 cells cultured in HA nanoparticles for 48 h. Microscope images (400×): (A) control, (B) 10 µg/mL, (C) 100 µg/mL, (D) 250 µg/mL, (E) 250 µg/mL + N-MPG, (F) 500 µg/mL, (G) percentage of apoptotic cells treated with HA. The white arrows show typical form of half-moon in apoptosis cells indicating chromatin condensation. *p < 0.05 compared with the control group, **p < 0.01 compared with the control group, ###p < 0.01 compared with 250 µg/mL HA group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
cultured with the HA (250 μg/mL) for 48 h was 51.94%. When pre-treated with ROS scavenger N-MPG, the viability of C6 cells was significantly increased to 85.14%. Then the treatment for 48 h was chosen for subsequent experiments on HA-induced cell death.

**FIGURE 4.** HA nanoparticles induced apoptotic cell death in C6 cells. The flow cytometry assay was carried out for detection of apoptotic cells cultured in HA for 48 h. A: Control, (B) 10 μg/mL, (C) 100 μg/mL, (D) 250 μg/mL, (E) 250 μg/mL + N-MPG, (F) 500 μg/mL, (G) the corresponding linear diagram of flow cytometry was shown. *p < 0.05 compared with the control group, **p < 0.01 compared with the control group, ***p < 0.01 compared with 250 μg/mL HA group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Morphology of apoptosis by hoechst 33342 staining**
The hoechst dye was able to diffuse through intact membranes of C6 cells and stain DNA. As shown in Figure 3, the nuclei exhibited dispersed and weak fluorescence in normal cells [Fig. 3(A)]. In contrast, cells treated with different
concentrations of HA showed marked condensed chromatin, some of which were assembled at the nuclear membrane, and showed typical form of half-moon [as white arrow pointed in Fig. 3(B–F)]. Figure 3(G) indicated that HA induced C6 cell apoptosis in a concentration-dependent manner. In addition, the percentage of apoptotic cells cultured with 250 μg/mL HA was 43.08%. When pre-treated with ROS scavenger N-MPG, the percentage of apoptotic cells was significantly decreased to 19.96%.

HA induced C6 cell apoptosis assessed by flow cytometry
The apoptosis of C6 cells was tested by flow cytometry, as shown in Figure 4. The apoptotic rate of C6 cells was 2.85% in control group. After incubated with different concentrations of HA (10, 100, 250, and 500 μg/mL) for 48 h, the rate of apoptosis increased to 19.25%, 31.47%, 41.28%, and 45.73%. However, a pre-treatment to cells using N-MPG reduced the cellular apoptosis from 41.28% to 21.23%. The results indicated that HA induced apoptosis of C6 cells in a concentration-dependent manner, but pre-treated with ROS scavenger N-MPG decreased the apoptotic rate of C6 cells.

Measurement of ROS generation
After the cells were treated with HA of different concentrations (10, 100, 250, and 500 μg/mL), the generation of ROS was elevated. As shown in Figure 5, the ratios of DCF-positive cells were 3.21%, 24.45%, 30.49%, 53.16%, and 74.46% at the concentrations of 0, 10, 100, 250, and 500 μg/mL, respectively.

T-SOD assay
The cellular level of T-SOD was significantly decreased when the cells were cultured in media containing different concentrations of HA for 48 h (Fig. 6). In contrast to the control group, different concentrations of HA (10, 100, 250, and 500 μg/mL) treated cells showed 4.40%, 12.43%, 19.71%, and 23.57% decrease in the T-SOD level, respectively.
exploring new medicine that could induce tumor cell apoptosis. Human cancer cells as the carriers for the controlled delivery of growth factors and drugs.37 Previous works have shown that HA nanoparticles could inhibit the proliferation of such cancer cells as liver, colon, stomach, and bone cancer cells.11,38 However, there were no related articles concerning the effects of HA on glioma cell proliferation. In this study, the viability of C6 cells incubated with different concentrations of HA was investigated. It was found that HA inhibited the proliferation of C6 cells in a concentration-dependent and time-dependent manner (Fig. 2).

Induction of apoptosis may account for the anti-proliferation effect. The detection of the cell apoptosis by flow cytometry showed that HA induced the apoptosis of C6 cells (Fig. 4). Meanwhile, characteristic apoptotic events such as morphological changes, including nuclear condensation and fragmentation, cell shrinkage, DNA fragmentation, which is a hallmark of cells undergoing apoptosis,39 were observed in HA-treated C6 cells (Fig. 3). Taken together, these results indicated that HA induced C6 cell apoptosis, and supported the concept that HA exerted the anti-proliferation effects by inducing apoptosis in C6 cells.

Programmed cell death is a highly conserved mechanism for removal of undesirable cells in vertebrate, and ROS plays a regulatory role in its signaling pathway. Human cancer cells with intrinsic oxidative stress are highly sensitive to ROS stress,40 and promoting ROS generation can effectively kill them.41 Studies have shown that diverse chemotherapeutic agents can induce apoptosis in glioma cell lines by elevating oxidative stress.19,21 In human gastric cancer cells, HA treatment resulted in an increased expression of Bax and decreased expression of Bcl-2 gradually in SGC-7901 cells.11 Shi et al. have showed that caspase-9 related intrinsic pathway might contribute to the HA-induced osteosarcoma cell apoptosis.32 ROS is crucial in cytochrome c-independent caspase-9 activation during apoptosis.15,43 As far as we know, there is no study on the role of ROS in HA-induced cell apoptosis. Therefore, the changes of ROS level were determined using ROS-sensitive DCFH-DA dyes (Fig. 5). It was found that after treated with different concentrations of HA, the contents of the ROS were increased significantly (p < 0.05, p < 0.01). Moreover, ROS scavenger N-MPG was used for further study. MTT results showed that when pre-treated with N-MPG, the cell viability increased from 51.94% to 85.14% in 250 μg/mL HA-treated group. These results were coincident with the following apoptotic tests. The detection of the cell apoptosis by hoechst 33342 staining and flow cytometry both showed that N-MPG could reduce the cell damage by HA (Fig. 3, p < 0.01; Fig. 4, p < 0.01). These results demonstrated that HA might induce the apoptosis of C6 cells by enhancement of intracellular ROS generation, while ROS scavenger N-MPG could improve the cellular viability and reduce the damage caused by HA.

Oxidative stress, where there is an imbalance between ROS and the cell’s antioxidant capacity,34 is one of the classical stress response mechanisms that modulate apoptosis and a critical path of therapeutic-agent induced stress in cancer cells.45 Oxygen species are regulated by SOD, an enzyme that changes superoxide into H2O2, or by catalase and glutathione peroxidase (GPx), which decompose H2O2 into H2O. Figure 5 showed that HA increased ROS level in C6 cells. Moreover, the increase of oxidative stress usually accompanied with the reduction of T-SOD activity (Fig. 6).

In conclusion, the present study indicated that HA was able to inhibit C6 cell growth and induce apoptosis accompanied with the elevated ROS level and decreased SOD level, which may add a novel targeted therapy for tumor treatment in the future.

REFERENCES


