Mitochondria-Dependent Apoptosis Induced by Nanoscale Hydroxyapatite in Human Gastric Cancer SGC-7901 Cells

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Nanoscale hydroxyapatite (nano-HAP) has been reported to exhibit anti-cancer effect on several human cancers, but the molecular mechanism of which remains unclear. The aim of this study was to explore the mechanisms by investigating the effects of nano-HAP on human gastric cancer SGC-7901 cells. Our results showed that nano-HAP significantly reduced cell viability, and induced apoptosis in SGC-7901 cells characterized by hypodiploid DNA contents, morphological changes and DNA fragmentation. The increase in apoptosis was accompanied with the increased expression of Bax, a pro-apoptotic protein, and decreased expression of Bel-2, an anti-apoptotic protein, the decrease of mitochondrial membrane potential and the release of cytochrome c from mitochondria into cytosol. Furthermore, the activation of caspases-3, and -9, but not activation of caspases-8 was induced by nano-HAP. Z-VAD-fmk, a universal caspase inhibitor, dose-dependently inhibited nano-HAP-induced apoptosis. This study demonstrates that nano-HAP inhibits the proliferation of SGC-7901 cells by inducing apoptosis, and the apoptotic pathway of nano-HAP-induced apoptosis is mediated through the mitochondrial-dependent and caspase-dependent pathway.

Key words nanoscale hydroxyapatite; mitochondrial membrane potential; apoptosis; caspase; cytochrome c; gastric cancer

Gastric cancer is one of the commonest malignancies worldwide and is the leading cause of cancer mortality in China.13 Despite advances in early diagnosis and treatment modalities, the side effects of chemotherapy and recurrence are still the problems. Therefore, there is a great need for identification of new and effective anti-cancer agents for reducing the mortality caused by gastric cancer.

Hydroxyapatite [HAP; Ca10(PO4)6(OH)2], one of major inorganic components of mammalian bones, has been used extensively and successfully as bone defect filling material, as well as medicine bearer and coating material.2–4 In recent years, nanoscale hydroxyapatite, a novel biomedical material, which diameter is less than 100 nm, has been reported that not only has better biocompatibility than HAP,5,6 but also anti-cancer activity. It has been shown to inhibit the proliferation of various tumors, such as hepatoma, colon cancer, and osteosarcoma.6–8 Moreover, the antiproliferation effect of nano-HAP has been shown to be due to induction of apoptosis.7–9 However, the actual molecular mechanism of nano-HAP-induced apoptosis remains unclear.

Apoptosis plays an important role in maintenance of homeostasis and eliminating damaged cells.10 It is well known that many chemotherapeutic agents take effect by inducing cancer cells apoptosis.11–13 Apoptosis may be initiated through the stimulation of death receptors located on the cell surface or through an intrinsic pathway involving the release of apoptotic signals from mitochondria.14,15 Both signals converge on a cascade of cysteine proteinases known as caspases, which are central to the initiation and execution of apoptosis. The stimulation of the death receptor pathway leads to the activation of caspase-8 following the recruitment of the pro-caspase to the death-inducing signaling complex.15 In contrast, the mitochondrial pathway requires the release of mitochondrial cytochrome c and the formation of a large multiprotein complex comprising cytochrome c, Apaf-1, and pro-caspase-9. Caspases-8 and-9 will then proteolytically activate downstream caspases, in particular caspases 3 and 7, which are responsible for the apoptotic destruction of the cell.10 Caspase-3, in particular, is an essential player in the DNA fragmentation process and other morphological changes associated with apoptosis.16,17 However, to the best of our knowledge, the apoptotic pathway involved in nano-HAP-induced apoptosis, and the effects of nano-HAP on human gastric cancer has not been investigated. Therefore, the objective of this study is to explore the apoptotic pathway involved in nano-HAP-induced apoptosis by investigating the effects of nano-HAP on cell proliferation and apoptosis in human gastric cancer SGC-7901 cells.

MATERIALS AND METHODS

Reagents RPMI-1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS) were purchased from Gibco, U.S.A. Propidium iodide (PI), ethidium bromide (EB), and phosphate buffered saline (PBS) were purchased from Sigma Co., U.S.A. DNA isolation kit, protein assay kit and caspase activity assay kits, including the fluorogenic substrates of caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC) and caspase-9 (LEHD-AFC), were purchased from R&D Systems, U.S.A. Benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was purchased from Enzyme System Products (Dublin, CA, U.S.A.). Western blot chemiluminescent detection system and the primary antibodies to cytochrome c, Bax, Bel-2 and β-actin were purchased from Santa Cruz, U.S.A. 3,3′-dihexyloxycarbocyanine (DiOC6) was purchased from Molecular Probe (Eugene, Oregon, U.S.A.). All other reagents are analytical or cultured grade purity.

Cell Culture and Preparation of Nano-HAP Human gastric cancer SGC-7901 cells were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were grown in monolayer culture in

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RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37 °C. For control, cells were incubated in nano-HAP-free medium. Nano-HAP (purity ≥99.9%, Φ=50 nm) was a kindly gift from the Institute of Biomaterials, East China University of Science and Technology and suspended steadily in RPMI 1640 with the final concentration of 400 μg/ml. The stock solution was diluted to the required concentration just before using.

**Cell Viability Assay** Cell viability was assessed with MTT assay. The exponentially growing cells (1×10⁵/well) were seeded into 96-well culture plates and incubated with various concentrations of nano-HAP (25, 50, 100, 150, 200 μg/ml) for 0, 24, 48, and 72 h. Four hours before termination, the supernatants were substituted with 90 μl fresh medium and 10 μl of MTT (1 mg/ml) solution. After incubated 4 h at 37 °C, the medium was aspirated and the formazan crystals were solubilized in acidified isopropanol. The reduction of absorbance was measured spectrophotometrically at 570 nm, and the cell viability was expressed as percentage over the untreated control.

**Flow Cytometry Assay** SGC-7901 cells were treated with 100 μg/ml nano-HAP for 0, 12, 24, and 48 h. Approximately 10⁶ cells per sample were harvested, washed with PBS and fixed in ice-cold 70% of ethanol at -20 °C before use. After resuspension, cells were washed and incubated with 100 μl PI (400 μg/ml) and 100 μl RNase I (1mg/ml) at 37 °C for 30 min. Cells were analyzed with flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) and the data were consequently evaluated by Cell Quest and Mod-Fit (Verity Software, Topsham, ME, U.S.A.).

**Transmission Electron Microscopy (TEM)** Untreated control and nano-HAP-treated (100 μg/ml, 48 h) SGC-7901 cells were harvested and washed with PBS. Cell pellets were fixed in 3% glutaraldehyde for 2 h at 4 °C and then incubated with 1% osmium tetroxide for another 1 h. After dehydration in series concentrations of ethanol and infiltration in propylene oxide, cells were embedded in Epon 812. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate, and then cell morphology was observed and photographed by TEM (Philips Tecnai 10).

**DNA Fragmentation Assay** SGC-7901 cells were treated with 100 μg/ml nano-HAP for 48 h. DNA of 3×10⁶ cells was extracted by the use of apoptotic DNA ladder kits according to the manufacturer’s instructions. Approximately 20 μg DNA was electrophoresed on 2% agarose gel at 1V/cm for 20 min. The DNA was visualized under UV light and photographed.

**Caspase-3, -8 and -9 Activity Assays** After treated with 100 μg/ml nano-HAP for 0, 12, 24, and 48 h, approximate 10⁵ SGC-7901 cells per sample were harvested, washed with PBS and lysed in radioimmunoprecipitation buffer (10 mM Phenylmethyl sulfonylfluoride, 1 μg/ml aprotinin, 100 mM EGTA, 100 mM sodium orthovanadate and 100 mM DTT, Sigma Co., U.S.A.). The supernatant was removed after centrifugation at 12000×g for 20 min. Activities of caspases-3, -8 and -9 were detected by measuring the proteolytic cleavage of the fluorogenic substrates, LEHD-AFC, IETD-AFC and DEVD-AFC, respectively. Fifty μg of each sample was incubated with caspase assay buffer and the appropriate substrate at 37 °C for 45 min. The caspase activity was measured using a spectrofluorimeter (LS-50B, Perkin Elmer, U.S.A.) set with an excitation at 400 nm and emission at 505 nm according to the manufacturer’s protocol.

**Caspase Inhibition Assay** SGC-7901 cells were treated with 100 μg/ml nano-HAP and Z-VAD-fmk (0, 25, 50, 70, 100, 125 μM). After 48 h, apoptotic cells were determined by Flow cytometry assay described above.

**Mitochondrial Membrane Potential** Changes of mitochondrial membrane potential (ΔΨm) have been considered to be an indicator of mitochondrial damage. Loss of mitochondrial transmembrane potential was monitored by flow cytometry. SGC-7901 cells were treated with 100 μg/ml nano-HAP for 0, 12, 24 and 48 h, and then incubated with DiOC6 (40 nm in culture medium) for 30 min prior to harvesting. The samples (10⁶ cells) were washed with PBS, and cellular uptake of DiOC6 was analyzed by flow cytometry. Histograms were analyzed using Cell Quest software, and compared with histograms of untreated control cells.

**Western Blot Analysis of Bcl-2, Bax and Cytochrome c** To further evaluate the mechanism of nano-HAP-induced apoptosis in gastric cancer cell, we examined the changes in protein expression of apoptosis-related genes, including cytochrome c, Bax, and Bel-2 by Western blot analysis using antibodies directed against the respective proteins. After treated with 100 μg/ml nano-HAP for 12, 24, and 48 h, approximately 10⁵ SGC-7901 cells per sample were harvested, washed twice with ice-cold PBS and re-suspended in ice-cold buffer A (250 mM sucrose, 20 mM HEPS, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 17 μg/ml Phenylmethyl sulfonylfluoride, 1 mM EGTA, 8 μg/ml aprotinin, and 2 μg/ml leupeptin, Sigma Co., U.S.A.). The re-suspended cells were homogenized with 10 strokes of Teflon homogenizer (BELLCO Biotechnology, Vineland, NJ, U.S.A.). The homogenates were centrifuged at 750×g for 10 min at 4 °C. The supernatants were centrifuged at 10000×g for 15 min at 4 °C to obtain the mitochondrial pellets. Cytosolic fractions were obtained after further centrifugation at 100000×g for 1 h at 4 °C. The total cellular protein was extracted from 10⁶ cells as previously described. Protein contents were quantified using the BCA protein assay kit and stored at −70 °C, respectively. Protein (25 μg per lane) was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (PVDF, Millipore, Bedford, MA, U.S.A.). The transblotted membrane was washed, blocked and incubated at 4 °C overnight with anti-Bcl-2 antibody, anti-Bax antibody, and anti-cytochrome c antibody, respectively. Immunodetection with the secondary peroxides-conjugated antibody and chemiluminescence were performed according to the manufacturer’s protocol. Equal protein loading was verified by probing with anti-β actin antibody.

**Data Analysis and Statistics** Results were expressed as means±standard deviation (S.D.). Statistical differences were evaluated using the two-tailed Student’s t-test and considered significant at the *p<0.05, **p<0.01 level.

**RESULTS**

Nano-HAP Inhibits the Proliferation of Human Gastric Cancer SGC-7901 Cells We first tested the antiprolifera-
tion effect of nano-HAP on human gastric cancer SGC-7901 cells. As shown in Fig. 1, the antiproliferation effect of nano-HAP was observed to be dose- and time-dependent manner.

**Nano-HAP Induces Apoptosis of SGC-7901 Cells**

As shown in Fig. 2A, in nano-HAP-treated cells, increased sub G1 hypodiploid cell population can be detected in a time-dependent manner. There was significant difference between nano-HAP-treated groups and untreated control in apoptotic DNA content ($p<0.01$). Under transmission electron microscope, characteristic changes of apoptosis: chromatin condensation and margination, cell blebbing and vacuoles could be observed in nano-HAP-treated cells (Fig. 2B-II) but none in control cells (Fig. 2B-I). The induction of apoptosis by nano-HAP was confirmed by electrophoresis of fragmented DNA. DNA extracts from SGC-7901 cells treated with nano-HAP displayed ladder patterns of discontinuous DNA fragments (Fig. 3) but no DNA fragments were detected in control group. All these results support the notion that nano-HAP induces apoptosis in SGC-7901 cells.

**Nano-HAP Activates Caspases-3 and -9, But Not -8**

Caspases, a family of cysteine proteases, are activated during the execution phase of the apoptotic process. Once activated, caspases cleave and activate downstream caspases, leading to apoptosis.19,20) Figure 4A showed a time-dependent activation of caspase-9 and caspase-3 in SGC-7901 cells treated with nano-HAP. However, caspase-8 activity did not change significantly. To determine whether that apoptosis induced by nano-HAP occurs via the caspase-dependent pathway, z-VAD-fmk, a universal caspase inhibitor was added with nano-HAP. As shown in Fig. 4B, z-VAD-fmk dose-dependently reduced the population of apoptotic cells induced by nano-HAP.

**Nano-HAP-Induced Apoptosis Is Associated with the Down-Regulation of Bcl-2, the Up-Regulation of Bax, the Decrease of Mitochondrial Membrane Potential and the Release of Cytochrome c from Mitochondria into Cytosol**

The decreasing of mitochondrial membrane potential ($\Delta \Psi_m$) causes the disruption of the outer mitochondrial membrane and contributes to the release of cytochrome c; therefore, the cytochrome c release results in the activation of caspase-9 and subsequently leads to apoptosis.19,21) As illustrated in Fig. 5A, exposure to nano-HAP induced a left shift and shrink of the DiOC6 fluorescence curves. This is indicative that the nano-HAP treatment induced a disruption of $\Delta \Psi_m$. Release of cytochrome c from the inter membrane spaces of mitochondria is important for the induction of apoptosis.21,22)
the mitochondria into the cytosol is a key event in apoptosis via the mitochondria-mediated pathway. As shown in Fig. 5C, cells exposed to nano-HAP had a significantly increased cytosolic fraction of cytochrome c with a loss of mitochondrial fraction of cytochrome c in a time-dependent manner.

Since the mitochondrial apoptosis is regulated by members of the Bcl-2 family, the signaling pathway upstream of the mitochondria was examined. During the process of apoptosis induced by nano-HAP, the expression of anti-apoptotic Bcl-2 was downregulated and the expression of pro-apoptotic protein Bax was upregulated in a time-dependent manner (Fig. 5B).

**DISCUSSION**

Previous works have shown that nano-HAP, which is a result of the combination of nanometer technology and modern medicine, could inhibit the proliferation of such cancer cells as liver, colon, and bone cancer cells. However, the effects of nano-HAP on gastric cancer cells proliferation and apoptosis haven’t been reported. In our work, we found that nano-HAP inhibited the proliferation of SGC-7901 cells in a dose- and time-dependent manner, consistent with other results. Induction of apoptosis maybe account for the antiproliferation effect. By flow cytometric analysis, we observed that sub G1 hypodiploid cell population increased in a time-dependent manner in nano-HAP treatment cells. Meanwhile, characteristic apoptotic events such as morphological changes, including chromatin condensation, cell shrinkage, nuclear fragmentation, and blebbing, and DNA fragmentation, which is a hallmark of cells undergoing apoptosis, were detected in

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**Fig. 4. Nano-HAP-Induced Cell Apoptosis Is Dependent of Caspase Activation**

(A) SGC-7901 cells were treated with 100 μg/ml nano-HAP for 0, 12, 24, and 48 h. The activity of caspase-3, -8 and -9, was estimated. The experiment was repeated three times with similar results and the caspase activity was expressed as fold activation over the untreated control. The mean fold activation was significantly higher than the control sample. (B) Z-VAD-fmk, a universal caspase inhibitor, dose dependently reduced the apoptosis induced by nano-HAP, and blocked the apoptosis at dose of 125 μg/ml (p<0.05). Data are presented as mean±S.D. of at least five replicates from three separate experiments.

**Fig. 5. Nano-HAP Induced ∆Ψm Alteration, Bcl-2 Down-Regulation, Bax Up-Regulation and Cytochrome c Release**

(A) Treatment of SGC-7901 cells with nano-HAP resulted in a decrease of mitochondrial membrane potential. SGC-7901 cells were treated with 100 μg/ml nano-HAP for 12, 24, and 48 h, and the relative ∆Ψm was measured by fluorescent emission. The MC represented the profile of control cells, and the ML indicated the treated cells. The numbers represented the mean of the relative fluorescent intensity. (B) Nano-HAP induced the down-regulation of Bcl-2 and Bax up-regulation. (C) Nano-HAP induced the release of cytochrome c from mitochondria into cytosol. Cells were treated with 100 μg/ml nano-HAP for 12, 24, and 48 h. Subsequently, cytosolic and mitochondrial fraction were isolated, and analyzed by Western blot as described in Methods. β-Actin was used as an internal control. The figure was obtained from at least three independent experiments with similar results.
nano-HAP treated SGC-7901 cells. Taken together, these results indicated that nano-HAP induced apoptosis in SGC-7901 cells, and supported the notion that nano-HAP exerted the antiproliferation effect by inducing apoptosis in cancer cells.

Although nano-HAP had been reported to induce apoptosis in other cancer cells, the molecular mechanism of the induction remains unclear. Then, we focused on it by investigating the apoptosis in SGC-7901 cells. Caspases, a family of aspartate-specific cysteine proteases, plays a pivotal role in the execution of apoptosis.20,24) There are at least two major apoptotic pathways such as the extrinsic pathways (death receptors) and intrinsic pathways (mitochondria), which are initiated by caspase-8 and caspase-9 respectively.19,20) It is feasible to differentiate them by evaluating the activities of caspase-8 and caspase-9. In this study, Caspase-9, the apical caspase in mitochondria-mediated apoptotic pathway, and caspase-3, but not caspase-8 was activated during the process of apoptosis induced by nano-HAP in SGC-7901 cells. Meanwhile, z-VAD-fmk, a universal caspase inhibitor significantly reduced the apoptosis induced by nano-HAP in a dose-dependent manner and blocked the apoptosis at dose of 125 μg/ml. These results indicated for the first time in human gastric cancer cells that nano-HAP-induced apoptosis was caspase-dependent, and suggested that mitochondria involved in the apoptosis induced by nano-HAP. It has been reported that Bcl-2, anti-apoptotic protein, and Bax, pro-apoptotic protein, resided in the mitochondrial outer membrane and involved in mitochondria-dependent apoptosis pathways by regulating mitochondrial membrane permeability.25,26) The proapoptotic proteins Bax induces cell apoptosis via mitochondrial membrane permeabilization (MMP) that leads to the release of cytochrome c.27) In this study, nano-HAP treatment resulted in increased expression of Bax and decreased expression of Bcl-2 gradually, suggesting that Bax and Bcl-2 play a role via mitochondria pathways in nano-HAP-induced apoptosis in SGC-7901 cells. At the same time, we found that the mitochondrial membrane potential of SGC-7901 cells decreased after nano-HAP treatment, and the cytosolic cytochrome c increased in a time-dependent manner. Disruption of mitochondrial membrane potential is considered to be an indicator of mitochondria damage and generally is defined as an early stage of apoptosis, preceding efflux of cytochrome c from the mitochondria and followed by caspase-9/caspase-3 cascade activation.26,28) These results demonstrated that nano-HAP has potency on changing mitochondrial membrane potential and triggering apoptosis in mitochondria pathways.

In conclusion, we have demonstrated in this study that nano-HAP conducts a caspase-dependent pathway through the collapse of the membrane potential of mitochondria and the mitochondrial release of cytochrome c. Further studies are still needed to understand the various mechanisms regulating the antiproliferation effect and apoptosis induced by nano-HAP. Further studies are also in progress to clarify whether nano-HAP-induced apoptotic response varies with cancer cells of different tissue origin. Nevertheless, our experiments for the first time demonstrated that nano-HAP inhibits the proliferation of SGC-7901 cells by inducing apoptosis and provide elementary in vitro evidence that nano-HAP-induced apoptosis is mediated through the mitochondrial-dependent and caspase-dependent pathway.

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