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Size effect of hydroxyapatite nanoparticles on proliferation and apoptosis of osteoblast-like cells

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Abstract

Nano-hydroxyapatite (nano-HAP) may be a better candidate for an apatite substitute of bone in biomedical applications than micro-sized hydroxyapatite (m-HAP). However, size control is always difficult when synthesizing well-defined nano-HAP particles. In this study, nano-HAP particles with diameters of ~20 nm (np20) and ~80 nm (np80) were synthesized and characterized. The size effects of these nano-HAPs and m-HAP were studied on human osteoblast-like MG-63 cells in vitro. Our results demonstrate that both cell proliferation and cell apoptosis are related to the size of the HAP particles. Np20 has the best effect on promotion of cell growth and inhibition of cell apoptosis. This work provides an interesting view of the role of nano-HAPs as ideal biomedical materials in future clinical applications.

Keywords: Size effect; Nano-hydroxyapatite; Osteoblast-like cells; Cell proliferation; Cell apoptosis

1. Introduction

Hydroxyapatite (HAP), with the structural formula of $Ca_{10}(PO_4)_6(OH)_2$, is the principal inorganic constituent of human bones and teeth [1]. Synthetic HAP crystals are now widely used in medical applications, e.g. as implants or coatings on prostheses [2–4]. Accordingly, the biological characteristics – e.g. non-immunogenicity, non-inflammatory behavior, good biocompatibility, high osteoconductivity and/or osteoinductivity [5] – of these synthetic HAPs have been extensively researched.

Recently, it was observed by atomic force microscopy (AFM) that plate- or ball-shaped minerals were densely packed into woven collagen fibrils in trabecular bone or vertebra [6]. These mineral plates in bone exhibited a large range of plate diameters from 30 to 120 nm. However, in other literature [7], ultrastructural examination of deproteinated bone reveals that individual 25–50 nm HAP crystals are the essence of bone in terms of mechanical properties and bioresorbability, and play an important role in biomineral formation. Thus, nanosized HAP particles (nano-HAP) have aroused intensive interest, and great efforts have been made in the last decade to study their synthesis, structure and properties [8–9]. These nano-HAPs have especial characteristics such as improved biocompatibility, good bioactivity and flexible structure [10–12], which are important for their potential applications in medicine. However, it is always difficult to synthesize nano-HAP particles with well-defined sizes [13], and the size effects and the biological effects of these nanoparticles are also not yet understood.

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In this study, HAP nanoparticles with diameters of 20 nm (np20) and 80 nm (np80) were synthesized and characterized, and the effect of these HAP particles on osteoblast-like MG-63 cells were also conducted to evaluate their cellular biocompatibility.

2. Materials and methods

2.1. Preparation of HAP particles

The chemicals were analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). The size-controlled nano-HAP particles were prepared as recently described [14]. Briefly, based on the theory of critical micelle concentration (CMC) [15], Hexadecyl (cetyl) trimethyl ammonium bromide (CTAB) was used to regulate the size of HAP particles. $CaCl_2$ (5.0 mM) solution (60 ml) was dropped into 240 ml Na_2HPO_4 (1.25 mM) in the presence of CTAB (6.0×10^{-4} or 12.0×10^{-4} mol l^{-1}). The pH of the reaction solutions was maintained at 10.0 ± 0.5 by using a dilute ammonia solution (0.1 mol l^{-1}). The reaction was suspended for 24 h when the drop-wise addition was finished. The precipitates were separated by centrifugation and filtration, and then were washed completely with ethanol to remove the residual CTAB molecules. Micro-sized HAP particles (m-HAP) were prepared regularly as described by Nancollas and Mohan [16].

After physico-chemical analysis of HAP particles, HAP films were obtained by slowly coating cover glasses (2 cm in diameter) with 1% HAP particle suspension and then drying these in air. Prior to the in vitro experiments, all films were sterilized by steam autoclaving at 120 °C for 30 min.

2.2. Physico-chemical characterization of HAP films

All obtained particles were characterized by transmission electron microscopy (TEM, JEM-200CX, Japan), X-ray diffraction (XRD, Rigaku D/max-2550 pc) with Cu K_α radiation, dynamic light scattering (DLS, NanoS ZEM 1600, Malvern, UK), and chemical analysis (atomic adsorption spectrometry for calcium and phosphate). Field emission scanning electron microscopy (FESEM, Hitachi 4700, Japan) was used to examine the morphologies of HAP films. Fourier transform infrared (FTIR) spectroscopy was used to detect typical CTAB bands in HAP nanoparticles. Data were analyzed based on six replicated tests.

2.3. Cell culture

Human osteoblast-like MG-63 cell line (American Type Culture Collection, VA, USA) was used in this study. Cells were seeded onto HAP films at a density of 3×10^4 per well in 12-well plates. Incubation was performed in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco), 100 μg ml^{-1} streptomycin and 100 U ml^{-1} of penicillin. The cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Parallel experiments on the sterile transparent cover glasses were also performed as controls.

2.4. Cell proliferation assay

To evaluate cell proliferation rate quantitatively, the MG-63 cells on three different films were cultured for up to 5 days. Ten different fields per sample were randomly chosen under a microscope, and normal cells were counted by two blind observers at days 1, 3 and 5, respectively.

2.5. Acridine orange staining

At day 5, acridine orange (AO) staining was performed to show cell numbers on the surface of films. Briefly, the cells were fixed in 95% cold ethanol for 20 min. After two washes with PBS, cells were stained by 0.01% AO (Sigma, USA) for 2 min at room temperature, and then observed by fluorescence microscopy (Olympus BX51, Japan). AO is a lipid-soluble dye and stains DNA green and RNA red.

Because cells were washed twice with PBS before AO staining, most of apoptotic or necrotic cells were washed off, and the photographs of AO staining show the number of normal cells.

2.6. FESEM examination

After 5 days' incubation on HAP films, MG-63 cells were fixed in 2.5% glutaraldehyde, treated with 1% osmium tetroxide and dehydrated. The samples were then subjected to critical point drying and coated with gold–palladium. The cell morphology was determined by FESEM. The FESEM images clearly represent the cellular morphology, including not only the normal cells but also the deformed cells.

2.7. TEM observation

The cells cultured for 5 days were detached by 0.25% trypsin–EDTA, and fixed with 2.5% glutaraldehyde. After treatment with 1% osmium tetroxide, the samples were dehydrated with a series of alcohols and infiltrated with epoxy resin. The resin sample block was trimmed, thin-sectioned and collected on formvar-coated copper grids. Thin sections were stained in uranylacetate and lead citrate for examination by TEM (TECNAL 10, Philips, Holland).

2.8. Flow cytometric detection

MG-63 cells were harvested and fixed with ice-cold 70% ethanol at −20 °C for 2 days. After washing twice with PBS, the cells were resuspended in PBS containing RNase A (100 μg ml^{-1}) and propidium iodide (PI) solution (50 μg ml^{-1}) for 20 min at room temperature. For each sample, a minimum of 10,000 events were scored by a FACScan (BD Biosciences) flow cytometer, and the per-

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