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RESEARCH ARTICLE

The pathway of HAP nanoparticle uptake into hepatoma carcinoma cells

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Abstract To study the pathway of hydroxyapatite (HAP) nanoparticle uptake into hepatoma carcinoma cells, by selecting bigger particles as contrast, HAP particles with 70.8, 1078.8, and 1906.6 nm mean diameters were prepared. Using TEM, the distribution conditions of the three kinds of HAP particles on the cell membrane surface and within the endochylema, as well as the change in lysosomes was observed after HAP particles were treated onto Bel-7402 cells for 2, 12, 24, and 48 h. The results showed that there were HAP nanoparticles adhering to the depressed part of the plasmalemma, and in the endochylema there were HAP nanoparticles which were enclosed in many vesicles with different sizes. The lysosome had no changes compared to the blank control. The same results were seen for bigger particle groups. Through contrast analysis, it was thought that HAP nanoparticles were internalized into the hepatoma carcinoma cells and then formed vesicles. HAP nanoparticles enclosed in vesicles did not undergo lysosomal digestion. These characteristics are in accord with non-clathrin-mediated endocytosis. Therefore, the pathway of HAP nanoparticle uptake into hepatoma carcinoma cells is possibly caveolae-mediated endocytosis and it has no relation to particle size. This result lays the foundation for further research into the anticancer mechanism of HAP nanoparticles, and plays an important role in the research on nanoparticles as carriers of anticancer drugs.

Keywords hydroxyapatite (HAP) nanoparticles, hepatoma carcinoma cell, endocytosis, caveolae

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1 Introduction

Nano-technology has been an emerging technology since the 1980s. In recent years, it has been gradually applied in the medical field and has opened up a new realm for the development of medicine. Cancer seriously harms human health, and the traditional anti-cancer drugs have serious side effects, therefore, looking for anti-cancer drugs with small and even no side effects is particularly important. In the chemical composition of calcium phosphate salts, synthetic hydroxyapatite (HAP) is similar to the bone tissue of organisms; consequently, it has good biocompatibility. In addition, nano-hydroxyapatites (nano-HAPs) possess unique functions. Researches have indicated that nano-HAP can be used as anti-cancer drug carriers, and have a better therapeutic effect than single anti-cancer drugs [1]. Also, by themselves they have inhibitory effects on a variety of cancer cells including liver cancer cells that they can inhibit significantly [2,3], although the mechanism of inhibition is not clear. In this study, selecting two kinds of large-size HAP particles with a higher electron density identified easily as the control, through TEM observation and analysis, the pathway of HAP nanoparticles into hepatoma carcinoma cells was researched. This lays a foundation for further research in the anticancer mechanism of HAP nanoparticles, and plays an important role in research on nanoparticles as carriers of anticancer drugs.

2 Materials and methods

2.1 Preparation of HAP nanoparticles

HAP_1 nanoparticle sol and large-size HAP_2 and HAP_3 particle powders were synthesized by the homogeneous

precipitation method and by controlling temperature. HAP_2 and HAP_3 particle suspensions were prepared with a stabilizer ultrasonically. All of the sol and the suspension were sterilized in an autoclave for 20 min above a temperature of 120°C and stored at 4°C. When the HAP_1, HAP_2 and HAP_3 particles were used for research, the stock suspensions of HAP_2 and HAP_3 particles still needed to be ultrasonicated.

2.2 Characterization of HAP nanoparticles

The sizes of HAP_1 nanoparticles, HAP_2 particles, and HAP_3 particles were measured by dynamic light scattering (Malvern Zetasizer) and their shapes were investigated by TEM.

2.3 Cells and cell culture

The Bel-7402 human hepatocellular carcinoma cell line from the Cell Bank of the Chinese Academy of Science was supplied by the China Center for Type Culture Collection (CCTCC) in Wuhan University. Cells were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 100 μg/mL penicillin and 100 μg/mL streptomycin at 37°C in the presence of a humidified atmosphere of air (95%) and CO_2 (5%). Cells in log phase were used in the experiment.

2.4 Bel-7402 cells treated with HAP particles

Bel-7402 cells were plated at a density of 5×10^5 cells/mL in culture flasks (four groups). After 24 h, in the HAP_1, HAP_2 and HAP_3 group, the Bel-7402 cells were treated respectively with HAP_1 nanoparticles, HAP_2 particles, and HAP_3 particles at the same final concentration of 0.56 mmol/L; in the blank control group the Bel-7402 cells were treated with nothing. The cells in the four groups were cultured in a humidified incubator containing 5% CO_2 and 95% air. Cells in every group were incubated for 2, 12, 24, and 48 h and were collected respectively in EP tubes and fixed in 2.5% glutaraldehyde. Then, cells pellets were dehydrated, cleared, embedded, and sectioned. The samples were observed under a transmission electron microscope.

3 Results and analysis

3.1 HAP nanoparticles characterization

The distribution of HAP_1 nanoparticles was uniform and steady in the prepared sol, with an average size of 70.8 nm (main range 44.6–86.8 nm). As shown by TEM, HAP_1 was rod-shaped and ball-shaped (Fig. 1). The average sizes of the HAP_2 and HAP_3 particles were, respectively, 1078.8 nm (main range 301.6–1339.3 nm) and 1906.6 nm (main range 843.2–2443.0 nm), with irregular and ball shapes (Figs. 2 and 3).

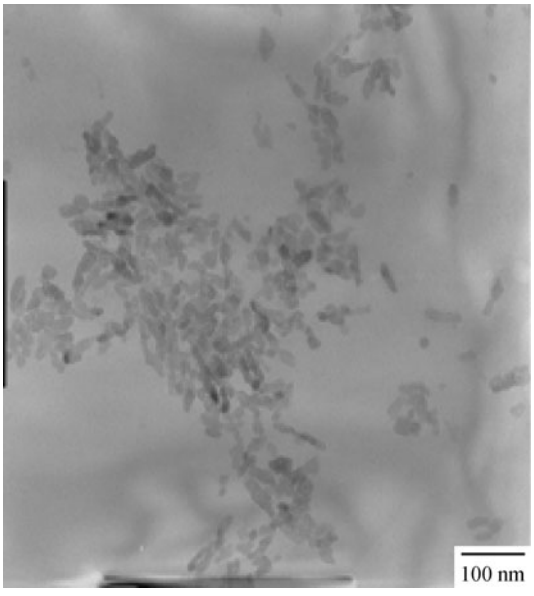

Fig. 1 TEM photo of HAP_1

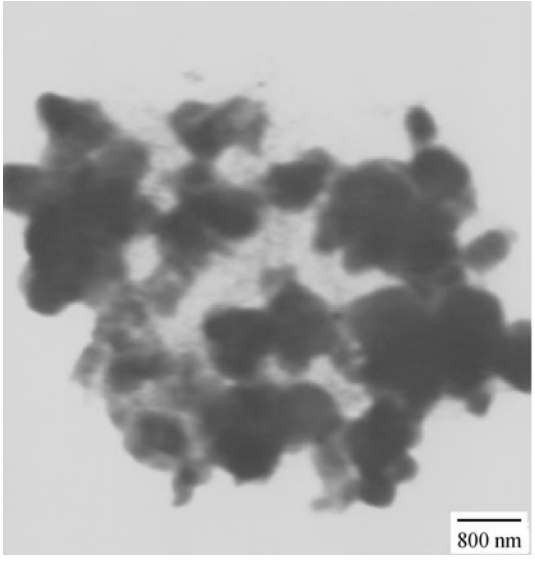

Fig. 2 TEM photo of HAP_2

3.2 HAP nanoparticles entering into Bel-7402 cell

With TEM observation of the Bel-7402 cells treated for 2, 12, 24, and 48 h, it was found that there were a number of granules on the cell membrane surface and in the endochylema in the HAP_1 group (Fig. 4). As these rod- and ball-shaped granules were electron-dense granules agglomerated together, they were easily identified as not

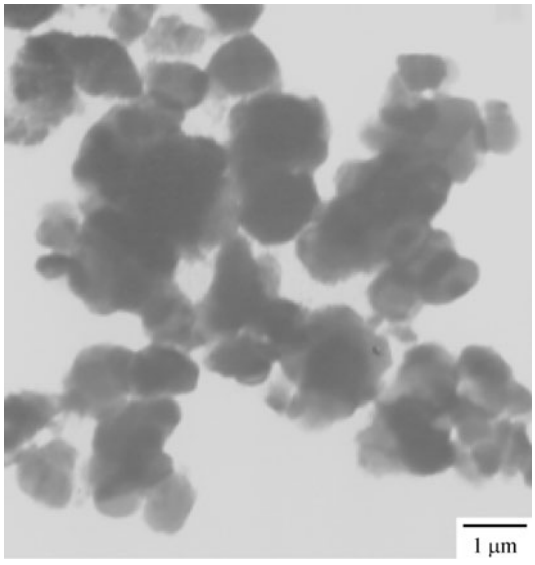

Fig. 3 TEM photo of HAP_3

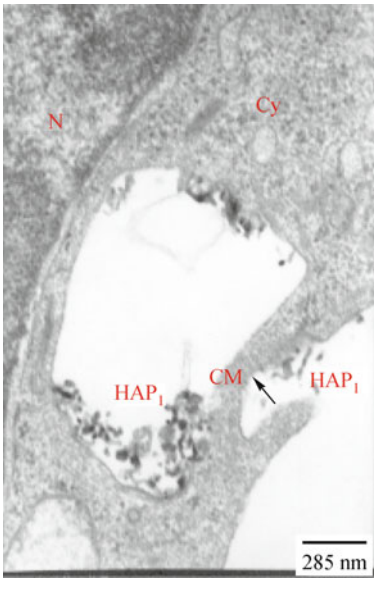

Fig. 4 TEM photo of Bel-7402 cells treated for 2 h showing cells internalizing HAP_1 nanoparticles (CM: cell membrane; MV: microvilli; Cy: cytoplasm; N: nucleus)

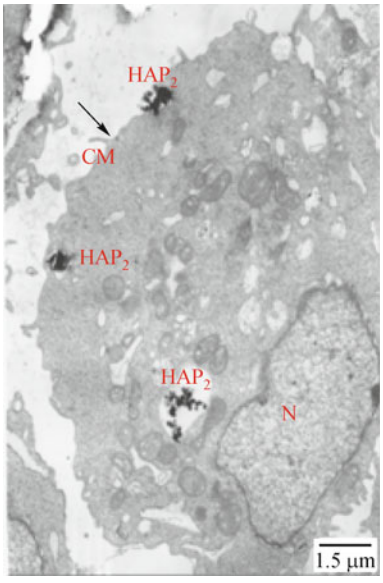

Fig. 5 TEM photo of Bel-7402 cells treated for 2 h showing cells internalizing HAP_2 nanoparticles (CM: cell membrane; MV: microvilli; Cy: cytoplasm; N: nucleus)

being cell components by comparing them with the blank control group. In the HAP_2 group or in the HAP_3 group, the same results were found, and compared to the HAP_1 group, bigger granules with denser electron densities were seen, and the number of granules on the membrane surface and in the endochylema was less (Fig. 5). These granules must have been HAP_2 particles and HAP_3 particles. Because the HAP_2 particles and HAP_3 particles were identified more easily, in the HAP_1 group the granules on the membrane surface and in the endochylema were identified as HAP_1 nanoparticles. Furthermore, the researchers also found that there were a number of electron-dense granules in hepatoma carcinoma cells treated with HAP nanoparticles, and through electron diffraction, the granules were confirmed as HAP nanoparticles [4].

The number of HAP_2 or HAP_3 particles on the membrane surface and in the endochylema was obviously less than that of the HAP_1 nanoparticles, which indicated that the HAP_1 nanoparticles linked (or adsorbed) more easily with the cell membrane of hepatoma carcinoma cells than the HAP_2 or HAP_3 particles.

Nano-material is tiny, but its large proportional atoms lie in the grain boundary, and it abounds in interfaces and free surfaces. Interaction exists among nano-elements. The pH value of the cellular culture medium easily affects the dispersal system of HAP_1 nanoparticles resulting in the reversible agglomeration of nanoparticles.

3.3 HAP nanoparticles in relation to the membrane of the Bel-7402 cell

3.3.1 Bel-7402 cells treated with HAP nanoparticles for 2 h

In the HAP_1 group, the TEM observation found that HAP_1 nanoparticles adhered to the depressed parts of the plasmalemma, and it also found that HAP_1 nanoparticles were enclosed in many vesicles in the endochylema. The number and size of the vesicles were different among cells, and in vesicles the number of nanoparticles was also different. In the HAP_2 group and in the HAP_3 group, the phenomena noted in the TEM observation were similar to that of the HAP_1 group.

3.3.2 Bel-7402 cells treated with HAP nanoparticles for 12, 24 and 48 h

Bel-7402 cells treated for 12, 24, and 48 h were observed respectively with TEM. In the HAP_1 group, the same results were found at the different time points, wherein HAP_1 nanoparticles existed only in many vesicles with different sizes in the cells, and these vesicles were almost near the nucleus. The observation results in the HAP_2 group and in the HAP_3 group were similar to those in the HAP_1 group.

It is thus evident that HAP_1 nanoparticles were taken up into the hepatoma carcinoma cells. HAP_1 nanoparticles adhered to the depressed parts of the plasmalemma and then were internalized quickly into the Bel-7402 cells, and a vesicle was formed. The vesicle was gradually transported toward the nucleus.

After the Bel-7402 cells were treated for 12–48 h, as the concentration of the HAP_1 nanoparticles in the culture fluid were reduced gradually, accordingly, the probability of internalization of HAP_1 nanoparticles degraded gradually and the process of internalization was quick, thus it was not easily found that HAP_1 nanoparticles adhered to the depressed membrane, and were just soundly internalized into the cells.

3.4 The lysosome in Bel-7402 cells treated with HAP nanoparticles

For the lysosomes in Bel-7402 cells treated for 2, 12, 24, and 48 h, under TEM observation it was found that there was no change in the HAP_1 group compared to the blank control, and the same result was seen in the HAP_2 group and in the HAP_3 group. It is thus clear that HAP_1 nanoparticles in hepatoma carcinoma cells were not undergoing lysosomal digestion.

4 Discussion

4.1 Pathways of substances into cells

The main structure of the plasma membrane is formed by a fluid phospholipid bilayer in which the hydrophobic fatty-acid-tails of both layers meet in the membrane center while the hydrophilic phosphate-glycerol-heads build the interfaces with the intracellular cytoplasm and the extracellular fluid. The phospholipid bilayer is reinforced by cholesterol and embedded with a large number of diverse proteins. Substances have to cross the cell membrane to enter into the cell. The main pathways of substances into the cell are diffusion, passive and active transport, and a number of endocytic mechanisms.

Uncharged molecular solutes such as O_2, N_2, CO_2 and ethanol as well as water (osmosis), can move down their concentration gradients directly across the lipid bilayer itself by simple diffusion. Most solutes with a size < 10 nm, however, can cross the membrane only if there is a membrane transport protein (channel or carrier protein) to transfer them. This applies to cations like K^+, Na^+, Ca^{2+}, anions like Cl^- and HCO^{3-}, as well as small hydrophilic molecules, e.g., glucose, and macromolecules such as proteins and RNA. By both carrier proteins and channel proteins, passive transport in the same direction as a concentration gradient occurs spontaneously, whereas when only using carrier proteins to transport against a concentration gradient, called active transport, an input of energy is required. Bigger particles are internalized by eukaryotic cells through endocytic pathways including phagocytosis, macropinocytosis, clathrin-mediated endocytosis and non-clathrin-mediated endocytosis such as internalization via caveolae [5]. Each endocytic mechanism is used by cells to accomplish different tasks.

4.2 Endocytic pathways

4.2.1 Phagocytosis

Phagocytosis is practiced only by specialized cells like neutrophils or macrophages to ingest particulate matter and large solid items such as bacteria from the extracellular fluid (ECF) in a sporadic manner. The formed endosomes are called phagosomes or vacuoles. The phagosomes deliver their contents to lysosomes which destroy and digest the enclosed contents, e.g., bacteria, by means of acids and degradative enzymes.

4.2.2 Pinocytosis

Pinocytosis (cell drinking) is applied by almost all cells for the ingestion of dissolved, fluid materials in a continuous manner. In this way, cells acquire the molecules and ions dissolved in the ECF and pick up critical components in the ECF that may be in scant supply.

4.2.3 Endocytosis mediated by clathrin

Macromolecules and bigger particles that bind to specific receptors on the cell surface which are concentrated in specialized regions of the plasma membrane, called clathrin-coated pits, are internalized via clathrin mediated endocytosis. After entering the cytoplasm, the endocytotic vesicle loses its clathrin coat and quickly fuses with other such vesicles (endosomes). The endosome finally fuses together with lysosomes (pH 5.0–5.5) to digest the enclosed substances and release them into the cytoplasm.

4.2.4 Endocytosis mediated by caveolae

In recent years, evidence has accumulated for clathrin-independent pathways that are mediated by caveolae or

lipid rafts [6–9]. Caveolae ("little cavities") were first identified in the 1950s by Palade and Yamada [10,11] due to their characteristic morphology observed by electron microscopy of thin sections. They typically appear as rounded plasma membrane invaginations of 50–80 nm diameter. Many cell types such as adipocytes, endothelia, and muscle cells contain caveolae with different compositions, appearances, and functions while others cell types like lymphocytes or many neuronal cells do not show any caveolae at all. Caveolae have long been implicated in endocytosis, transcytosis, and cell signaling. Unlike clathrin-mediated endocytosis, internalization through caveolae is a triggered event that involves complex signaling. Pelkmans et al. [7] described the process of internalization via caveolae for the uptake of SV40 particles. After binding to the membrane, the virus particles are mobile until trapped in caveolae. In the caveolae, the particles trigger a signal transduction cascade. Virus-loaded caveolae vesicles are now released from the membrane and can move into the cytosol. After internalization, caveolae can deliver their contents either to endosome-like compartments or to the plasma membrane on the opposite side of a polarized cell (transcytosis process). As caveolae enclosed substances do not undergo lysosomal digestion, the caveolae mediated pathway may provide a safe gateway for viruses and bacteria into the cell [12,13].

4.3 Pathway of HAP nanoparticles into hepatoma carcinoma cell

Analyzing from the angle of material into cells, HAP nanoparticles are not micromolecules or materials (ions or macromolecules) with < 10 nm diameter, or dissolved matter. HAP nanoparticle entry into hepatoma carcinoma cells, therefore, is not by simple diffusion, or membrane protein transportation, or pinocytosis. Analyzing from the angle of the cell to intake material, hepatoma carcinoma cells are not specialized cells like neutrophils or macrophages practicing phagocytosis. Phagocytosis, accordingly, is not the mechanism used by the hepatoma carcinoma cells to intake HAP nanoparticles. Analyzing the results of TEM observation in this study, after the hepatoma carcinoma cells internalize HAP nanoparticles, there are vesicles formed in the cytoplasm, which corresponds to both endocytosis mediated by caveolae and endocytosis mediated by clathrin, but HAP nanoparticles in the vesicles did not undergo lysosomal digestion in this study, thus the pathway does not correspond to endocytosis mediated by clathrin. Therefore, HAP nanoparticle uptake into the hepatoma carcinoma cells were mediated by the caveolae.

The typical caveolae that have been reported appear as rounded plasma membrane invaginations with 50–80 nm in diameter. They must contain only one or two HAP nanoparticles. However, in this research, the diameter of the plasma membrane invaginations was observed to be about 200–1000 nm. The possible reason is that HAP nanoparticles formed reversible agglomerations in the cellular culture medium. It is thus clear that the size of the caveolae is related to the substance internalized. In addition, we still need to elevate the magnification of TEM to observe plasma membrane invaginations ingesting single nanoparticles which probably remain in the hepatoma carcinoma cells.

5 Conclusions

Through TEM observation of Bel-7402 cells treated respectively with three kinds of HAP particles (70.8, 1078.8 and 1906.6 nm in mean diameter), it was found that HAP nanoparticles were the easiest to bind onto the cell surface. The pathway of HAP nanoparticles into hepatoma carcinoma cells is endocytosis mediated by caveolae, and the mechanism has no relation to the size of the particles.

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