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JBR

The Journal of Biomedical Research, 2015, 29(6):486-490

Original Article

Epirubicin-gold nanoparticles suppress hepatocellular carcinoma xenograft growth in nude mice

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Abstract

We sought to investigate the effects of epirubicin-nanogold compounds (EPI-AuNP) on hepatocellular carcinoma xenograft growth in nude mice. EPI-AuNP was prepared and hepatoma xenograft model was established in nude mice. The mice were then randomly divided into four groups: the control group with injection of saline, the AuNP treatment group, the EPI treatment group and the EPI-AuNP treatment group. After two weeks, the hepatoma weight and volume of the xenografts were assessed. Our transmission electron microscopy revealed that epirubicin-gold nanoparticles caused significantly more structural changes of hepatocellular carcinoma cells HepG2. The tumor weight in the Epi-AuNP treatment group (0.80±0.11 g) was significantly lower than that of the control group (2.48±0.15 g), the AuNP treatment group (1.67±0.17 g), and the EPI treatment group (1.39±0.10 g) ($P<0.01$). Furthermore, the tumor volume of mice in the EPI-AuNP treatment group (0.27±0.06 cm^3) was significantly smaller than that of the control group (2.23±0.34 cm^3), the AuNP treatment group (1.21±0.25 cm^3) and the EPI treatment group (0.81±0.11 cm^3) ($P<0.01$). In conclusion, epirubicin-nanogold compounds (EPI-AuNP) have significant inhibitory effects on the growth of hepatocellular carcinoma cells *in vivo.*

Keywords: epirubicin, nanogold, hepatoma xenograft, HepG2 cells, HUVEC

Introduction

Epirubicin is an anthraquinone antitumor drug. It has great potential in cancer treatment but lacks specificity. The complications of myocardial and bone marrow toxicity have limited its clinical application[1]. Epirubicin could be combined with a nano-carrier, forming a synthetic drug delivery system, to improve the specificity of the drug. This system improves epirubicin targeting and prolongs *in vivo* bioavailability, thereby enhancing the drug's efficacy[2]. Furthermore, epirubicin has the special property of being fluorescent under ultraviolet light. When combined with nanoparticles, this fluorescence is decreased and, hence, is an effective way to identify successful combination of the compound.

Gold nanoparticles are spherical, with a mean diameter of 10 nm. They have the advantages of having a well-established synthesis process, good biocompatibility, optimal tissue permeability, and relatively easy modification of the surface[3]. Preliminary studies showed that gold nanoparticles possess an anti-angiogenesis effect by itself[4]. The proposed mechanism is binding by gold nanoparticles to the heparin receptor sites and vascular endothelial growth factor (VEGF165), which would in turn block the activation

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Received 07 March 2014, Revised 29 April 2014, Accepted 11 October 2014, Epub 05 August 2015

CLC number: R735.7 Document Code: A

The authors reported no conflict of interests.

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doi: 10.7555/JBR.29.20140044

of VEGFR2 and hence inhibit the activation and proliferation of tumor vascular endothelial cells. As a result, gold nanoparticles prohibit angiogenesis[5]

In addition, gold nanoparticles can serve as a "drug delivery system". It can be combined with anti-tumor drugs by means of physical adsorption, ionic or covalent bonding[6]. This system would have the functions of anti-angiogenesis as well as anti-tumor cell proliferation. Hence, it can exert its anti-tumor effect *via* multiple levels and multiple targets. In the current study, we first synthesized gold nanoparticles and then investigated the *in vivo* effect of epirubicin-gold nanoparticles on human hepatocellular carcinoma xenografts in nude mice.

Materials and methods

Reagents and instruments

Epirubicin was commercially available (Sigma, St. Louis, MO, USA). Gold nanoparticles were prepared with gold chloride and sodium citrate (Sinopharm Chemical Reagent Co., Ltd.). RPMI-1640 medium, *L*-glutamine, penicillin and streptomycin (Hyclone, USA) were purchased. M199 medium, fetal bovine serum, and trypsin (Gibco Le.) were commercially available. Human hepatocellular carcinoma cell line HepG2 was maintained in our own laboratory. Human umbilical vein endothelial cells (HUVECs) were obtained by primary isolation and culture[7]. Ultraviolet-visible (UV-Vis) spectrophotometer (Lambda 45, PerkinElmer, USA) and transmission electron microscope (TECNAI 10, Philips Ltd.), full wavelength multifunctional micro-plate reader (Safire II, Bio-Rad, Hercules, CA, USA), atomic force microscopy (American Thermo-microscope, Ltd.) and micro-titer plates (Costar) were used for the *in vitro* study.

Animals

Six to eight week-old BALB/c nude mice with body weight ranging from 15-22 g were purchased from the Sun Yat-sen University Laboratory Animal Center (Guangzhou, China) and kept in the Laboratory Animal Center of Jinan University under conditions recommended by the Environmental Standards of China GB14925-2007, at 20-27 °C and the IS07 level of cleanliness. The study protocol was approved by the local institutional review boards and animal study was carried out in accordance with the established institutional and state guidelines on experimental use of animals.

Synthesis and identification of gold nanopar–ticles

Gold chloride was mixed with citric acid and Au^{3+} in gold chloride was reduced to Au. All glassware was cleansed in aqua regia (3 parts HCl and 1 part HNO_3). Then, 5 mL of sodium citrate (38.8 mmol/L) was added to 50 mL of boiling gold chloride (1 mmol/L) solution. The resultant suspension was then cooled down to room temperature. It was subsequently filtered with a 0.22 μm filter to remove large aggregates and then stored at 4 °C.

Synthesis and identification of epirubicin–gold nanoparticles

Four mL of the gold nanoparticle solution was diluted with 8 mL of ultrapure water. Sodium hydroxide (1 mol/L NaOH) solution was added and titrated to pH 8.0. Then, 2 mL of epirubicin was added. The solution was placed overnight in an air shaker (172 g) at 37 °C. After centrifugation (28,720 g) for 40 minutes, the supernatant was discarded and the epirubicin-gold nanoparticles were washed thrice with 0.01 mol/L phosphate buffered saline with 0.2% bovine serum albumin and then analyzed[8] by UV-Vis absorption spectrophotometry, fluorescence quenching, dynamic laser light scattering (DLS), and zeta potential analyzer.

Atomic force microscopy (AFM)

Cellular suspensions of HepG2 cells and HUVECs at 1×10^4 cells/mL were plated in 6-well plates with 22 mm × 22 mm sterile cover slips, respectively, and then cultured for 24 hours. The cover slips, adsorbed with HepG2 cells and HUVECs, were gently rinsed with PB solution (0.1 mol/L, pH=7.4) three times for 5 min each. Then, they were immersed in 4% paraformaldehyde for 15 minutes and subsequently rinsed three times with ultrapure water. They were then air-dried at room temperature. Finally, AFM was used for scanning. The setting of 100 μm scanner, contact mode, and silicon probe, with resonance frequency of 250-320 Hz was used. The images were enhanced using Proscan Image Processing Software Version 2.1.

Mouse hepatocellular carcinoma xenograft model

HepG2 cells in 0.2 mL (containing 5×10^6 cells) were implanted subcutaneously in the right axilla of six to eight-week-old BALB/c nude mice. When the transplanted tumor diameter reached 6 - 8 mm, the nude mice were randomly divided into four groups of eight and marked for identification. The four groups were processed as: (1) the control group (normal saline), (2) the AuNP (2.5 μg/kg) treatment group, (3) the EPI (5 mg/kg) treatment group and (4) the EPI- AuNP (AuNP 2.5 μg/kg + EPI 5.0 mg/kg) treatment group. The drugs were injected as 0.2 mL solution *via* the tail vein every other day for two consecutive weeks.

For measurement of tumor volume and weight, the nude mice were sacrificed and the tumor xenografts were removed en bloc and measured using the Vernier caliper by one person to eliminate observer variation. The longest diameter (a) and perpendicular diameter (b) were measured and the tumor volume was calculated (tumor volume $= ab^2/2$). The weight of the tumor xenografts were measured with electronic analytical balance.

Statistical analysis

Continuous variables were checked for normal distribution and presented as mean and standard deviation or median and range as appropriate. Comparison of continuous variables was performed by using Student's t test for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables. One-way analysis of variance (one-way ANOVA) was used to compare means of two or more samples with least significant difference (LSD) as post hoc test. All tests of significance were at the 5% significance level. Analyses were conducted using SPSS version 11.0.

Results

Epirubicin–gold nanoparticles caused apparent structural changes of hepatocellular carcinoma cells

Transmission electron microscopy (TEM) (***Fig. 1***) revealed that epirubicin-gold nanoparticles were spherical, uniform in size and about 10 nm in diameter. UV-Vis spectrophotometry displayed the maximum absorption peak of gold nanoparticles at 520 nm, which was markedly reduced when epirubicin-gold nanoparticles were used. DLS demonstrated that gold nanoparticles were (14.34 ± 0.75 nm), which was significantly smaller than epirubicin-gold nanoparticles (18.54 ± 1.84 nm). Zeta potential analyzer showed that gold nanoparticles had a zeta potential of -21.19 ± 0.64 mV, which was markedly different from that of epirubicin-gold nanoparticles (-15.34 ± 0.72 mV).

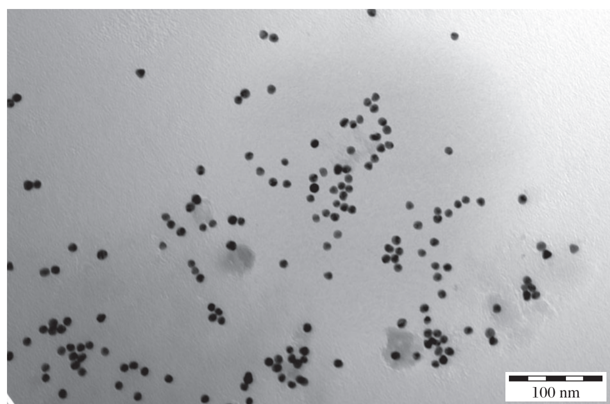

Fig. 1 **Transmission electron microscope images of epirubicin–nanogold compounds.** Nanogold compounds were prepared as described in Methods and they appear spherical, uniform in size and about 10 nm in diameter. (Bar=100 nm)

AFM was used to visualize the morphological changes in the hepatoma xenografts and in turn compared the treatment effect. As shown in ***Fig. 2***, the surface of normal HepG2 cells was smooth with turgid cytoplasm. In comparison to the control group, in AuNP-treated cells, there was invagination of cell membrane and there was an, increase in surface pore size with the more rough surface morphology. EPI treated HepG2 cells showed obvious holes of varying sizes on the cell surface, which may signify cellular injury due to EPI. This was more apparently aggravated in EPI-AuNP treated HepG2 cells whose cell membrane appeared rougher with uneven larger pores with the maximum diameter of up to about 2 μm.

Epirubicin–gold nanoparticles markedly suppress hepatocellular carcinoma xenograft growth in nude mice

We observed significant difference in the tumor weight of hepatocellular carcinoma xenograft in the different groups of nude mice (***Fig. 3***). The tumor weight in the Epi-AuNP treatment group (0.80 ± 0.11 g) was significantly less than that of the control group (2.48 ± 0.15 g) and the AuNP treatment group (1.67 ± 0.17 g), and the EPI treatment group (1.39 ± 0.10 g)($P<0.01$). Furthermore, there were significant differences in the tumor volume of hepatocellular carcinoma xenograft in the different groups of nude mice. The tumor volume of mice in the EPI- AuNP treatment group (0.27 ± 0.06 cm^3) was significantly smaller than that of the control group (2.23 ± 0.34 cm^3), the AuNP treatment group (1.21 ± 0.25 cm^3) and the EPI treatment groups (0.81 ± 0.11 cm^3) ($P<0.01$).

Discussion

Epirubicin is an effective antitumor drug but its lack of specificity has prohibited its widespread use. The introduction of nano-biotechnology may provide a possible solution for its use as a targeted therapy. In the current study, we successfully created epirubicin nanogold particles. The special nature of epirubicin being fluorescent under ultraviolet light is also a convenience. When combined with nano-particle, this fluorescence is quenched. This can serve as a simple and effective way to confirm success in the creation of the compound.

Gold nanoparticle itself can prohibit angiogenesis. When combined with epirubicin, apart from the anti-tumor effect, it can increase the uptake of epirubicin into tumor cells. In turn, epirubicin will act by

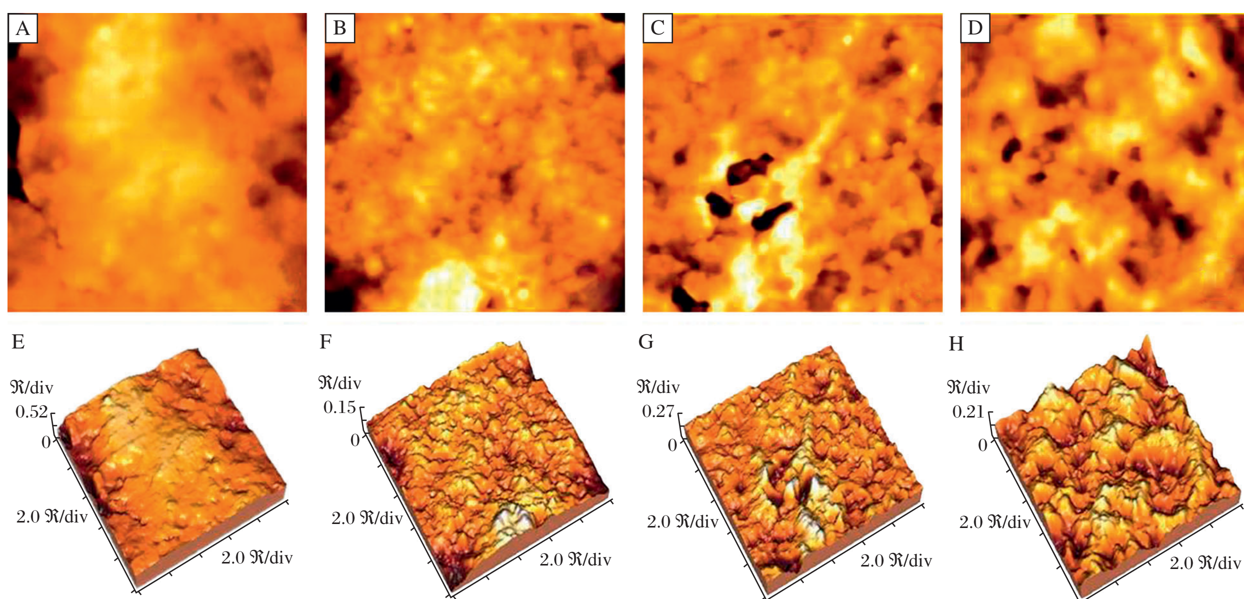

Fig. 2 **AFM image of HepG2 cell surface morphology** A: Control; B: AuNP (nanogold compounds) treatment group; C: EPI (epirubicin) treatment group; D: EPI-AuNP (epirubicin-nanogold compounds) treatment group. Photos (E) to (H) are corresponding three-dimensional reconstruction (image size: 10 μm × 10 μm).

intercalating DNA strands, resulting in a complex formation which inhibits DNA and RNA synthesis. In addition, by triggering DNA cleavage by topoisomerase II, cytotoxic effects and free radicals wil all contribute to the cytotoxic effect. In our study, we confirmed the successful synthesis of EPI-AuNP compound by several ways. Firstly, ultraviolet-visible absorption spectrophotometer (UV-Vis) can measure the absorbance of light in visible and ultraviolet ranges in organic compounds. A shift in the maximum absorption denotes successful combination of compounds, altering the permittivity, which is how the electric field is affected by a dielectric medium. Secondly,

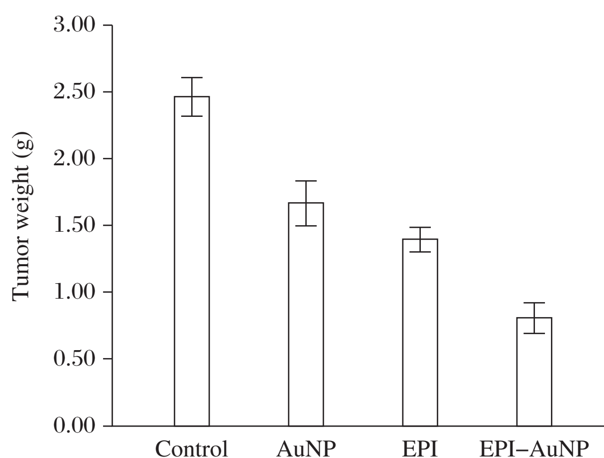

Fig. 3 **Effect of treatment in tumour weight in nude mice liver carcinoma xenograft.**

fluorescence spectroscopy can detect the fluorescence intensity of EPI, which is greatly diminished in EPI-AuNP. Thirdly, DLS analyzes the Brownian motion of particles or molecules in suspension that causes laser light to be scattered at different intensities. These intensity fluctuations yield the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship. Finally, Zeta Potential Analyzer measures the electro-kinetic potential of colloid. Colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

In the results of the *in vivo* effect on hepatoma xenografts in nude mice, there was a clear anti-tumor effect both in terms of weight and volume as compared to the control for all three limbs. There is also an increase in anti-tumor effect in an ascending order for AuNP, EPI and EPI-AuNP. EPI-AuNP can evade phagocytosis of the reticuloendothelial system and can maintain epirubicin biological activity until it reaches the tumor site[9]. Through vascular permeability and, also, intercellular communication channels, EPI-AuNP can concentrate at the tumor cells and decrease the side effects on the heart and the bone marrow. This targeted therapy will increase the safety of its use.

After the release of epirubicin, AuNP itself could still have an anti-tumor angiogenesis effect by reducing the tumor's nutrient and oxygen supply, and prohibiting tumor growth. Furthermore, it is capable of binding to VEGF165, thereby blocking signal

transduction and, in the end, inhibiting endothelial cell proliferation and tumor angiogenesis. Finally, it can inhibit human hepatoma cells HepG2 for VEGF expression and secretion.

AuNP is a multifunctional inorganic nano-particle for imaging, targeting, and drug delivery[10]. It can efficiently carry anticancer drugs, plasmid DNA or small interfering RNA to reach therapeutic targets. It has the potential to play a significant role in cancer chemotherapy[11], radiation therapy[12] or even gene therapy. Furthermore, AuNP can function as a new generation of contrast agents for diagnostic imaging of tumor morphology. In addition, it can be combined with tumor marker antibody and may serve as a sensitive diagnostic modality[13].

In conclusion, *in vitro* studies of epirubicin-nanogold compound (EPI-AuNP), when compared to AuNP alone or EPI alone, showed an increase in morphological changes signifying cellular damage in HepG2 cells and HUVECs. EPI-AuNP also had an increase in accumulation in cells compared with epirubicin alone. Furthermore, *in vivo* studies of hepatoma xenografts in nude mice showed that EPI-AuNP has enhanced antitumor effects, when compared to AuNP alone or EPI alone in both tumor volume and weight.

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