Chitosan-coated doxorubicin nano-particles drug delivery system inhibits cell growth of liver cancer via p53/PRC1 pathway

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Abstract
Background: Nano-particles have been widely used in target-specific drug delivery system and showed advantages in cancers treatment. This study aims to evaluate the effect of chitosan coated doxorubicin nano-particles drug delivery system in liver cancer.

Methods: The chitosan nano-particles were prepared by using the ionic gelation method. The characterizations of the nano-particles were determined by transmission electron microscopy. The cytotoxicity was detected by MTT assay, and the endocytosis, cell apoptosis and cell cycle were examined by flow cytometry. The protein level was analyzed with western blot. The dual luciferase reporter assay was performed to assess the interaction between p53 and the promoter of PRC1, and chromatin immunoprecipitation was used to verify the binding between them.

Results: The FA-CS-DOX nano-particles were irregular and spherical particles around 30–40 nm, with uniform size and no adhesion. No significant difference was noted in doxorubicin release rate between CS-DOX and FA-CS-DOX. FA-CS-DOX nano-particles showed stronger cytotoxicity than CS-DOX. FA-CS-DOX nano-particles promoted the apoptosis and arrested cell cycle at G2/M phase, and they up-regulated p53. FA-CS-DOX nano-particles inhibited cell survival through p53/PRC1 pathway.

Conclusion: Chitosan-coated doxorubicin nano-particles drug delivery system inhibits cell growth of liver cancer by promoting apoptosis and arresting cell cycle at G2/M phase through p53/PRC1 pathway. © 2017 Elsevier Inc. All rights reserved.

1. Introduction

Cancer is one of the leading causes of death all over the world, and it is universally regarded as a difficult medical problem. The anti-cancer drugs currently in use are unable to be delivered targetedly due to its slightly solubility, or they are decomposed in advance during the delivery, largely reducing the efficiency of treatment [1]. In addition, majority of drugs showed non-specific cytotoxicity to normal cells, leading to serious adverse effect in patients. Hence, targeted therapy of drugs has drawn increasing attentions in recent years [2]. Nano-particles refer to the solid colloidal particles made of natural or synthetic high polymer materials with particle size at 1–1000 nm, and varieties of nanoparticles made of various organic or inorganic materials have been developed for target-specific drug delivery system [3]. Nano-particles exhibits clear advantages in protecting chemotherapeutic drugs from degradation in vivo, locating targets for drugs and controlling drug release at the target site [4]. Even with substantial researches, the way from nano-therapy to clinical products is still with a sea of troubles. Doxil® is the first cancer nano-medicine approved by FDA for treating certain cancers, which is a liposomal nano-particles formulation of doxorubicin that releases encapsulated doxorubicin to targets cells [5]. The success of nanoparticles in delivering doxorubicin shed light on many other drug delivery systems.

Chitosan is the unique alkaline polysaccharide exists in the nature, which is characterized as non-toxicity, biodegradability, bio-compatibility and tumor suppressive activity [6]. Chitosan nano-particles have shown good biocompatibility and absorption properties as well as enhanced permeability and retention (EPR) effects and increased blood circulation times, which enhance drug delivery efficiency to the tumour sites [7]. Chitosan nanoparticles drug delivery system has been considered a promising strategy to deliver chemotherapy directly to target organs. Doxorubicin is the most common and important anticancer drug used to treat cancers
since long, with broad-spectrum antineoplastic activity and high therapeutic index [8]. The exact mechanism of action of doxorubicin is complicated, and it interacts with DNA for inhibiting macromolecular biosynthesis of DNA or DNA-dependent RNA; another mechanism is related to generate free radicals that lead to DNA and cell membrane damage [9]. Despite its high antitumor activity, adverse effects such as cardiotoxicity have limited the utility of doxorubicin in clinical practice, and tremendous efforts have been made to improve the condition.

In addition, the studies on targeted drug delivery system mediated by folate receptor have becoming mature. The folate receptor is over-expressed in many cancer cells but nearly non-expressed in normal organs, which devotes to transferring folate or folate-conjugate into cells via endocytosis; the specific combination between folate and folate receptor achieves the targeted delivery of folate-conjugate and thus protecting normal cells from damaged [10]. The liver is a critical target tissue for drug delivery because many fatal conditions including chronic hepatitis, enzyme deficiency, and hepatoma occur in hepatocytes [11]. Hence, we undertook this study to evaluate the effect and action mechanism of chitosan coated doxorubicin nano-particles drug delivery system in hepatoma cell.

2. Materials and methods

2.1. Preparation of nano-particles

2.1.1. Preparation of chitosan (CS) nano-particles

To prepare CS solution (0.5 mg/ml), 0.25 g CS was dissolved in 500 mL 2% acetic acid and the pH value was adjusted to 4.7 with 20% NaOH, and then the solution was heated in water bath at 60 °C for 10 min and filtered at 0.45μm to remove the impurities. The tripolyphosphate (TPP) stock solution (0.5 mg/ml) was filtered at 0.45μm to remove the impurities. The chitosan nano-particles were prepared by using the ionic gelation method [12]. With 20 ml CS (0.5 mg/ml) as base solution, 3 ml TPP (0.5 mg/ml) was quickly drop-wise added into and stirred for 60 min, and then it was centrifuged (16000 rpm, 20min) and freeze-dried to get the CS nona-particles.

2.1.2. Preparation of CS-DOX nano-particles

Under low temperature, with 20 ml CS (0.5 mg/ml, 10 mg, pH = 4.7) as base solution, 3 ml DOX (1 mg/ml, 3 mg) solution was added into, and 3 ml TPP (0.5 mg/ml) was quickly drop-wise added into and stirred for 60 min. And the CS-DOX nona-particles were obtained by centrifugation (16000 rpm, 20min) and freeze-drying to get the CS nona-particles.

2.1.3. Preparation of FA-CS nano-particles

10 mg CS nona-particles were dissolved in 10 ml phosphate buffer solution (PBS, pH = 7.4) to get the PBS suspension, and 2 mg FA dissolved in 10 ml NaOH (20%) was added into the suspension, stirring continuously for 30 min. Then it was washed by deionized water, centrifuged (16000 rpm, 20min) and freeze-dried to get the FA-CS nona-particles.

2.1.4. Preparation of FA-CS-DOX nano-particles

10 mg CS-DOX nano-particles were dissolved in 10 ml phosphate buffer solution (PBS, pH = 7.4) to get the PBS suspension, and 2 mg FA dissolved in 10 ml NaOH (20%) was added into the suspension, stirring continuously for 30 min. Then it was washed by deionized water, centrifuged and freeze-dried to get the FA-CS nona-particles.

2.2. Characterization by transmission electron microscopy (TEM)

The microstructures of the samples were observed by transmission electron microscopy (TEM, Philips-XL30). The size of particles was measured by TEM. The stock solution of 80 mg/L was prepared in Milli-Q water and sonicated (Sonics & Material Inc., Newtown, CT) for 5 min with 45/15s on/off pulse to obtain a homogeneous suspension for TEM analysis on a formvar coated copper grid at 80 kV. The particle size distribution and Zeta-potential of nano-particles solutions were analyzed through Zeta-sizer (Malvern Instrument Ltd., Worcestershire, UK).

2.3. In vitro release of doxorubicin from the FA-CS-DOX nano-particles

In vitro release profile of doxorubicin from chitosan coated nano-particle containing doxorubicin (FA-CS-DOX) were examined at 37 °C for 7 days under protection from light. The amount of released doxorubicin at 16 h, 32 h, 48 h, 64 h and 80 h was determined using a fluorometer with an emission wavelength of 480 nm and an excitation wavelength of 590 nm. The initial amount of doxorubicin and 5-FU used in this study was 5 and 10 mg.

2.4. Cell culture

HepG2 cells were cultured in a 24-well plate at a density of 4 × 10⁴ cells/well in DMEM medium supplemented with 10% FBS, 100U/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO₂ for 24 h. Then the ultraviolet sterilized nano-particles were added into medium and cultured for more than 24 h for cell tests.

2.5. Detection of cytotoxicity with MTT assay

Cell viability, as a testing endpoint of cytotoxicity, was determined with MTT assay [15]. HepG2 cells were seeded into 96-well plates at a density of 1 × 10⁴ cells per well in DMEM (10% FBS) and incubated for 12 h. The CS or FA-CS nano-particles were firstly dissolved in dimethyl sulfoxide (DMSO), and then diluted in culture medium with final DMSO concentration of 0.5% (v/v). After that, the cells were exposed to doxorubicin with concentration of 0.1, 0.2, 0.3, 0.4, and 0.5 (mg/ml) for 48 h. Medium containing 0.5% DMSO was used as negative control. After treatment, the treatment medium was replaced with fresh culture medium without FBS containing MTT at a concentration of 0.5 mg/ml, and the cells were further incubated for 4 h at 37 °C. The medium was removed and the formazan crystals were dissolved in DMSO. The optical density (OD) was measured at 490 nm using a Microplate Reader (MD SpectraMax M5).

2.6. Detection of endocytosis

HepG2 cell suspension (1 × 10⁶/ml) was seeded in a 24-well culture plate. After 24 h of growth, cells were incubated with CS (NPs concentration 50 μg/ml), or FA-CS (NPs concentration 50 μg/ml), and FITC labeled FA-CS with initial folic acid (2 mM) was pre-treated for blocking of folate receptor of HepG2 cells (folate receptor was blocked by treatment of folic acid (2 mM)). Subsequently, the cells were lifted using a FACS Calibur flow cytometer (BD Biosciences) for FITC.
2.7. Cell apoptosis detection

Cell apoptosis was measured with Annexin V-PE/7AAD staining (Southern Biotech, Birmingham, AL) according to the manufacturer’s instructions. After washed twice with 37 °C PBS and stained with Annexin V-PE/7AAD, cells were analyzed by flow cytometry apparatus [FACScan, BD Biosciences], and apoptotic fractions were recorded with CELL Quest 3.0 software.

2.8. Cell cycle assay

After co-cultured with FA-CS-DOX nano-particles, the cell cycle of HepG2 cells was evaluated. Flow cytometry was operated with FACSCalibur (BD Biosciences), and the cell fraction in each cell cycle phase was determined by the cell cycle analysis platform in FlowJo V10 software (Tree Star Inc.).

2.9. Western blot assay

After cell lysed in RIPA lysis buffer (Aidlab Biotechnology) with protease inhibitors (Roche) on ice, ultrasonication, and centrifugation, the whole protein was isolated. The protein was separated with 12% SDS-PAGE and then transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore). The transferred membrane was maintained in antibodies against Bax, Bcl-2, p53, PRC1, c-caspase 3, and β-actin (Abcam) which served as loading control. After washed and incubated in HRP-conjugated secondary antibodies, the protein transferred to membranes was defined with chemiluminescence detection kit (Aidlab Biotechnology) and quantified by Image Lab 4.0 software (Bio-Rad).

2.10. Dual luciferase reporter assay

For finding out the interaction between p53 and PRC1, the PRC1 DNA promoter segments containing the predicted p53 binding site was inserted into pGL3-Basic vector (Promega). The pGL3-PRC1 recombinant vector was transfected into HepG2 cells using Lipofectamine2000 (Invitrogen). The luciferase activity was investigated by the dual luciferase reporter assay system (Promega) according to the manufacturer’s instruction.

2.11. Chromatin immunoprecipitation (ChIP) assay

Experiments were performed with the ChIP kit (Upstate Biotechnology) according to the manufacturer’s specifications [16]. Briefly, HepG2 cells cultured at 37 °C for 2 days were cross-linked with 0.37% formaldehyde and the genomic DNA was sheared by sonication. The samples were then immunoprecipitated with antibody against p53 (Abcam). Normal rabbit IgG (Abcam) was used as a negative control. The immune complex was heated at 65 °C for 4 h to revert the crosslinking between DNA and proteins. The purified bound DNA was dissolved in 20 ml of Tris buffer (10 mM Tris, pH 8.5). The input DNA was diluted 100 × prior to PCR. The bound and the input DNA were analyzed by PCR and the PCR products were visualized on a 2% agarose gel stained with ethidium bromide. The intensity of the PCR products was scanned with a BioRad Fluoro-S Multimager System and quantified by the Quantity One Program.

2.12. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from HepG2 cells by using Trizol reagent (Invitrogen) according to the manual. The total RNA was transcribed with a cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was quantified by qRT-PCR on an ABI 7300-fast Real Time PCR system (Applied Biosystems) with SYBR Select Master Mix (Applied Biosystems). Primers were designed and synthesized by Sangon Biotech (Shanghai, China). GAPDH served as the internal control and the relative expression was analyzed with 2 -ΔΔCt method.

2.13. Statistical analysis

Data were represented as mean ± standard deviation (SD), and statistically analyzed by SPSS 21.0 software (SPSS Inc.) Comparisons between two groups were completed by Student’s test, and comparisons among multi-groups were achieved by One-way ANOVA. P < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of FA-CS-DOX nano-particles delivery system

The molecule structure of FA-CS-DOX nano-particles was showed in the TEM images, and they were irregular and spherical particles around 30~40 nm, with uniform size and no adhesion. It’s well-known that cell cycle arrested at the G2/M phase is closely associated with p53 [15531928], which was determined in previous study. The doxorubicin release rate of CS-DOX and FA-CS-DOX at different times was examined, and no significant difference was noted between them (Fig. 1D).

3.2. Cytotoxicity and endocytosis of nano-particles

The cytotoxicity of CS and FA-CS was determined, and no significant difference was found between them, and the cell growth was not influenced with increasing amount of nano-particles (Fig. 2A). The detection of cell endocytosis indicated that after folate receptor was blocked (FITC-CS + FA blocking), the positive rate of FITC was decreased (Fig. 2B), suggesting it’s easier for FA-CS entering cells than CS. The survival of HepG2 cells was declined when co-cultured with FA-CS-DOX, compared with CS-DOX co-culture (Fig. 2C), implying the stronger cytotoxicity of FA-CS-DOX than CS-DOX.

3.3. Effects of nano-particles on cell apoptosis and cell cycle

Compared with control HepG2 cells, the apoptosis of HepG2 was markedly promoted when co-cultured with FA-CS-DOX (10 µg/ml) particles (Fig. 3A), showing the role of FA-CS-DOX in stimulating apoptosis. When co-cultured with FA-CS-DOX nano-particles (10 µg/ml), the cell cycle was arrested at the G2/M phase (Fig. 3B). It’s well-known that cell cycle arrested at the G2/M phase is closely associated with p53 [15531928], which was determined in expression level, as well as molecules related to cell apoptosis. It showed that p53 was obviously up-regulated while the PRC1 was down-regulated in HepG2 cells co-cultured with FA-CS-DOX nano-particles, and the Bak was dramatically up-regulated and it repressed Bcl-2 significantly, while the c-caspase 3 was evidently up-regulated (Fig. 3C), agreeing with the promotion of apoptosis.

3.4. FA-CS-DOX nano-particles inhibited cell survival through p53/PRC1 pathway

The Fig. 4A indicated that the promoter activity of PRC1 was suppressed by p53, and it also inhibited when cells were co-cultured with FA-CS-DOX nano-particles (Fig. 4A). In the previous step, we found that p53 was obviously up-regulated in HepG2 cells co-cultured with FA-CS-DOX. Then the binding between p53 and PRC1 was detected by ChIP and the binding site demonstrated in previous study was also verified [17] (Fig. 4B). The cell viability detection showed that cell survival decrease was reversed by...
inhibition of p53 (Fig. 4C), and expression of PRC1 mRNA was also reversed by inhibition of p53 (Fig. 4D). With p53 inhibited, expression of PRC1 protein was increased, and the apoptosis-associated factor c-caspase 3 was decreased (4E). These findings revealed that FA-CS-DOX nano-particles inhibited cell survival through p53/PRC1 pathway.

4. Discussion

The folate-conjugated and chitosan-coated doxorubicin nanoparticles (FA-CS-DOX) were successfully prepared with the ionic gelation method, with excellent dispersiveness and uniform size. The FA-CS-DOX nano-particles showed higher cytotoxicity to
hepatocytes, and promoted apoptosis and arrested cell cycle at G2/M phase through p53/PRC1 pathway; thus inhibiting cell growth of liver cancer. Compared with CS-DOX nano-particles, the FA-CS-DOX nano-particles drug delivery system showed stronger inhibitory effect on cell growth of tumor.

The development of nano-particles drug delivery system clearly improves the water solubility, bioavailability, and pharmacokinetics feature of chemotherapeutic drugs, enhancing efficiency of antitumor and reducing side effects to organism to some extent [18]. Chitosan has been widely used in the field of medical material due to its excellent biocompatibility, biodegradable and biosecurity, and it also strengthens anti-tumor effect of drugs [14], acting as an optimal carrier for developing the targeted drug delivery system. Nano-particles based on chitosan have been considered as promising vehicles for drug delivery, primarily due to their ability to contact targeted sites intimately and negligible toxic effect. For instance, chitosan-modified cobalt oxide nano-particles stimulated TNF-α mediated apoptosis in human leukemic cells [19], and chitosan-coated curcumin nano-particles served as a therapy for local treatment of oral cancer, with decreased cytotoxicity [20]. In this study, nano-particles based on chitosan showed similar properties in size, structure, zeta potential and DOX release, but distinct effect on cell behaviors. Compared with FA-CS-DOX, CS-DOX showed lower cytotoxicity to hepatocytes, in accord with the property of chitosan. But for developing the targeted drug delivery system by combining folate and chitosan, more efforts should be made in minimizing cytotoxicity.

As the most important anticancer drug, doxorubicin is mainly used in chemotherapy by intravenous administration, which is not the preferred method for maintaining sustained drug

Fig. 3. Effects of nano-particles on cell apoptosis and cell cycle. (A) The cell apoptosis was detected with flow cytometry. (B) The cell cycle was analyzed by flow cytometry. (C) The expression levels of the proteins were analyzed by western blot. Asterisk** indicates a statistically significant difference at p < 0.01.
concentration and diminishing side effects [9,21]. Compared with free doxorubicin, liposomal nano-particles formulation of doxorubicin showed improved circulation, tumor persistence and half-life, fully reflecting the advantages of nano-particles in target therapy [22]. And DOX-loaded nano-particles make it possible to take DOX by oral administration, with improved suitability and reduced cost of therapy for patients. It’s a noteworthy finding that preparation of the polyelectrolyte complex (poly and chitosan) nano-particles loaded DOX exhibited merits in easy preparation, good stability, organic solvent or surfactant-free, and pH controlled release for DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX, implying the potential application of chitosan coated nano-particles in controlling DOX. As a cell cycle-dependent agent [24], we showed that DOX in the FA-CS-DOX nano-particles arrested the cell cycle at the G2/M phase, which largely affected cell cytokinesis, proliferation and tumor progression.

The G2/M phase is a pivotal checkpoint that correctly controls cell division, and it’s closely associated with p53 [25]. P53 is an important gatekeeper to maintain cell homeostasis, and it’s also the most tumor suppressor devoting in blocking malignant transformation [26]. Correspondingly, p53 mutation is the most common phenomenon found in cancer, being changed in nearly 50% of human malignant neoplasms; and p53 served as a valid target in treating cancers [27]. Previous study has indicated that Hedgehog pathway activation induces tumorigenesis by evasion of p53-mediated tumor-suppressive activity and G2/M cell cycle check-points [28], implying that p53 and cell cycle arrest at G2/M are critical in tumorigenesis. The protein regulator of cytokinesis (PRC1) was identified as a target of p53, and suppressive transcription of PRC1 by p53 induced G2/M arrest of MCF-7 breast cancer cells [17], elucidating the mechanism of cell cycle G2/M arrest induced by p53. PRC1 is a cell cycle protein that plays key roles in the process of cytokinesis, by participating in microtubule formation, and it promoted cancer proliferation and tumourigenesis in hepatocellular carcinoma [29]. In this study, we demonstrated that p53 protein was up-regulated and the PRC1 protein was down-regulated in FA-CS-DOX nano-particles treated hepatoma cell, and the negative regulation between was verified, along with increased apoptosis and G2/M arrest. The effects of p53 and PRC1 on hepatoma cell growth were identified by FA-CS-DOX nano-particles treatment, conforming to the previous cognition of their roles in cancer.

In conclusion, with FA-CS-DOX nano-particles prepared and its influence on cell apoptosis and cell cycle arrest of hepatoma cell evaluated, we confirmed that chitosan-coated doxorubicin nano-particles drug delivery system inhibits cell growth of liver cancer by promoting apoptosis and arresting cell cycle at G2/M phase through p53/PRC1 pathway. But the increased cytotoxicity of the FA-CS-DOX nano-particles should be improved in the future application.

Conflict of interest

The authors declare no conflict of interest.

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