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Research paper

Pharmacodynamics of cisplatin-loaded PLGA nanoparticles administered to tumor-bearing mice

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ABSTRACT

Biodegradable poly (lactic-co-glycolic) acid (PLGA) nanoparticles incorporating cisplatin have been developed to evaluate its *in vivo* efficacy in tumor-bearing mice.

In vitro study proved two mechanisms of action for cisplatin depending on the dose and the rate at which this dose is delivered. *In vivo* study, 5 mg/kg of cisplatin nanoparticles administered to mice, exhibited a tumor inhibition similar to free cisplatin, although the area under cisplatin concentration–time curve between 0 and 21 days (AUC_{0-21}) had lower value for the formulation than for drug solution ($P < 0.05$). This result was associated with a higher activation of apoptosis in tumor, mediated by caspase-3, after nanoparticles administration. Toxicity measured as the change in body weight, and blood urea nitrogen (BUN) plasma levels showed that cisplatin nanoparticles treatment did not induce significant changes in both parameters compared to control, while for free drug, a statistical ($P < 0.01$) increase was observed. In addition, a good correlation was found between time profiles of tumor volume and vascular endothelial growth factor (VEGF) plasma levels, suggesting that its expression could help to follow the efficacy of the treatment. Therefore, the PLGA nanoparticles seem to provide a promising carrier for cisplatin administration avoiding its side effects without a reduction of the efficacy, which was consistent with a higher activation of apoptosis than free drug.

1. Introduction

Cisplatin (CDDP) is widely used as a chemotherapeutic agent to treat a variety of solid tumors [1]. However, its therapeutic effect is dose limiting due to the presence of side effects such as gastrointestinal disturbance and specially nephrotoxicity [2]. In order to avoid the toxicity caused by its systemic administration, different types of controlled release formulation have been designed by several authors to enhance the drug levels in the tumor tissue [3–5]. Particularly, polymeric nano-sized carriers have shown a high tumor-targeting ability [5]. This preferential drug transport of these carriers is explained by the so-called "enhanced permeability and retention" effect, which is caused by the disorganized and increased vascularization of the tumor area [6,7]. Although, intratumoral administration is the best alternative to assure that the compound is effective, in most of the situations that is not possible. In this way, locoregional chemotherapy for adjuvant treatment of colorectal cancer seems to be a promising approach. In this malignant disease, patients have tumors that may spread with metastases in liver and peritoneal cavity [8]. In addition, the administration of the drug in the tumor area could help to increase its uptake due to the angiogenesis process. Vascular endothelial growth factor (VEGF) is a protein directly implicated in this process, especially in colorectal carcinoma. In fact, some authors suggest its expression as a useful marker to follow the tumor behaviour [9].

Controlled release formulations could be an emergent method to improve the delivery of the drug in the tumor target modifying the toxicities associated with a specific drug systemically administered [10]. However, the development of polymeric systems represents a serious problem in relation to the limitation for drug encapsulation. For cisplatin, this process shows several difficulties due to its physico-chemical characteristics, which make that to formulate systems in order to maintain adequate concentrations of this agent for 2 weeks or more, being very difficult [11]. In the last year, Moreno et al. [12] have reported a protocol to develop different PLGA systems for cisplatin using the emulsion solvent evaporation method. These systems were able to induce a different molecular mechanism in relation to the cell cycle, apoptotic factors and their cytotoxic profiles in comparison with free cisplatin in colon cancer cell line, suggesting that these formulations could play a significant role in the efficacy of cisplatin in this type of tumor. Thus, treatments with these formulations were able to induce cell

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arrest in the phase G2/M of the cell cycle followed by a gradual activation of caspase-3, the main apoptotic protein implicated in the cytotoxicity of cisplatin [13–15]. However, the cell arrest after high doses of free drug was in phase G1, which is associated, in part, with resistance phenomenon because some of these cells could be able to re-enter in a new cycle [15].

Therefore, in the present work, the objective was to explore the antitumor efficacy of PLGA nanoparticles incorporating cisplatin in tumor-bearing mice. That efficacy was related to the tumor size, the activation of apoptosis in tumor target and the presence of adverse effects. In addition, the correlation between the expression of VEGF protein and tumor size was also investigated.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymer with a molecular weight (Mw) of 12,000 Da and a co-polymerization rate 50:50 (lactic/glycolic) (Resomer 502H) was purchased from Boehringer Ingelheim (Germany). Cisplatin (cis-diamminedichloride platinum (II)), polyvinyl alcohol (PVA 87–89% hydrolyzed, Mw 13,000–23,000) and Trizma hydrochloride were obtained from Sigma–Aldrich (Madrid, Spain).

2.2. CDDP nanoparticles preparation

PLGA nanoparticles (NP) loaded with cisplatin were prepared by a water–oil–water (w/o/w) emulsion solvent evaporation method [12]. A solution of cisplatin in Tris–HCl (1.67 mg/mL) was emulsified in 0.5 mL of chloroform containing 100 mg of PLGA, using a microtip probe sonicator (Microson XL 2000, Misonix Incorporated, USA) set at level 20 for 5 s. This primary (w/o) emulsion was added to 2 mL of PVA 9% containing 1 mg/mL of cisplatin and mixed by the Ultra-Turrax system (T 20 b, Ika Labortechnik, Germany) at 21,500 rpm for 20 s. The second w/o/w emulsion was transferred drop wise to 8 mL of PVA 9% saturated also with cisplatin (1 mg/mL) and agitated by magnetic stirrer for 3 h at room temperature (RT) until complete evaporation of the organic solvent. NP were collected by ultracentrifugation (40,000*g* for 10 min), washed with water, freeze-dried and stored at −20 °C until use.

To prevent the NP aggregation and retain their redispersibility after lyophilization, a suitable lyoprotectant (mannitol 6% [wt./vol.]) was added before lyophilization [16]. The developed process to formulate these nanoparticles was reproducible because in all preparations with 100 mg of polymer, the final amount of nanoparticles obtained was 72 ± 2.1 mg.

2.3. Characterization of nanoparticles

The size and zeta potential (ζ) of NP were determined by laser diffractometry using a Zetasizer Nano Series (Malvern Instruments, UK) after resuspension in 1 mL of PBS.

The morphology of NP was studied by scanning electron microscopy (SEM). Particles were attached to a double-sided tape and were spray coated with gold prior to inspection under electron microscope.

2.4. In vitro CDDP release profile

The loading of cisplatin in the NP was also quantified by high-performance liquid chromatography (HPLC) method. Weighed samples (5 mg) of NP were dissolved in 1 mL of NaOH (1 N) and kept at RT overnight in continuous agitation by a magnetic stirrer. Afterwards, the solutions were centrifuged at 18,000*g* for 10 min, and the supernatants were analyzed.

Samples of NP (5 mg) dissolved in 1 mL of PBS were placed in a microcentrifuge tube and maintained at 37 °C with constant stirring. At predetermined time intervals, between 0 and 35 days, an aliquot of the supernatant was collected and centrifuged (18,000*g*, 10 min) for analysis, and equal volume of fresh buffer was replaced. Samples were analyzed by HPLC following the method previously described by Moreno et al. [12]. Both studies were carried out by triplicate using three independent experiments.

2.5. In vitro cytotoxicity characterization

2.5.1. Antiproliferative effect

DHD/K12PROb adenocarcinoma colon cell line, obtained from BDIX rats, was grown as adherent monolayers in 25-cm^2 culture flasks at 37 °C in a 5% CO_2 humidified atmosphere and maintained in a mixture of Dulbecco's modified Eagle's and Ham's F-10 medium supplemented with 10% fetal bovine serum and 0.01% gentamicin.

Cells were seeded into 96-well microtiter plates at a density of 20×10^3 cells/well/200 μL and incubated in 5% CO_2 humidified atmosphere at 37 °C for 24 h. To evaluate the dependence of drug activity on concentration and exposure time, a design previously used in this cell line was followed [12]. The plates were treated with serial concentrations (2.5, 10, 18 or 50 μM) of free or encapsulated cisplatin (NP) and incubated for 3, 10, 24, 48, 72 and 144 h. After each treatment, the plates were washed twice with PBS, and the survival cells were quantified using the neutral red assay [17]. Optical density was read at 540 nm using a microtiter plate reader (Labsystems iEMS Reader MF). Wells with untreated cells were also included in each plate. Two linear standard curves, where the number of cells (from 5×10^3 to 100×10^3 and from 1×10^3 to 10×10^3 cells/well) were related to the absorbance measurements, were previously generated.

2.5.2. Cell cycle analysis

DHD/K12PROb cells (25×10^4 cells/well) were seeded into 6-well culture plates following the protocol described earlier. In each plate, wells with untreated cells (control) were also included. After each exposure time, the plates were washed with PBS, and the cells were collected in microtubes and washed by centrifugation (320*g*, 5 min). The pellets were incubated for 30 min at 37 °C, with 100 μL of Tween 20 (0.2% PBS) and 20 μL of ribonuclease type IIA (45 U/mL). Afterwards, the cells were stained with propidium iodide (25 μg/mL) for 10 min in darkness. The analysis of each sample by flow cytometry was performed with FACScan flow cytometer (Becton Dickinson) and CellQuest acquisition/analysis software.

2.5.3. Determination of VEGF and caspase-3 activity

Following the protocol described earlier, DHD/K12PROb cells (20×10^3 cells/well) were seeded into 96-well culture plates (MicroWell™ white-walled plates for luminescence). After each treatment, samples were split up in two parts: supernatants and pellets or cells.

VEGF levels were measured in the supernatant samples using the Quantikine Mouse VEGF Immunoassay Kit (R&D Systems, Inc., Minneapolis, USA) and following the manufacturer's protocol. Microtiter plate reader (Labsystems iEMS Reader MF) was used to measure the samples at 450–540 nm.

Caspase-3 activity was measured in the cell samples using the Caspase-Glo® 3/7 Luminometric Assay Kit (Promega Corporation, Madison, USA) [18]. Samples were washed twice with PBS and then mixed with the reagent provided by Promega, Caspase-Glo® 3/7 (100 μL per well) for 30 min. The RLU (relative light units) was quantified using a microplate Luminometer Orion II (Berthold sys-