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Dual-function baicalin and baicalin-loaded poly(lactic-*co*-glycolic acid) nanoparticles: Immune activation of dendritic cells and arrest of the melanoma cell cycle at the G2/M phase

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ABSTRACT

Accumulating evidence suggests that the flavone glycoside baicalin has immunomodulatory effects and antitumor potential. However, its weak stability in solution, poor absorption, and low bioavailability limit its clinical application. To overcome these disadvantages, we developed baicalin-loaded poly(lactic-*co*-glycolic acid) nanoparticles (PLGA-B) of small size. Next, we evaluated the dual function of immunotherapy and chemotherapy for PLGA-B using immune-related cells and tumor cells. Results showed that PLGA-B were spherical, with a particle size ~120 nm and narrow size distribution with an excellent polydispersity index of 0.103. In vitro experiments revealed that baicalin and PLGA-B could activate dendritic cells (DCs) to have higher expression of surface marker molecules and costimulatory molecules than those of control cells. Baicalin and PLGA-B could trigger apoptosis in melanoma (B16) cells via cell-cycle arrest at the G2/M phase. These data suggest that PLGA-B have important roles in activating DCs and killing melanoma cells. Our study could lay a foundation for melanoma treatment through a combined strategy of immunotherapy and chemotherapy.

Introduction

Traditional Chinese medicines (TCMs) are used widely as adjuvants or as the primary treatment for disease in many hospitals in China (Gao, Morgan, Sanchez-Medina, & Corcoran, 2011). In recent years, several TCMs with potential anti-tumor activities have been reported (Kato et al., 1998; Lian et al., 2003; Yin, Zhou, Jie, Xing, & Zhang, 2004).

The root of *Scutellaria baicalensis* Georgi (Labiatae) is an important TCM. When it is prescribed together with other herbs can work as a diuretic, laxative, febrifuge, an antipyretic, or anti-tumor agent (Chen, Kuo, Tzeng, & Tsai, 2012). Baicalin is from the dry roots of *S. baicalensis*, and has several pharmacologic effects: antibiosis, antiviral, antioxidant, anti-inflammation, anti-tumor, and antibacterial (Coler et al., 2011; Gao et al., 2011; Li-Weber, 2009).

Recently, several studies have suggested that baicalin could elicit immune activation and have anti-tumor effects (Chen et al., 2001; Gao et al., 2011; Nayak et al., 2014; Orzechowska et al., 2014). The actions of many herbal medicines are dose-dependent and high doses can be toxic to healthy cells (Shieh, Cheng, Yen, Chiang, & Lin, 2006). Baicalin is a small-molecule flavone glycoside with low hydrophilicity and lipophilicity which is insoluble in water at room temperature (Wei et al., 2014). The low water-solubility and poor absorption of baicalin have resulted in limited efficacy in various clinical studies (Zhang, Zhao, Chu, Han, & Zhai, 2014), so alternative strategies must be developed.

Nanoparticles, liposomes, microemulsions, and polymeric implantable devices have been proposed to deliver drugs into the systemic circulation (Bansal, Goel, Aqil, Vadhanam, & Gupta, 2011). Nanoparticles can be used as drug carriers upon which various therapeutic agents are "loaded". Nanoparticles can be taken

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up by cells readily and circulate in blood for a long time. Thus, nanoparticles can increase drug accumulation in tumor sites, which further improves the efficacy of cancer treatments (Cao, Zeng, & Zhao, 2016). In addition, motivated by the pivotal role of dendritic cells (DCs) in vaccine-induced immune responses, researchers have designed various nanoparticle drug-delivery systems based on DCs to increase cellular uptake by immune cells as DCs (Guo et al., 2016). Therefore, nanoparticles can improve the efficacy of cancer treatment by immunotherapy and chemotherapy.

Several types of materials are used for nanoparticle fabrication: polysaccharides, proteins, lipids, and synthetic polymers (Qian et al., 2016; Wang, Wu et al., 2014; Yue et al., 2012). Among them, poly(lactic-*co*-glycolic acid) (PLGA) is an US Food and Drug Administration-approved synthetic biodegradable polymer that has gained considerable attention. It has also been used for nanoparticle formation and applied in drug delivery extensively (Cao et al., 2016). In recent years, PLGA nanoparticles have been developed as drug-delivery systems owing to their excellent biocompatibility and biodegradability, good stability, low toxicity, good absorption, and because they can be administered in various forms (Cao et al., 2016; Liu et al., 2015; Luan, Zheng, Yang, Yu, & Zhai, 2015). The efficacy of nanoparticles is influenced by various physicochemical characteristics: particle size, shape, polydispersity index (PDI), encapsulation efficiency (EE), and drug-loading efficiency (LE) (Danhier et al., 2012; Zhao et al., 2013; Zhang, Wang et al., 2014).

We developed baicalin-loaded PLGA nanoparticles (PLGA-B) of small size for immune activation and apoptosis of tumor cells. The nanoparticle system was fabricated to deliver baicalin to DCs and melanoma cells. These PLGA-B nanoparticles were incubated with DCs, and the immunoregulatory activity of nanoparticles on DCs studied. Also, the toxicity of PLGA-B towards melanoma cells was investigated by an apoptosis assay and cell-cycle analyses. These studies could lay a foundation for melanoma treatment through a combined strategy of immunotherapy and chemotherapy.

Materials and methods

Materials, reagents and animals

Baicalin (purity >98% as assessed by high-pressure liquid chromatography (HPLC)) was purchased from Shanxi Anshun Biotechnology (Shanxi, China). PLGA (unit number ratio of lactic acid to glycolic acid = 75/25, weight average molecular weight M_w = 70 kDa) was obtained from Lakeshore Biomaterials (Birmingham, AL, USA). Dichloromethane (analytical reagent (AR) grade) was from Beijing Chemical Reagent Company (Beijing, China). Sodium cholate hydrate from ox bile or sheep bile (purity ≥ 99%) was supplied by Sigma–Aldrich (Saint Louis, MO, USA). Methanol (guaranteed reagent (GR) grade), acetonitrile (GR grade), and phosphoric acid (AR grade) were purchased from Beijing Science Experiment Instruments (Beijing, China). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were supplied by Gibco (Grand Island, NY, USA). Muse™ Count and Viability kit, Muse™ Annexin V and Dead Cell kit and Muse™ Cell Cycle kit were provided by Merck Millipore (Billerica, MA, CA, USA).

Fluorochrome-labeled major histocompatibility complex (MHCII, MHCI), cluster of differentiation (CD86, CD40, and CD11c) antibodies were purchased from eBioscience (San Diego, CA, USA). Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL-4) were obtained from PeproTech (Rocky Hill, NJ, USA). Mouse cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience.

Male C57BL/6 mice (4–6 weeks) were purchased from the Beijing Laboratory Animal Center (Beijing, China) and used for DC isolation. All animals were treated according to regulations set by the Chinese government and the Experimental Animal Ethical Committee of the Chinese Academy of Sciences (Beijing, China).

Preparation and characterization of PLGA nanoparticles

PLGA nanoparticles were fabricated using an ultrasonic double-emulsion method with modification, as described previously (Ho et al., 2008). Briefly, 200 μL of phosphate-buffered saline (PBS; Invitrogen, Carlsbad CA, USA) containing 200 μg of baicalin (internal water phase) was added to 1 mL of dichloromethane dissolving 20 mg of PLGA. A primary water in oil (W/O) emulsion was formed under sonication (120 W; Digital Sonifier 450; Branson Ultrasonics, Danbury, CT, USA) in a tube with an ice bath for 40 s at a duty cycle of 50% (4-s on and 2-s off). The primary W/O emulsion was added to 5-mL of an external water phase containing 2% (w/v) sodium cholate to form a double emulsion (W/O/W) using sonication in a tube with an ice bath for 2 min at a duty cycle of 50%. The double emulsion was magnetically stirred at 450 rpm for 4 h to solidify the nanoparticles.

The mean size and PDI of PLGA nanoparticles were measured by a Nano-ZS Zeta Sizer (Malvern Instruments, Malvern, UK). The morphology of PLGA nanoparticles was characterized by scanning electron microscopy (SEM) using a JEM-6700F (Jeol, Tokyo, Japan) system.

The baicalin content encapsulated into nanoparticles was measured by incubating ~5 mg of freeze-dried nanoparticles in 1 mL of acetonitrile at room temperature for 2 min to degrade the nanoparticles, and the amount of baicalin in the supernatant was assayed using HPLC. A LC-20AT HPLC system equipped with a SPD-M20A PDA detector (Shimadzu, Kyoto, Japan) was employed for sample analysis. The quantitative analysis of baicalin was achieved with a mobile phase consisting of an aqueous phase (0.2% phosphoric acid solution, 47% v/v) and organic phase (100% methanol, 53% v/v) using a C18 analytical column (4.6 × 250 mm, 5 μm; Purosphere, Merck, Darmstadt, Germany) at the flow rate of 1 mL/min and column temperature of 30 °C. Analysis was carried out at 280 nm. The drug-loading efficiency (LE) and encapsulation efficiency (EE) of baicalin in PLGA nanoparticles were calculated as follows:

$$\text{LE}\ (\%) = (\text{weight of baicalin in nanoparticles} / \text{weight of nanoparticles}) \times 100,$$

$$\text{EE}\ (\%) = (\text{actual weight of baicalin encapsulated in nanoparticles} / \text{accrual weight of baicalin added into the system}) \times 100.$$

Activation and maturation of bonemarrow dendritic cells (BMDCs) stimulated with baicalin and PLGA-B in vitro

BMDCs were cultured from bone-marrow cells according to an established protocol (Torres et al., 2011). In brief, bone-marrow cells were isolated from the femurs and tibias of C57BL/6 mouse, and then cultured in RPMI medium 1640 supplemented with GM-CSF and IL-4 (10 and 50 ng/mL, respectively) for 6 days at 37 °C to harvest immature DCs. Immature DCs were cultured further with baicalin and PLGA-B at 37 °C for a specific time.

BMDCs viability was determined using a Muse Cell Analyzer. A Muse Count and Viability kit was applied to assess the viability of

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