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Enhanced anti-tumor activity and reduced toxicity by combination andrographolide and bleomycin in ascitic tumor-bearing mice

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ABSTRACT

Bleomycin (BLM) is an effective anti-carcinogen. With the main detrimental effects of inducing pulmonary fibrosis on patients, its clinical use is limited. Developing agents that enhance the efficacy and attenuate the side effects of cancer chemotherapy are critical. Andrographolide (Andro), an active diterpenoid labdane component extracted from *Andrographis panicula*, is generally prescribed for treatment of inflammatory associated diseases. The study showed that BLM combined with Andro was significantly more effective than BLM alone on inhibiting the tumor growth, arresting the cell cycle at G0/G1 phase, promoting the capase−3 and capase−8 activity to induce cancer cell apoptosis. The underlying mechanisms may be related to the transcriptional regulation of P53/P21/Cyclin pathways. Moreover, BLM induced pulmonary fibrosis in tumor-bearing mice, but BLM combined with Andro dramatically alleviated the lesion in pulmonary fibrosis by activating the SOD, suppressing MDA and HYP production, in the meanwhile attenuating the IL−1β, TNF- α, IL−6 and TGF-β1 level. These mechanisms were associated with its effect on inhibition of protein expression of TGF-β, α-SMA, p–Smad2/3, enhanced expression of Smad7. Thus, it demonstrated that Andro might be a potential adjuvant therapeutic agent for BLM.

1. Introduction

Chemotherapy has been commonly used for treating cancer, which is the second cause of death in the world. So far, despite the chemotherapeutics possess excellent anticancer activity, the clinical use is often limited due to their undesirable side effects on patients (De Besi et al., 1984; Fey, 2014). Therefore, developing agents that enhance the efficacy and attenuate the side effects of cancer chemotherapy is an important area in medical study (Hait et al., 1988; Leonetti et al., 2003; Zhang et al., 2013). Bleomycin (BLM), produced as metabolites of *Streptomyces vertidllus*, is effectively against a broad spectrum of cancers, such as esophageal and squamous cell carcinomas, lymphomas, testicular tumors and malignant pleural effusions(Edsmyr et al., 1973; Erikci et al., 2013; Nikbakhsh et al., 2011). Many studies have confirmed that BLM inhibits the cancer cells proliferation by breaking the double helical structure of DNA or inducing apoptotic body (Antholine et al., 1981). However, with its inducement of dose-dependent pulmonary fibrosis through causing inflammation upon long-term administration, treatment with BLM is very risky, because pulmonary fibrosis is a progressive, increasingly fatal damage and untreatable disease (Chandler, 1990; Jules-Elysee and White, 1990; Sleijfer, 2001).

Andrographolide (Andro), an active diterpenoid labdane component extracted and purified from *Andrographis panicula*, is generally prescribed for treatment of inflammatory associated diseases, including asthma, laryngitis, upper respiratory tract infection and rheumatoid arthritis (Jayakumar et al., 2013). Andro has been found to protect lung from LPS or BLM induced injury

Abbreviations: BLM, bleomycin; Andro, andrographolide; LPS, lipopolysaccharides; IL−1β, interleukin−1β, TGF-β1, transforming growth factor-β1; TNF-α, tumor Necrosis Factor-α; IL−6, interleukin−6; HYP, hydroxyproline; CMC-Na, carboxyl methyl cellulose-Na; MDA, malondialdehyde; SOD, superoxide dismutase; α-SMA, α-smooth muscle actin

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and fibrosis (Zhu et al., 2013a; Zhu et al., 2013b). In addition, this compound has been suggested to have great potential for cancer treatment because it can directly inhibit tumor cells growth through induction of cell cycle arrest, apoptosis and differentiation, it also can indirectly suppress cancer development through immune stimulating, anti-inflammatory, anti-angiogenic and chemo protective properties (Sheeja and Kuttan, 2007; Shi et al., 2008; Zhou et al., 2006). Based on the evidence, we hypothesized that Andro might improve the antitumor ability of BLM, and in the meanwhile, relieve the pulmonary fibrosis induced by BLM side effect. The study was aimed to evaluate whether the Andro enhanced the efficacy of BLM treatment and reduced its major toxicity in ascitic tumor-bearing mice.

2. Materials and methods

2.1. Chemical and reagents

Andrographolide (Andro), with 98% purity, was purchased from Sigma-Aldrich (St. Louis, MO, US). Bleomycin (BLM) hydrochloride was purchased from Nippon Kayaku type strain (Japan). Mouse interleukin−1β (IL−1β), transforming growth factor-β1 (TGF-β1), tumor Necrosis Factor-α (TNF- α), interleukin−6 (IL−6) and hydroxyproline (HYP) enzyme-linked immunosorbent assay kits were offered by eBioscience (CA, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were obtained from Gibco (Grand Island, NY, US). Phosphate buffered saline (PBS), penicillin and streptomycin were offered by Hyclone (Logan, Utah, US). All other reagents and chemicals used in the study were of analytical grade.

2.2. Animals

Male Kunming (KM) mice (18–22 g), were obtained from the Medical Laboratory Animal Center of Guangdong Province, China (Certificate No. SCXK2013–0085). The animals were kept in a controlled environment with temperature (24 ± 1 °C) and relative humidity (50 ± 10%) in an alternation cycle of 12 h light and dark. All animals care and experimental protocols were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care.

2.3. Cell culture

H22 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured with RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 IU/mL streptomycin, 100 IU/mL penicillin at 37 °C in a humidified atmosphere containing 5% CO2. Cells were passaged every 3−4 days.

2.4. Experimental design

KM mice were randomized into A-I groups. The animals were intraperitoneal injected with H22 cells (2×10^6 cells/mouse) to establish the ascitic tumor-bearing model, with the exception of group A (Untreated control), which received normal saline. After 5 days, both group A and B were given 0.5% sodium carboxyl methyl cellulose-Na (CMC-Na), once daily for 7 days. Group B served as the tumor control (Vehicle control). Moreover, Andro was freshly prepared in 0.5% CMC-Na. Groups C–I were respectively injected with BLM alone (15 mg/kg, ip, BLM group), BLM (15 mg/kg, ip) combined with low-dose Andro (25 mg/kg, ig, BLM+Andro-L group), BLM (15 mg/kg, ip) combined with medium-dose Andro (50 mg/kg, ig, BLM+Andro-M group), BLM (15 mg/kg, ip) combined with high-dose Andro (50 mg/kg, ig, BLM+Andro-H group), low-dose Andro alone (25 mg/kg, ig, Andro-L group), medium-dose Andro alone (50 mg/kg, ig, Andro-M group), High-dose Andro alone (100 mg/kg, ig, Andro-H group) by once every day for 7 days. The abdominal diameter and weight were measured every day. All rats were killed at 24 hours after the last dose, the ascites and lung tissues were collected for detection.

2.5. Determination of antitumor activity

After the volume of ascites was recorded, ascites from each group were centrifuged at 4 °C 3000 rpm, 3 min, then washed 2 times by PBS. Cells viability was analyzed using 0.2% Trypan Blue staining and detected by Countstar Automated Cell Counter (Shanghai, China) and the cells were harvested to examine biochemical index.

2.5.1. Flow cytometry analysis of cell apoptosis

Cellular apoptosis was evaluated by using annexin V-FITC apoptosis detection kit (MultiSciences Biotech, Hanzhou, China) and flow cytometry. Briefly, after treatment, cells in all groups were collected, centrifuged and washed twice with ice-cold PBS, and then resuspended in binding buffer. Finally, cells were subjected to Annexin V-FITC-propidium iodide (PI) double staining as described by the manufacture's instruction, and analyzed by flow cytometry (BD Biosciences, CA, USA). Data were analyzed by using CXP software (BD Biosciences, CA, USA). Quadrants were positioned on Annexin V-FITC/PI dot plots, allowing living cells (Annexin V-FITC−/PI−), early/primary apoptotic cells (Annexin V-FITC+/PI−), late/secondary apoptotic cells (Annexin V-FITC+/PI+) and necrotic cells (Annexin V-FITC−/PI+) to be distinguished. The cell populations of annexin V-FITC+/PI– and Annexin V -FITC+/PI+were calculated to represent apoptotic cells.

2.5.2. Cell cycle analysis

The stages of the cell cycle were tested by flow cytometry according to the protocol provided by the Cell cycle staining kit (MultiSciences Biotech, Hanzhou, China). Briefly, the 1 × 106 cells were centrifuged and washed twice with PBS, then resuspended in 1 mL PBS, and pipetted slowly into 3 mL absolute ethanol while vortexing at top speed, finally collected by centrifuging. After washed with PBS, cells were blended with 1 mL reagent A and 10 μl reagent B for 5−10 s, incubated for 30 min and detected by flow cytometry (BD Biosciences, CA, USA). Propidium iodide (PI) staining data were analyzed by using FlowJo software 7.6.2. Thereafter, some other cells were centrifuged and resuspended in fresh buffer, suspension was dropped in microscope slide and covered with a coverslip. The fluorescein changes in cells were observed by fluorescence microscopy (Olympus, Japan).

2.5.3. Measurement of caspase−3, caspase−8 activity

Caspase−3 and Caspase−8 activity under various experimental conditions were respectively measured in cells extracts using colorimetric assay kits from Byotime Biotechnology (Shanghai, China). The levels of chromophore *p*-nitroanilide (*p*NA), after cleavage from the labeled substrate of caspase−3 (acetyl-Asp-Glu-Val-Asp*p*-nitroanilide, Ac-DEVD-*p*NA) or the labeled substrate of caspase−8 ((acetyl-Ile-Glu-Thr-Asp*p*-nitroanilide, Ac-IETD-*p*NA), were respectively determined by using a spectrophotometry (λ=405 nm).

2.5.4. Quantitative real-time PCR

Ascitic cells from tumor-bearing mice in each group were treatment with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from 1.5 μg of total RNA of each cell sample using a high capacity cDNA reverse transcription Kit (Applied Biosystems, Branchburg, NJ, US) according to the