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Lipopolysaccharides-stimulated macrophage products enhance Withaferin A-induced apoptosis via activation of caspases and inhibition of NF-κB pathway in human cancer cells

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ABSTRACT

Macrophages, as a major cellular component in tumor microenvironment, play an important role in tumor progression. However, their roles in modulation of cytotoxic chemotherapy are still not fully understood. Here, we investigated the influence of Lipoplysaccharides (LPS)-stimulated macrophage products (LSMP) on Withaferin A (WA), a natural compound that derived from the medicinal plant Withania somnifera, as an antitumor agent in human breast cancer cells MDA-MB-231 and prostate cancer cells PC-3. Our results revealed that LSMP may enhance WA-induced apoptosis in both cell lines, the underlying mechanisms of which are closely associated with activation of caspase-8, −9 and −3, cleavage of poly ADP-ribose polymerase (PARP), as well as specifically inhibiting the translocation of nuclear factor-κB (NF-κB) and down-regulation of anti-apoptotic proteins X-linked inhibitor of apoptosis protein (XIAP) and inhibitor of apoptosis protein (cIAP1/2). These findings demonstrate that macrophages in tumor microenvironment can modulate tumor responses to chemotoxic agents, providing an effective strategy that targets macrophages to enhance the antitumor efficacy of cytotoxic chemotherapy.

1. Introduction

Macrophages in tumor microenvironment, commonly termed tumor-associated macrophages (TAMs), are believed to be closely implicated in tumor progression, like angiogenesis, immunosuppression, invasion, and metastasis (De Palma and Lewis, 2013), which are mediated by expressing numerous cytokines, chemokines, polypeptide growth factors, hormones, proteolytic enzymes (Gordon and Martinez, 2010; Murray and Wynn, 2011). Usually, the presence of macrophages in tumor microenvironment represents an unfavorable prognosis for cancer patients (Clear et al., 2010; Lewis et al., 2016). Because of highly functional and phenotypic plasticity, macrophages are generally classified into M1 and M2 phenotypes (Mosser and Edwards, 2008). M1 macrophages are classically activated by interferon-gamma (IFN-γ), Lipoplysaccharides (LPS) or tumor necrosis factor-alpha (TNF-α). They exert host defense functions through their bactericidal and tumoricidal activity and pro-inflammatory cytokine production such as interleukin(IL)-6, IL-12, IL-23 and TNF-α (Pahl et al., 2014; Ricardo et al., 2008). In contrast, M2 macrophages are involved in anti-inflammatory response, angiogenesis and tumor progression. Of course, that's a more complex process that the phenotype can vary in different tumor microenvironment (Ricardo et al., 2008; Mantovani et al., 2004). Generally, macrophages in tumor microenvironment display M2 phenotype with tumor-promoting activity (Biswas and Mantovani, 2010).

The macrophages activated with LPS can be implicated in antitumor activities. The research suggested that human macrophages induced by LPS + IFN-γ exerted direct antitumor activity against osteosarcoma cells (Pahl et al., 2014). Another research confirmed that LPS-stimulated macrophages showed significantly higher expression of M1 phenotype marker human leukocyte antigen-DR (HLA-DR) (Kumar et al., 2015). Bacillus Calmette-Guíerin (BCG)-induced macrophage could have cytotoxic effect on the bladder cancer cells through direct effector-target cell contact and macrophages releasing soluble cytotoxic factors, such as TNF-α, IFN-γ and nitric monoxide (NO), and TNF-α plays a positive

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role in tumoricidal activity of BCG-induced macrophage (Luo and Knudson, 2010). Therefore, targeting macrophages is an attractive strategy to complement current antitumor treatments.

Withaferin A (WA), a steroidal lactone purified from medicinal plant Withania somnifera, has been identified as a biologically active substance with anti-inflammation, antitumor (Vanden Berghe et al., 2012; Li et al., 2015; Lee et al., 2012; SoRelle et al., 2013), immunomodulation (Agarwal et al., 1999), cardioprotection (Gupta et al., 2004) and neuroprotection (Ahmad et al., 2005). It's antitumor activity has been well-documented in a wide spectrum of tumor cells, and the underlying mechanisms of which are involved in inducing apoptosis by inhibiting the nuclear factor-κB (NF-κB) activation (Mohan et al., 2004; Jackson et al., 2015), chymotrypsin-like activity of proteasome (Yang et al., 2007), reactive oxygen species (ROS) generation (Li et al., 2015) and p38 MAP kinase activation (Mandal et al., 2008).

Our previous research showed that TNF-a could enhance human breast cancer cells MDA-MB-231 to WA-induced apoptosis (Lu et al., 2014). LPS-stimulated macrophages have tumoricidal activity through releasing pro-inflammatory cytokines such as IL-6, IL-12, IL-23 and TNF-α (Chanmee et al., 2014). In the present study, we therefore investigated the effects of LSMP on WA-induced apoptosis in human breast cancer cells MDA-MB-231 and prostate cancer cells PC-3 and the underlying molecular mechanisms. The results demonstrate that the effects of macrophages on the antitumor efficacy of cytotoxic agents, which provide an effective strategy that targets macrophages to complement current antitumor treatments.

2. Materials and methods

2.1. Reagents

WA and LPS were purchased from A. G. Scientific, Inc. (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and Phosphate Buffer Saline (PBS, pH7.5), respectively. 50 mM stock solutions were prepared and aliquots were stored at −20 °C for further use in the experiments. L15 (Leibovitz's L-15 with L-glutamine), RPMI 1640 medium, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Gibco Technology (Brasil, USA). Bisbenzimide, methylene blue, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT), RNaseA, reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) kits were purchased from Promega (San Luis Obispo, CA, USA). Mouse monoclonal antibodies against human poly ADP-ribose polymerase (PARP), ubiquitins (Ubs), NF-κB inhibitor alpha (IκBα), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), caspase-3, caspase-9, X-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein (cIAP1/2) as well as secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

2.2. Macrophage products preparation

Female Balb/c mice, aged 4–6 weeks, were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China) and maintained in a room with constant temperature. All the experiments were conducted in accordance with institutional guidelines and were approved by Guangzhou Medical University Ethics Committee. In the sterile environment, the mice were killed by rapid cervical dislocation after general anesthesia, followed by disinfection with 75% alcohol. 5 ml of ice-cold PBS was injected into the peritoneal cavity of each mouse. After the peritoneal cavity was massaged to dislodge loosely adherent peritoneal cells, the cell suspension was then collected from the peritoneal cavity with a needle and syringe (Zhang et al., 2008). The suspension was centrifuged at 161 × *g* for 5 min to obtain cell pellet, which was then resuspended in 1 ml RPMI 1640 medium containing 10% FBS and inoculated in 60 mm dishes at 3×10^6 cells/dish. The supernatant was discarded following 2 h culture and the adherent cells were macrophages. The macrophages were incubated in complete medium (RPMI 1640 plus 10% FBS) that containing LPS with a final concentration of 10 μg/ml for 6 h at 37 °C with 5% CO_2. Finally, the collected supernatants were LSMP.

2.3. Cell culture and whole cell extract preparation

Human breast cancer MDA-MB-231 cells and human prostate cancer PC-3 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MDA-MB-231 cells and PC-3 cells were cultured in L15 and RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, respectively. MDA-MB-231 cells were maintained at 37 °C without CO_2 atmosphere and PC-3 cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Whole cell extracts were prepared as described previously (Xiao et al., 2008). Briefly, cells were harvested, washed with PBS and homogenized for 30 min at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). The homogenates were immediately centrifuged at 13,523 × *g* for 12 min at 4 °C and the supernatants were collected as whole cell extracts. The bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein concentration and bovine serum albumin (BSA) were employed as a standard.

2.4. Cell proliferation assay

MTT assays were performed to evaluate the effect of WA or LSMP on cell proliferation and determined using the MTT assay as described elsewhere (Lu et al., 2011). Briefly, 3.5×10^3 MDA-MB-231 cells and PC-3 cells were seeded into 96-well plates, respectively. After incubation overnight, the cells were treated with indicated concentrations of WA (1, 2 μM), indicated concentrations (final concentrations was 10%, 30%, 50%, 100%) of LSMP or WA plus LSMP for 48 h and the DMSO treated cells were taken as controls. The absorbance was measured with a microplate reader (Sunrise, Tecan) at 540 nm, and cell viability was expressed relative to control cells treated with DMSO. The experiments were performed in quadruplicate and repeated at least three times to assess for consistency of response. The IC50 was calculated by Origin 7.0 software (OriginLab, Northampton, MA, USA).

2.5. Clonogenic assay

The assay was performed as previously described (Lu et al., 2011). Briefly, MDA-MB-231 cells or PC-3 cells were exposed to different concentrations of WA, different concentrations of LSMP or WA plus LSMP for 24 h. Subsequently, the cells were suspended in 30% agarose supplemented with 20% FCS and 50% L15 (for MDA-MB-231 cells) or RPMI-1640 medium (for PC-3 cells), and then cultured in 60 mm dishes for 10 days. The colonies larger than 60 μm in diameter were calculated and images were taken. The experiments were performed in triplicate.

2.6. Scratch wound-healing assay

For a scratch wound healing assay which was performed as mentioned before (Lu et al., 2014), MDA-MB-231 cells or PC-3 cells