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Research paper

Resveratrol inhibits lung cancer growth by suppressing M2-like polarization of tumor associated macrophages

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ABSTRACT

In cancer, tumor associated macrophages (TAMs) play an important role in the cancer progression, evasion of immunity and dissemination of cancer cells. Inhibition of the activation or the M2 polarization of TAMs is an effective therapy for cancer. In the present study, we investigated the ability of resveratrol (RES) to inhibit lung cancer growth using *in vitro* and *in vivo* studies, and examined the underlying mechanisms. We demonstrated that M2 polarization of human monocyte derived macrophage (HMDMs) induced by the lung cancer cells conditioned medium was inhibited by RES. Additionally, RES exhibited inhibitory function in lung cancer cells co-cultured with human macrophages. The activity of signal transducer and activator of transcription 3 (STAT3) was significantly decreased by RES. Moreover, in a mouse lung cancer xenograft model, RES significantly inhibited the tumor growth, which was associated with inhibition of cell proliferation and decreased expression of p-STAT3 in tumor tissues. Further, RES inhibits F4/80 positive expressing cells and M2 polarization in the tumors. These results suggest that RES can effectively inhibit lung cancer progression by suppressing the protumor activation of TAMs.

1. Introduction

Despite advances in the clinical management of lung cancer, lung cancer remains as the leading cause of cancer-related deaths worldwide [1]. It is increasingly recognized that the interaction between tumor and immune effector cells enhances tumor growth, invasion and local immunosuppression, to evade from antitumor immune responses, which is also essential to achieve effective extravasation at prospective metastatic sites [2]. Thus primary tumors, and possibly, circulating tumor cells (CTCs), are capable of recruiting immunosuppressive cells particularly of the myeloid-macrophage lineage. These immunosuppressive cells undergo functional polarization driven by tumor-derived factors [3]. These "tumor-educated" macrophages are involved in all stages of cancer metastasis: cancer invasion, intravasation, survival in the circulation, and sustainable growth at secondary lesions are all promoted by these macrophages [4]. Because of their existence as a major cellular component of murine and human tumors, they are commonly termed tumor-associated macrophages (TAMs). Originally, TAMs are proposed to be involved in anti-tumor immunity, whereas recent evidences unveiled the tumor-promoting effect of TAMs [5,6]. Epidemiological evidences indicate a strong link between increased macrophage infiltration and poor prognosis in lung cancer [7]. TAM density was found to be negatively correlated with poor patient survival and frequent recurrence [8]. The median survival of patients with high macrophages density was significantly shorter than those who had a lower macrophages density [7]. These studies suggested that TAMs play a crucial role in cancer development.

Plasticity and flexibility are key indicators of the activation states of macrophages. Whether TAMs assume tumor-preventing or tumor-promoting role is dependent on their polarization statues [9]. The activation states, M1 and M2, denote classical activation and alternative activation of macrophages, respectively. In benign or regressive tumors, the majority of TAMs are classically activated macrophages (M1-like), which exert pro-inflammatory activity by antigen-presenting and promoting tumor lysis [10]. Conversely, TAMs in malignant tumors resemble alternatively activated macrophages (M2-like), enhancing tumor angiogenesis, migration and invasion. M2-like macrophages suppress antitumor immune responses. Thus, M2-like TAMs are promising targets for adjuvant anticancer therapies. Encouraging anti-tumor efficacy has been achieved by recent M2-TAM-targeting approaches. For example,

Abbreviations: TAMs, tumor associated macrophages; RES, resveratrol; STAT3, signal transducer and activator of transcription 3.

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targeted delivery of peptide to M2-like TAM demonstrated improvement in the survival of tumor bearing mouse [11]. In another study, *in vitro* co-culturing of cancer cells with M2 macrophages showed activation of signal transducer and activator of transcription 3 (STAT3), which is an important modulator of tumor progression and chemo-resistance in cancer cells [12]. M2 macrophages activated by cancer cells secrete several cytokines, such as IL-6 and IL-10, which in turn promote cancer progression [13,14]. Therefore, inhibiting macrophage polarization into the M2 phenotype, and blocking the tumor-macrophage interaction could be a viable approach toward efficient cancer therapy.

Resveratrol (RES) possess multiple pharmacological activities in extending longevity and treatment of cardiovascular disease, diabetes and cancer [15]. RES is a polyphenol that naturally exists in grapes and red wine. The investigation of RES originated from studies on 'French Paradox', which describes beneficial cardiovascular outcomes of a high-fat diet in French cuisine [16]. Its anti-cancer effects have been well documented in a variety of cancers, whereby RES regulated cell division, growth, angiogenesis and metastasis [17]. In lung cancer, RES was shown to promote premature senescence in cancer cells (A549 and H460 cells) via inducing NAPDH oxidase-5 (Nox5) [18], which resulted in inhibition of cancer cell proliferation and survival [19]. Recent studies showed that RES exerts anti-tumor effects by regulating the release of cytokines. RES selectively inhibits or activates the release of cytokines. RES-suppressed cytokines include IL-6, IL-12 and TNF-α [20,21]. However, the beneficial effects of RES in lung cancer remain to be clarified.

In the present study, we investigated the ability of resveratrol to inhibit lung cancer growth using *in vitro* and *in vivo* models. Given that M2 macrophages are closely associated in cancer progression, we also investigated the relevance between macrophage polarization and the antitumor effects of RES.

2. Materials and methods

2.1. Cell culture

Human lung cancer cells, A549 and H1299, were acquired from the American Type Culture Collection (Manassas, VA, USA). Lewis lung cancer (LLC) cells were obtained from the Cell Bank of the China Science Academy (Shanghai, China). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and regularly checked for *Mycoplasma* contamination. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Peripheral blood mononuclear cells were collected from healthy volunteer donors, with written informed consent filed for all sample collection. Subsequent analysis was performed on all healthy donors. Experiments regarding human studies were performed according to protocols approved by the Tianjin Huanhu Hospital Review Board. $CD14^+$ monocytes were isolated from the peripheral blood mononuclear cells using magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) based on positive selection, followed by co-culturing with GM-CSF (10 ng/ml, Wako, Tokyo, Japan) or M-CSF (50 ng/ml, WAKO) for a week for macrophage differentiation. The differentiated macrophages were then used as human monocyte-derived macrophages (HMDMs) in the present study, as described previously [22].

2.2. Preparation of the tumor conditioned medium (TCM)

The tumor conditioned medium was acquired from the supernatant of A549 cultured medium after 72 h of incubation. The conditioned media was collected from the Transwell insert systems, followed by filtration through 0.2 μm pores (Sartorius Stedim Biotech, Germany). The TCM was stored at −80 °C until further usage in the ELISA assays.

2.3. Cytokine ELISA kit

HMDMs were seeded in 12-well plates at the density of 3×10^5 cells/well. HMDM cells were subjected to lipopolysaccharide (LPS) (100 ng/mL, Sigma, Louis, MO, USA) stimulation for 24 h, followed by incubation with TCM or with RES-containing TCM (Sigma, Louis, MO, USA) for 24 h. ELISA was used to determine IL-10, IL-12 and TNF-α secretion according to manufacturer's guidelines (eBioscience, San Diego, CA, USA).

2.4. Cell proliferation assay

Viability assay was performed on A549 and H1299 cells using the Cell-Light EdU Apollo488 *In Vitro* Imaging Kit (RiboBio) in accordance to the manufacturer's protocol. Briefly, cells incubated with 10 μM EdU for 2 h were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.3% Triton X-100 and stained with EdU. Cell nucleus were stained by 10-min-incubation with 5 μg/mL DAPI (4′,6-diamidino-2-phenylindole). Number counting of Edu-positive cells was counted under a light microscope based on five random fields (×100). All assays were performed in triplicate independently.

2.5. MTT assay

MTT assay was used to determine the effect of RES on the viability of A549 and H1299 cells. Briefly, cells were treated with RES or STAT3 inhibitor (WP1066) (Sigma, Louis, MO, USA) for 48 h. At the end of treatment, 50 μL of 5 mg/mL MTT were added and the resulting formazan crystals were dissolved in 100 μL of DMSO, followed by measuring absorbance at 570 nm by an Automated Microplate Reader (Bio-Tek, USA). The cell viability was calculated using the following equation: cell viability (%) = (OD of treated cells/OD of control cells) × 100. All assays were performed in triplicate with at least 3 independent experiments.

2.6. RNA isolation, reverse transcription and qRT-PCR

Total RNA from BMDM was extracted and purified using the Easy Pure RNA Kit (Transgen Biotech Co., Ltd), followed by cDNA synthesis. Quantitative real time-PCR (qRT-PCR) was carried out using SYBR Premix ExTaq (TaKaRa, Dalian, China) on a Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., CA, USA). All mRNA levels were normalized to the GAPDH level. The quantification of mRNA levels were performed using the $2^{-\Delta\Delta CT}$ method and normalized to GADPH. PCR reactions were performed in triplicates independently. Primers used in PCR reaction were as follows: *MRC1*, forward primer: 5′-AGG GAC CTG GAT GGA TGA CA-3′; and reverse primer: 5′-TGT ACC GCA CCC TCC ATC TA-3′; *CCL24*, forward primer: 5′-TGT CTG CAG TTG AGC CTA CG-3′; and reverse primer: 5′-GTT CGG GAC CCT GGA GTT AG-3′; *chil3*, forward primer: 5′-CAT GAG CAA GAC TTG CGT GAC-3′; and reverse primer: 5′-GGT CCA AAC TTC CAT CCT CCA-3′; *Retnla*, forward primer: 5′-CCC TGC TGG GAT GAC TGC TA-3′; and reverse primer: 5′-TGC AAG TAT CTC CAC TCT GGA TCT-3′; *IL-10*, forward primer: 5′-ACT GGC ATG AGG ATC AGC AG-3′ and reverse primer: 5′-CTC CTT GAT TTC TGG GCC AT-3′; *Arg1*, forward primer: 5′-GAT TAT CGG AGC GCC TTT CT-3′ and reverse primer: 5′-CCA CAC TGA CTC TTC CAT TCT-3′; *CD206*, forward primer: 5′-CTG CAG ATG GGT GGG TTA TT-3′ and reverse primer: 5′-GGC ATT GAT GCT GCT GTT ATG-3′; *GAPDH*, forward primer: 5′-CAG CCT CAA GAT CAT CAG CA-3′ and reverse primer: 5′-TGT GGT CAT GAG TCC TTC CA-3′.