Carnosic acid enhances the anti-lung cancer effect of cisplatin by inhibiting myeloid-derived suppressor cells

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[ABSTRACT] Cisplatin and other platinum-based drugs are used frequently for treatment of lung cancer. However, their clinical performance are usually limited by drug resistance or toxic effects. Carnosic acid, a polyphenolic diterpene isolated from Rosemary (Rosemarinus officinalis), has been reported to have several pharmacological and biological activities. In the present study, the combination effect of cisplatin plus carnosic acid on mouse LLC (Lewis lung cancer) xenografts and possible underlying mechanism of action were examined. LLC-bearing mice were treated with intraperitoneal injection with cisplatin, oral gavage with carnosic acid, or combination with cisplatin and carnosic acid, respectively. Combination of carnosic acid and cisplatin yielded significantly better anti-growth and pro-apoptotic effects on LLC xenografts than drugs alone. Mechanistic study showed that carnosic acid treatment boosted the function of CD8+ T cells as evidenced by higher IFN-γ secretion and higher expression of FasL, perforin as well as granzyme B. In the meantime, the proportion of MDSC (myeloid-derived suppressor cells) in tumor tissues were reduced by carnosic acid treatment and the mRNA levels of iNOS2, Arg-1, and MMP9, which are the functional markers for MDSC, were reduced. In conclusion, our study proved that the functional suppression of MDSC by carnosic acid promoted the lethality of CD8+ T cells, which contributed to the enhancement of anti-lung cancer effect of cisplatin.

[KEYWORDS] Lung cancer; Carnosic acid; Cisplatin; MDSC; CD8+ T cell

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Introduction

Lung cancer, including non-small-cell lung cancer (NSCLC) or small-cell lung cancer, is a concerned public health challenge for its high morbidity and mortality. American Cancer Society estimated that there will be about 23,4030 new cases and 15,4050 deaths for lung cancer in the USA in 2018 [1]. Surgery, radiation, chemotherapy as well as targeted therapies are used for lung cancer treatment [2]. Platinum-based chemotherapy consists the main part among systemic therapy for lung cancer. Unfortunately, serious side effects like peripheral neuropathy as well as the intrinsic or acquired resistance limit the applications of cisplatin. Therefore, novel agents with less toxicity and fewer side effects for lung cancer treatment are so much impending [3-4].

In recent years, immunotherapy has shown exciting outcomes for lung cancer treatment. Immune checkpoint inhibitors (Nivolumab, Keytruda, and Atezolizumab) have been approved by US FDA for NSCLC treatment after failure of the first treatment. In September 2017, the combination of chemotherapy and immunotherapy (Keytruda) as a first line treatment for metastatic, nonsquamous, non-small cell lung cancer patients was also approved. These advancements have suggested that immunotherapy should be a powerful weapon for lung cancer therapy. Nowadays, more new strategies for boosting anti-tumor immunity are being developed. Besides immune checkpoint on T cells, MDSC (Myeloid-derived suppressor cells), NK cells, Treg cells, and IDO1 are hot targets, which are being developed in preclinical and/or clinical trials [5-9].

Carnosic acid, a naturally polyphenolic diterpene derived from the rosemary plant, has drawn tremendous attention for its pharmacological activities, including anti-oxidation, anti-in-
flammation, anti-tumor activities with good tolerance and safety.\textsuperscript{10-12} An increasing amount of evidence has shown the inhibitory effect of carnosic acid on tumor. Carnosic acid inhibits the proliferation and migration capacity of human colorectal cancer cells\textsuperscript{13}. In addition, carnosic acid induces apoptosis of hepatocellular carcinoma cells via ROS-mediated mitochondrial pathway\textsuperscript{14} as well as endoplasmic reticulum stress mediated apoptosis in human renal carcinoma Caki cells\textsuperscript{15}. It can also induce autophagic cell death through inhibition of the Akt/mTOR pathway in human hepatoma cells\textsuperscript{16}. Furthermore, carnosic acid could enhance the anti-tumor effect of other regents, such as adriamycin, fisetin, and tamoxifen\textsuperscript{17-19}.

In the present study, we demonstrated that carnosic acid inhibited infiltration and function of MDSC and enhanced function of CD8\textsuperscript{+} cytotoxic T cells, leading to enhanced anti-tumor immune-response and boosted the anti-lung cancer effect of cisplatin.

Materials and Methods

Reagents

Mouse Lewis lung cancer (LLC) cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) containing 10\% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and antibiotics (100 U⋅mL\textsuperscript{-1} of penicillin and 100 U⋅mL\textsuperscript{-1} of streptomycin) in a humidified atmosphere of 5\% CO\textsubscript{2} at 37 °C. 4'-Diamidino-2-phenylindole (DAPI) and carnosic acid (CA) were purchased from Sigma Aldrich (St. Louis, MO, USA). TUNEL assay kit was bought from Vanzyme Biotech Co., Ltd. (Nanjing, China). Immunohistochemical analysis KIT was bought from Gene Tech Co., Ltd (Shanghai, China). CD45.2-PE, Gr1-FITC, CD11b-APC, CD8-FITC, and IFN-γ-PE were obtained from eBioscience (San Diego, CA, USA). The antibodies against Granzyme B (#17215) was bought from Cell Signaling Technology Inc (Danvers, MA, USA). The antibodies against PCNA (sc-56) and β-actin (sc-517582) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

C57BL/6 mice (6-8 weeks old) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China), which were maintained with free access to pellet food and water in plastic cages at room temperature of 21 ± 2 °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States). The protocol was approved by the Nanjing University Animal Care and Use Committee (NJU-ACUC). All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

\textit{In vivo} tumor growth assay

Mouse LLC (Lewis lung cancer) cells (5 × 10\textsuperscript{6} cells in 0.1 mL) were injected into the right flank of C57BL/6 mice. Once the tumor reached an average size of 50 mm\textsuperscript{3}, the mice were distributed into 4 groups (n = 8 per group). Carnosic acid (CA) was suspended in 0.9\% carboxymethylcellulose sodium (CMC-Na) solution and administered at the dose of 10 mg⋅kg\textsuperscript{-1} per day by gavage once daily for 16 days. Cisplatin (1 mg⋅kg\textsuperscript{-1}) were given every three days via intraperitoneal injection. The vehicle group was administered with 0.9\% CMC-Na solution. Tumor volumes were measured every 2 days and calculated using the following formula: 0.5 × L × W\textsuperscript{2}, where L and W are the long and short diameters of the tumor. On the 16th day, the mice were sacrificed and solid tumors were separated.

\textbf{Histological analysis}

Tumor tissue samples were formalin-fixed, paraﬁn embeed, cut into sections and stained with hematoxylin and eosin (H&E) following a standard protocol\textsuperscript{20}. The section slides were visualized by IX73 microscopy (Olympus, Tokyo, Japan).

\textbf{Immunofluorescence}

Paraﬁn-embedded tumor sections were de-waxed and washed with PBS and then incubated with 3\% goat serum in PBST (PBS containing 0.1\% Triton X-100). After incubation, the cells were stained with anti-cleaved caspase-3 antibody or isotype control rabbit IgG (1 : 50) and detected with a secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1 : 300) (Thermo Fisher Scientiﬁc, MA, USA). The images were observed under ﬂuorescence microscopy (IX73; Olympus, Tokyo, Japan).

\textbf{Immunohistochemistry}

Paraﬁn-embedded tumor sections were stained with PCNA antibody (1 : 400). Detection was carried out by using GTVisin\textsuperscript{TM} immunohistochemical analysis KIT (Gene Tech Co., Ltd., Shanghai, China). The images were observed under a microscope (IX73; Olympus, Japan).

\textbf{TUNEL assay}

Paraﬁn-embedded tumor sections were stained with TUNEL-FITC (1 : 50) and then the nuclei were stained with DAPI (2 min). Fluorescence images were acquired under an IX73 microscope (Olympus, Japan).

\textbf{Quantitative real-time PCR}

Total RNA was extracted from cells using Trizol reagent and reversed to cDNA and subjected to quantitative PCR with the BioRad CFX96 Touch\textsuperscript{TM} Real-Time PCR Detection System (BioRad, CA, USA) using iQ\textsuperscript{TM} SYBR® Green Supermix. The threshold cycle numbers were calculated using BioRad CFX Manager software. The program for amplification was 1 cycle of 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 95 °C for 10 s. The primer sequences were listed as follows:

- \textit{Perforin}
  - Forward: 5'-CGTGAAGTCAACAAAAGAAG-3';
  - Reverse: 5'-CGTGCCTTCCATCCATCCAG-3';
- \textit{Granzyme B}
  - Forward: 5'-CACTCCAGTTGACGACT-3';
  - Reverse: 5'-GGAAGATAGGCCTGTGGTAAG-3';
- \textit{Carnosic acid}
  - Forward: 5'-CTGCTAAAGCTGAAGTAAGG-3';
  - Reverse: 5'-ACCTCCTTGTACGTTCATTGAG-3';
Arg-1 forward 5′-AACACGGCAGTGGCTTTAACC-3′;
Arg-1 reverse 5′-GGTTTTCATGRRGCGCATT-3′.
MMP9 forward 5′-CATCGAATTGGACACTGA-3′;
MMP9 reverse 5′-AGCCACGAACCATACAGATAC-3′.
iNOS2 forward 5′-CGAAACGCTTCACTTCCAA-3′;
iNOS2 reverse 5′-TGAGCCTATATTGCTGTGGCT-3′.
β-actin forward 5′-TGCTGTCCCTGTATGCCTCT-3′;
β-actin reverse 5′-TTTGATGTCACGCACGATTT-3′.

Isolation of tumor-infiltrating lymphocytes
Tumor tissues were minced and digested with 0.5 mg·mL⁻¹ of collagenase IV (Sigma-Aldrich, MO, USA) and 0.01 mg·mL⁻¹ DNaseI (Roche, Basel, Switzerland) in RPMI 1640/5% FCS for 1 h at 37 °C. The cell suspension was then filtered through a 70 μm nylon mesh, layered on a Percoll gradient (30%−70%) and centrifuged at 800 g for 20 min. The separated tumor infiltrating lymphocyte (TIL) fraction was washed twice with PBS before use. The CD45.2⁺ cells represented the total leukocyte population.

FACS analysis
Splenocytes and tumor-infiltrating lymphocytes were incubated with specific surface-binding antibodies for 15-20 min at room temperature. For intracellular IFN-γ staining, the cells were stimulated with 100 ng·mL⁻¹ of PMA (Sigma-Aldrich) and 500 ng·mL⁻¹ of ionomycin (Sigma-Aldrich) in the presence of monensin (Becton, Dickinson and Company, NJ, USA) for 4 h. The washed cells were then fixed and permeabilized using a Cytofix/Cytoperm Kit (BD Pharmingen) after labeling with surface marker antibodies, followed by anti-IFN-γ mAb staining. The samples were analyzed by flow cytometry on a FACSscan flow cytometer (Becton, Dickinson and Company, NJ, USA).

Statistical analysis
All the data were represented as means ± SEM of three independent experiments. Comparisons were made by one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

Results
Carnosic acid in combination with cisplatin treatment suppresses LLC growth in vivo
To examine the antitumor activity of carnosic acid in combination with cisplatin in vivo, a mouse xenograft model of LLC cells growth in C57BL/6 mice was employed. As shown in Fig. 1A, 1 mg·kg⁻¹ of cisplatin led to significant decrease in the growth of LLC xenografts, while 10 mg·kg⁻¹ carnosic acid had minor effects. Interestingly, combination of 1 mg·kg⁻¹ of cisplatin with 10 mg·kg⁻¹ of carnosic acid had significantly better effect than 1 mg·kg⁻¹ of cisplatin (Fig. 1A). Specifically, the mean tumor weight dropped from 0.57 g (vehicle control group) to 0.25 g (carnosic acid + cisplatin) while cisplatin group decreased to 0.40 g (Fig. 1C). The representative pictures of tumor after dissection also showed the shrink of tumor volume by combination treatment (Fig. 1D). It should be noted that cisplatin significantly decreased the body weight of LLC-bearing mice while carnosic acid alleviated the loss of body weight induced by cisplatin (Fig. 1B). These results implied that carnosic acid enhanced the anti-growth effects of cisplatin on LLC xenografts and reduced the side effects of cisplatin.

Fig. 1  Carnosic acid in combination with cisplatin treatment suppressed LLC growth in vivo. LLC cells were injected into the right flank of C57BL/6 mice. 72 h after tumor cell transplantation, the mice bearing tumors were distributed into 4 groups with 8 mice per group. Each group was treated with PBS, cisplatin (1 mg·kg⁻¹), CA (10 mg·kg⁻¹) or cisplatin (1 mg·kg⁻¹) plus CA (10 mg·kg⁻¹) for 16 days, respectively. (A) The tumor volumes were monitored and recorded every two days. (B) Bodyweight of mice was measured every two days. (C) Tumor weight. (D) Photos of tumor tissues. Results were shown as mean ± SEM, * P < 0.05, ** P < 0.01 vs control.
Carnosic acid enhances cisplatin-induced tumor proliferation inhibition and apoptosis in tumor tissue

To further verify the inhibition effect of carnosic acid in combination with cisplatin, the proliferation and apoptosis in tumor tissues from each group were detected. A typical pathological characteristic of malignancy was shown H&E stained-section from control group while cisplatin treatment caused massive cancer cell damage evidenced by condensation of cytoplasm and pyknosis of nuclei. Carnosic acid in combination with cisplatin led to greater extent of cell damage (Fig. 2A). Next, we detected the levels of several markers for tumor growth and apoptosis. Proliferating cell nuclear antigen (PCNA) is a marker for tumor growth. Immunohistochemistry stain showed that cisplatin combined with carnosic acid significantly decreased the protein levels of PCNA compared with cisplatin treatment alone (Fig. 2B). Meanwhile, TUNEL staining implied that cisplatin combined with carnosic acid induced more apoptosis than each drug alone (Fig. 3A). Caspase-3, the executor of apoptotic process [21], was also significantly elevated in the combination treatment group (Fig. 3B).

Carnosic acid treatment promotes CD8+ T cells-mediated antitumor immune response

The CD8+ T cells are the final executor of antitumor immunity for its direct cytotoxicity on tumor cells via secreting IFN-γ, triggering Fas-mediated cell toxicity or perforin-induced cytolysis [22]. The intracellular staining showed a significantly increase in IFN-γ expression in CD8+ T cells in carnosic acid and carnosic acid plus cisplatin treatment group (Fig. 4A). As shown in Fig. 4B, the mRNA levels of the functional marker of CD8+ T cells, including Perforin, Granzyme B and FasL, and protein level of Granzyme B (Fig. 4C) were also increased in the combination treatment group. These evidence suggested that carnosic acid treatment enhanced infiltration and function of CD8+ T cells.

Carnosic acid treatment decreases function and accumulation of MDSCs in tumor tissue

As carnosic acid treatment could enhance CD8+ T cells-mediated antitumor immune response, next we aimed to find out the underlying mechanisms. It is reported that the MDSC accumulation in tumor microenvironment would suppress the functions of T cells and benefit tumor growth while MDSC depletion and down-regulation of function have been proven to be effectively for boosting antitumor immunity [23-24]. To test our hypothesis, we examined the infiltration of MDSCs in
Fig. 3  Carnosic acid enhanced cisplatin-induced apoptosis in tumor tissue. (A) Paraffin sections of tumor tissue from each group were stained with TUNEL-FITC and photographed by fluorescence microscopy. (B) Paraffin sections of tumor tissues from mice were analyzed by immunofluorescence of cleaved-caspase-3 (scale bar: 50 μm)
tumors. As shown in Fig. 5A, a high percentage of CD11b+ Gr1+ MDSCs was emerged in tumor tissues while carnosic acid treatment significantly reduced this propotion compared with that of controls (Fig. 5A). MDSCs hampered the immune system dependent on the enzyme activity of Arg-1, iNOS-2, and MMP9 [5]. The mRNA level examined by quantitative PCR manifested that these enzyme expressions were also markedly down-regulated by carnosic acid, leading to the function disability of MDSCs (Fig. 5B).

Discussion

Nowadays, chemotherapy is still the main strategy used for tumor treatment although it would usually lead to immunosuppressive effects. Cisplatin, a chemotherapy drug frequently used to treat lung cancer, is prone to drug resistance and uncomfortable side effects during long-term application. Recent years, tremendous advance has been made in the area of immunotherapy [25-30]. More and more attentions are paid out to find new strategies for immunotherapy.

It is the truth that most chemotherapeutic agents including cisplatin and 5-FU were regarded as immunosuppressants, combinations between immunotherapy and chemotherapy were once considered as inappropriate. However, the emergence of the concept of immunogenic cell death [27], the observations that some chemotherapies can eliminate regulatory immune cell subsets [28-29] and some clinical trials results showing that patients treated first with immunotherapy, followed by chemotherapy demonstrated better clinical outcomes than patients that have received chemotherapy alone [30-31], have prompted scientists and physicians to reassess the potential of combination therapies between chemotherapy and immunotherapy [32-34].

Here in our study, we found out that carnosic acid could enhance the anti-lung cancer effect of cisplatin by inhibiting MDSC, suggesting a new combination therapy for lung cancer.

MDSCs, acting as one of the major immunosuppressive cells hindering the antitumor immune response in human body, are a heterogeneous population derived from the myeloid lineage and accumulate abnormally in peripheral blood and lymphoid organs in tumor patients [35-37]. MDSCs could not only inhibit anti-tumor immune reactions but also promote tumor growth and metastasis. Therefore, strategies targeting MDSC can potentiate the efficiency of cancer immunotherapy [38-40].
Besides surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy, an increasing number of patients also select naturopathic therapy with traditional Chinese medicine (TCM) for adjuvant treatment. TCM treatment is considered to be multicomponent affecting multi-targets to restore the normal balance and flow in whole-body system. In consideration of these, naturopathic therapy is believed to reinforce the endogenous resistance of the body to diseases [41]. Several herbal medicine-derived drugs such as temsirolimus, ixabepilone, and trabectedin, have been approved for clinical use [42]. In the present study, we found that carnosic acid, an agent which has antioxidative and antimicrobial properties, could inhibit function of MDSC in tumor model and enhance anti-lung cancer activity of cisplatin.

Several mechanisms have been reported for the role of MDSC play in suppression of host immune responses such as high expression level of Arg1 (arginase 1) and production of nitric oxide and immune-suppressive cytokines [43-44]. Arg1 converts l-arginine to urea and l-ornithine, while over consumption of l-arginine inhibits T-cell proliferation [45-46]. NO generated by iNOS can also suppress T-cell function through various mechanisms including STAT5 inhibition [47] and the induction of T-cell apoptosis [48]. Furthermore, MDSCs secrete proteinases such as MMP9 that induce the mobilization of pro-angiogenic molecules residing in the extracellular matrix of the tumor microenvironment [49]. Substantial MDSCs were present in the untreated tumor tissue, while after carnosic acid treatment, the ratio was lowered and their functions were also down-regulated, as evidenced by decreased expression of Arg-1, iNOS2, and MMP9.

With the help of activated CD4⁺ T cells, CD8⁺ T cells can be activated and migrate to the tumor site, producing a specific cytotoxic effect such as releasing Perforin and Granzymes [50]. Perforin forms pores in the cell membrane of the target cells, creating an aqueous channel through which the Granzymes enter, which leads to cell apoptosis via degradation of DNA or initiation of the FasL/Fas apoptotic pathway [51-52].

In our experiments, the CD8⁺ T cells were found to largely present in the tumor tissues after carnosic acid treatment. At the same time, the mRNA expression of Perforin, Granzym B, and FasL were all boosted, which might suggest that carnosic acid promoted the functions of CD8⁺ T cells and contributed to tumor suppression together with cisplatin.

Collectively, our present study provided evidences supporting that carnosic acid promoted the infiltration and functions of CD8⁺ cytotoxic T cells through inhibiting the MDSC in tumor-bearing mice and thus elevated the anti-cancer effect of cisplatin. Our data suggested carnosic acid may be a new candidate for lung cancer combination therapy. Meanwhile, several issues need to be clarified in future studies, including the detailed mechanism for carnosic acid on MDSC function as well as on other immune cells like DC or macrophages.

References


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