



Research Paper

Irradiation enhanced the effects of PD-1 blockade in brain metastatic osteosarcoma



Liming Xia*, Hao Wu, Wenkang Qian

Department of Musculoskeletal Cancer Surgery, Zhejiang Cancer Hospital, Hangzhou 310000, People's Republic of China

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ABSTRACT

Brain metastasis of osteosarcoma are rare but carry a dismal prognosis. Despite the advances in both systemic immunotherapy and localized radiation, it is still difficult to treat brain metastasis, with less than 12 months of survival from the time of diagnosis for most patients. Currently, there is interest in combining strategies to take advantage of the potential synergy. In this study, the mouse model of metastatic osteosarcoma to brain was used to explore the ability of local radiation and anti-PD-1 blockade to induce beneficial anti-tumor immune responses against distant, unirradiated brain metastatic tumors. Immune markers from the peripheral blood and tumor tissue were analyzed by flow cytometry, real-time PCR and western blot. The combination treatment produced a stronger systemic anti-tumor response than either treatment alone, shown by the reduced tumor burden and larger numbers of cytotoxic CD8⁺ T cells in the unirradiated tumors, indicating an abscopal effect. These data suggested that combination treatment of irradiation with anti-PD-1 immunotherapy can induce abscopal anti-tumor responses and improve both local and distant control.

1. Introduction

Osteosarcoma is one of the most common type of malignant bone tumors in children and young adults with devastating clinical consequences. Brain metastasis in osteosarcoma is frequently preceded by lung metastasis [1]. Although considerable progress had been made, survival rates remain dismal for those patients with advanced metastatic diseases and recurrence [2]. Osteosarcoma is considered as one of the classic chemo- and radio-resistant brain metastasis, therefore it is important to explore the use of multiple modalities in order to improve the therapeutic ratio and overcome the inherent resistance [1,3].

Even though surgery and radiotherapy are major treatment modalities with successful control of local lesions and may have a role in the management of metastatic diseases [4], patients with osteosarcoma frequently progress systemically or become failure in distant brain metastasis. By boosting patients' own anti-tumor immune responses, immunotherapies have revolutionized the treatment of cancer [5]. Among these, immune-checkpoint inhibitors targeting on programmed cell death-1 (PD-1) and its ligand (PDL-1) have become important strategies to control advanced tumors due to their efficacy in clinical use [6,7]. PD-1/PDL-1 blockade prevents T cell inhibition and results in dramatic successes in the treatment of patients with cancer. However, the percentage is still small for patients with cancer who benefit from checkpoint inhibitors. Combination treatments appear to be more

potent in treating cancer [8]. Therefore, optimizing the use of immune-checkpoint inhibitors in combination with other treatments may yield stronger effects against metastatic osteosarcoma.

In this study, the mouse-bearing brain and flank tumor model was used, representing modeling brain metastasis and extracranial disease. Mice received irradiation to the flank tumor alone or in combination with anti-PD-1 antibody. Results showed that combination treatment of irradiation and anti-PD-1 resulted in the decrease of tumor burden, indicating that anti-PD-1 treatment combining with irradiation of local lesion can cause a beneficial immune response to affect unirradiated distal tumors. Our data suggested that these two common treatments for metastatic osteosarcoma would have synergistic effects in the clinic.

2. Materials and methods

2.1. Cell line

The K7M2 cell line was obtained from the American Type Culture Collection. K7M2 cells were maintained in RPMI with 10% fetal bovine serum and 100 u/ml penicillin/streptomycin in an incubator with 5% CO₂ at 37 °C.

* Corresponding author.

E-mail address: Xlf1787@163.com (L. Xia).

2.2. Tumor model development and treatment

Six-week old female Balb/c mice were purchased from Vital River Lab (Beijing, China) and maintained under specific pathogen-free conditions. Mice had free access to food and water during the whole experimental period. All animal experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals and approved by the Ethical Committee on Animal Care and Use of Zhejiang Cancer Hospital.

5×10^5 K7M2 cells were injected into Balb/c mice subcutaneously and 4×10^5 tumor cells were injected into the right frontal lobes of the same mice. Tumor volume was measured with a caliper twice per week for the duration of the experiment. When the tumor volume reached 50–100 mm³, mice were divided into four groups (five mice per group) and tumor-bearing mice were treated with anti-PD-1 antibody (5 mg/kg, once a week, I.P.), irradiation (2 Gy x 4, five consecutive days), or the combination. Mice treated with IgG were used as the negative control (five mice per group). For the irradiation, mice were immobilized after anesthesia and irradiation was delivered to the flank tumors using a Pantak X-ray irradiator. Lead shielding was used to limit radiation exposure to other areas of the body.

2.3. Analysis of immune markers from peripheral blood

Peripheral blood was obtained from tumor-bearing mice and normal mice. Blood was stained with anti-CD4 FITC, anti-CD8 Percp, anti-Ly6G AF700, anti-Ly6C APC, anti-CD11b APC–Cy7 antibodies for flow cytometry analysis (BD FACSCalibur). M-MDSC was defined as CD11b⁺Ly6C⁺Ly6G⁻ and G-MDSC was defined as CD11b⁺Ly6G⁺Ly6C⁻.

2.4. Analysis of immune markers from tumor tissue

Tumor tissue was harvested and cut into small fragments followed by digestion with tumor disassociation kit for 30 min (Miltenyi Biotec, USA), and then filtered by 70 μm cell strainers. Mononuclear cells were enriched by percoll gradient centrifuging of the single cell suspension. Cells were washed with PBS and stained with Live/Dead dye, anti-CD4 FITC, anti-CD8 PE-Cy7, anti-Ki67 APC, anti-IFN γ PE-CF594, anti-FoxP3 Percp and anti-granzyme B PE antibodies, along with appropriate isotype controls (all from BD) for flow cytometry analysis (BD FACSCalibur).

2.5. Quantitative real-time RT-PCR

The total RNA was extracted from the tumor samples with 1000 μl TRIzol reagent. 0.4 μg total RNA was used to generate complementary DNA with SuperScript master mix. Quantitative PCR was performed using SYBR green supermix (Bio-Rad, CA) with comparative Ct value method to quantify the expression of genes of interest in different samples. The mRNA levels were normalized to the housekeeping gene *Gapdh*.

2.6. Western blot

Protein was extracted from the tumor samples and quantified by BCA method. After boiling, equal amounts of protein (40 μg) were subjected to electrophoresis on a 4–12% (v/v) SDS-polyacrylamide gel. Protein was then electroblotted to the polyvinylidene difluoride membrane from gel. The membrane was blocked with phosphate buffered saline containing 5% non-fat milk at room temperature for 1 h, and incubated with indicated primary antibodies at 4 °C overnight, followed by incubating with the goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. Membrane was washed three times, and visualized by the enhanced chemiluminescence system.

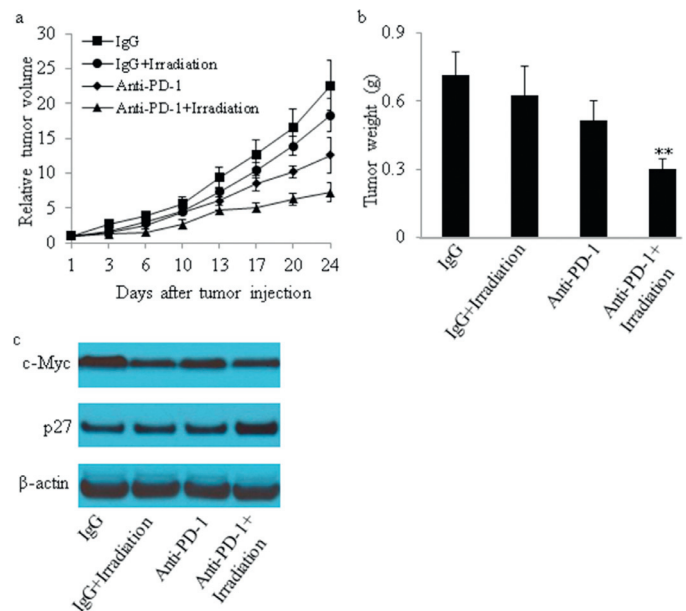


Fig. 1. Tumor-bearing mice were treated with IgG, IgG + Irradiation, anti-PD-1 antibody or the combination. The combination treatment delayed the tumor growth of the flank tumors (a), and induced a systemic anti-tumor response by decreasing the tumor burden of distal, non-irradiated brain metastatic tumors (b) through increasing the protein expression of p27 and decreasing the protein expression of c-Myc (c). Data were expressed as mean \pm SD. ** $p < 0.01$ vs IgG group.

2.7. Statistical analysis

Data were expressed as mean \pm SD and analyzed by one-way ANOVA with SAS 9.1 software. Values were expressed as mean \pm SD. $p < 0.05$ was considered as significant difference.

3. Results

3.1. Combination treatment induced a systemic anti-tumor response

In order to cause a systemic anti-tumor immune response that could affect the growth of distal, non-irradiated tumors (abscopal effect), the combination treatment of the irradiation with anti-PD-1 antibody was used in this study. Treatment of tumor-bearing mice with either irradiation or anti-PD-1 antibody afforded modest effect. However, the irradiation improved the efficacy of the immune checkpoint blockade by decreasing the tumor burden of tumor-bearing mice through increasing the protein expression of p27 and decreasing the protein expression of c-Myc ($p < 0.01$) (Fig. 1).

3.2. Combination treatment induced immune responses in peripheral blood of tumor-bearing mice

Whether irradiation would enhance immune responses was tested in the mouse tumor model. After treatment, mice were sacrificed to collect blood for analysis. Combination treatment significantly increased CD4 as well as CD8 T cells in peripheral blood and decreased MDSCs in peripheral blood ($p < 0.01$) (Fig. 2).

3.3. Combination treatment induced immune responses in tumor of tumor-bearing mice

In order to determine whether the delayed tumor burden in non-irradiated tumors was due to anti-tumor immune responses, tumors in the brains were harvested and analyzed. Results showed combination treatment significantly increased CD4 as well as CD8 T cells infiltration

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