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Original article

Axitinib augments antitumor activity in renal cell carcinoma via STAT3-dependent reversal of myeloid-derived suppressor cell accumulation

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MDSC, Myeloid-derived suppressor cell
RCC, Renal cell carcinoma
VEGFR, Vascular endothelial growth factor receptor
STAT3, Signal transducer and activator of transcription 3

ABSTRACT

Axitinib, a selective inhibitor of vascular endothelial growth factor receptor (VEGFR), is used as an anti-angiogenic agent for the treatment of metastatic renal cell carcinoma (RCC). However, the effect of axitinib on antitumor immunity has remained largely unexplored. In this study, we show that while axitinib (25 mg/kg) significantly suppresses tumor growth and metastasis, thus prolonging life span in murine RCC xenografts, the treatment leads to a major decrease in the number of myeloid-derived suppressor cells (MDSCs) in the spleens and tumor beds of animals, which in turn promotes antitumor responses of CD8+ T-cells *in vivo*. Moreover, as one of the main transcription factors that regulate MDSC function, Signal transducer and activator of transcription 3 (STAT3) was also significantly inhibited in the Renca tumor-associated MDSC and tumor tissues. These results suggest that axitinib has the potential to modulate antitumor immunity by downregulating STAT3 expression and reversing MDSC-mediated tumor-induced immunosuppression. The study reveals the unique antitumor mechanism of axitinib and provided useful information for its clinical application.

1. Introduction

Axitinib is a potent and selective inhibitor of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3 [1]. Compared with sorafenib, sunitinib and pazopanib, which are multi-targeted tyrosine kinase inhibitors, axitinib exhibits a higher potency (in the picomolar range) against VEGFRs and greater selectivity for VEGFRs [1,2]. In animal studies, axitinib produced dose-dependent blockade of VEGFR-2 phosphorylation, reduction in vascular permeability and angiogenesis and induction of tumor cell apoptosis, providing evidence for therapeutic potential [1]. Based on these observations, axitinib has been evaluated in the clinic as an anti-angiogenic agent, and has been approved for use in patients with metastatic renal cell carcinoma (RCC) who have failed to respond to a previous treatment [1,3]. While axitinib has been explored in the clinic mainly for its anti-angiogenesis effects, its response to the antitumor immune system has remained largely unexplored.

Accumulating evidence suggests that myeloid-derived suppressor cells (MDSC) are elevated in cancer patients and tumor-bearing hosts, and inhibit both adaptive and innate immunity [4,5]. MDSC have been identified as a population of immature myeloid cells that consists of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cell (DC), with the ability to suppress T-cell activation in humans and mice [6–8]. They notably have the capacity to inhibit CD8+ T-cell antigen-specific

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reactivity by different mechanisms, mainly through their capacities to produce nitric oxide and radical oxygen species [9–11]. In mice, MDSCs are characterized by the co-expression of the myeloid lineage differentiation antigen Gr1 and CD11b [12]. Elimination of MDSC in mouse tumor models has been shown to enhance antitumor responses, resulting in tumor regression. In this regard, strategies aimed at depleting MDSC *in vivo* using agents that target MDSC have been shown to be very promising [13–15].

Signal transducer and activator of transcription 3 (STAT3) is constitutively activated in diverse cancer cell types, including RCC [16,17]. Frequent STAT3 activation in tumor cells is largely due to the fact that STAT3 is a point of convergence for numerous tyrosine kinases, including VEGFR, PDGFR, EGFR, among many others [18]. Recently, it was documented that STAT3 was critically involved in tumor accumulation of MDSCs, which plays an important role in suppressing antitumor immune responses [19]. MDSCs from tumor-bearing mice have markedly increased levels of phosphorylated STAT3 compared with immature myeloid cells from naive mice [20]. Moreover, ablation of STAT3 expression through the use of conditional knockout mice or selective STAT3 inhibitor (JSI-124) markedly reduced the expansion of MDSCs, promoted accumulation of dendritic cells and increased T-cell responses in tumor-bearing mice [20,21]. Herein, activated STAT3 has been proposed to be the main regulator of MDSC expansion.

In the present study, other than the widely known anti-angiogenesis activity, we assessed the effect of axitinib on MDSCs in a tumor microenvironment. The findings may suggest that axitinib-based therapy has the potential to modulate antitumor immunity by reversing MDSC-mediated tumor-induced immunosuppression.

2. Materials and methods

2.1. Cell culture

Murine renal cancer cell lines Renca was kindly given by Prof Can Zhang (China Pharmaceutical University, Nanjing, China), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal calf serum (Gibco, USA) under a humidified 5% (v/v) CO_2 atmosphere at 37 °C.

2.2. Animals

Six-week-old female Balb/c mice were purchased from Academy of Military Medical Sciences (Beijing, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of Nanjing University. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

2.3. Reagents

Axitinib (purity > 99%) was purchased from Hubei Xinyinhe Chemical Engineering Company (Wuhan, China) as a white to light yellow crystalline powder and stored at 4 °C in the dark. For *in vivo* experiments, axitinib was formulated in a homogeneous suspension of 0.5% carboxylmethyl cellulose (CMC; ICN Pharmaceuticals SA, France). DMEM, RPMI-1640 and fetal bovine serum were purchased from Life Technologies (Carlsbad, CA). Antibody to CD31 was purchased from BD Biosciences (San Jose, CA). TUNEL assay kit was purchased from Vazyme Biotech (Nanjing, China). Antibodies to PCNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Real Envision Detection kit was from GeneTech Company (Nanjing, China). Percoll was purchased from Sunshine Biotechnology (Nanjing, China). Antibodies to CD11b-PE, Gr1-FITC, CD8-FITC, CD45.2-APC, p-STAT3-PE were purchased from eBioscience (San Diego, CA). Antibodies to p-STAT3, STAT3, Bcl-xL, CyclinD1, Survivin, Arg-1 and ROS were obtained from Santa Cruz Biotechnology (St. Louis, MO).

2.4. Mouse xenograft model

Mice were inoculated subcutaneously (right flank) with Renca cells (6×10^5/mouse, 100 μL in PBS). When the tumor size reached 80 to 100 mm^3 (5 days after inoculation, D6), mice were randomly distributed into two groups and administrated with axitinib (25 mg/kg) or vehicle (0.5% CMC). Drugs were intragastric administrated once a day for 20 days. The tumor size and body weight of mice were monitored everyday until the end of experiment (D25). Tumor size was caculated as follows: tumor volume (mm^3) = length (mm) × width^2 (mm^2) × (π/6). When the experiment was terminated, the mice were euthanized by cervical dislocation. The tumor tissue of each mouse was excised, and weighed. Paraffin section of every one was taken for further experiments. For survival assay, mice were monitored till the end of their life.

2.5. Immunohistochemistry (IHC)

Paraffin-embedded lung/tumor sections were heat-fixed, blocked with 3% H_2O_2, and incubated with specific antibodies overnight at 4 °C. Detection was done using Real Envision Detection kit from GeneTech Company (Shanghai, China) according to the manufacturer's instructions. Images were acquired using fluorescence microscopy (200×, Olympus).

2.6. Cell isolation

Preparation and staining of single cell suspensions of spleen, or tumor tissues were as described previously [22]. Tumor tissues were digested in digestive buffer, which contains RPMI-1640 (10% FBS), collagenase IV (1 mg/mL), DNAase (100 ng/mL), and FACS–EDTA (2% FBS, 0.1% NaN_3, 5 mM EDTA in PBS) for 1 h at 37 °C with pipetting at an interval of 15 min Digestion was stopped with RPMI-1640 complete medium and the cell suspension was filtered through the gauze to harvest single cell suspension followed with centrifugation. The precipitation was washed with FACS–EDTA once, then following the instruction of percoll protocol.

2.7. Flow cytometry analysis of cell surface molecules

Cells (5×10^5) suspension were added into FACS tubes and washed with PBS once. Then, cells were stained with fluorescent antibody for 20 min, and resuspended with 400 μL PBS for flow cytometry analysis.

2.8. Western blot

Western blot analysis was performed as described earlier [23]. Proteins were extracted in a lysis buffer (30 mmoL/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl-fluoride (PMSF), 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The protein sample was electrophoresed by SDS–PAGE and transferred onto nitrocellulose membranes. After blocking with 5% non-fat milk, the membrane was incubated with a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. Detection was carried out using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).