Experimental Cell Research 351 (2017) 142–149

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Interactions between interleukin-6 and myeloid-derived suppressor cells drive the chemoresistant phenotype of hepatocellular cancer

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ARTICLE INFO

Keywords:
MDSC
IL-6
Chemotherapy
Hepatocellular cancer

ABSTRACT

Emerging evidence implicates an important role for myeloid-derived suppressor cells (MDSCs) in tumor growth, angiogenesis and metastasis. However, limited knowledge is known about the function of MDSCs in response to chemotherapies. In this study, we find that drug-resistant hepatocellular cancer (HCC) cells-derived conditioned medium significantly enhances the expansion and immunosuppressive function of MDSCs compared to their parental sensitive cells, which is demonstrated by increased level of arginase, nitric oxide (NO), and reactive oxygen species (ROS). Next, we reveal that drug-resistant HCC cells-derived IL-6 activated MDSCs, which is demonstrated by using an anti-IL-6 neutralizing antibody that caused a reduced MDSC immunosuppressive activity. More importantly, the depletion of MDSC via the administration of anti-Gr-1 antibody or the blockade of IL-6 signaling sensitized 5-FU-resistant H22 hepatoma to chemotherapy in the immunocompetent C57BL/6N mice. In primary human HCC, IL-6 expression levels strongly correlate with an MDSC phenotype and chemotherapy response in HCC patients. In conclusion, these results describe a role of IL-6 in the drug resistance in HCC chemotherapy and suggest that MDSC-targeting treatments may be potential therapeutic strategy for HCC chemoresistance.

1. Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor and the second leading cause of cancer-related deaths worldwide, with 700,000 cancer deaths and 750,000 new cases reported each year [1]. Although significant progresses in early diagnosis, surveillance programs in high risk patients and therapeutic approaches in recent year, the clinical outcome of HCC patients remains poor [2–5]. Notably, increasing incidence of chemoresistance certainly contributes to the poor prognosis of HCC patients [6,7]. Therefore, it is imperative to explore the factors involved in the outcome of chemotherapy and the mechanisms of chemoresistance.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells derived from progenitor cells in bone marrow, which are substantially expanded in various diseases including cancers, and are capable of supporting tumor progression by damping T-cell immunity and promoting angiogenesis through remodeling of the tumor microenvironment [8]. In mice, MDSCs are characterized by co-expression of Gr-1 and CD11b cell surface markers and lack of the markers of mature macrophages (MΦ) and dendritic cells (DC). In human cancer patients, MDSCs are defined as co-purification with the mononuclear cell fraction and have a phenotype of CD33 and CD11b [9]. MDSCs are functionally characterized by suppression of T-cell responses via Arginase, nitric oxide (NO), reactive oxygen species, and other mechanisms [10]. The immunosuppressive function of MDSCs and their accumulation in tumor microenvironment is induced by multiple soluble mediators, such as cytokines and chemokines derived from tumor cells [11,12]. Recently, emerging evidence have demonstrated that tumor cells-induced MDSCs remarkably contributes to the cancer-induced immune suppression, and therefore multiple approaches have been exploited to disrupt the functional crosstalk between cancer cells and the MDSCs [12–14].

It has been reported that hypoxia induces MDSC recruitment to HCC through chemokine (C-C motif) ligand 26, and MDSCs inhibit natural killer cells in HCC patients via the NKp30 receptor [15,16]. Importantly, the roles of MDSCs in supporting tumor initiation,

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http://dx.doi.org/10.1016/j.yexcr.2017.01.008
Received 7 December 2016; Received in revised form 12 January 2017; Accepted 17 January 2017
Available online 19 January 2017
0014-4827/

progression, angiogenesis and metastasis have been well studied [17]. However, little is known about mechanisms underlying the role of MDSCs in chemotherapeutic resistance.

In this study, we demonstrated for the first time that IL-6 secreted by drug-resistant HCC cells enhanced the expansion of MDSCs, as well as its immunosuppressive activity as revealed by Arginase, NO and ROS production. Moreover, by an *in vivo* experimental model in immunocompetent C57BL/6N mice, we certified that novel treatment strategies targeting MDSCs or IL-6 were able to overcome the chemoresistance of HCC. Collectively, our results suggested that IL-6 acts as a main driver of cytokines that orchestrate the expansion and the immunesuppressive function of MDSCs in HCC.

2. Materials and methods

2.1. Cell culture

Human HCC cell line BEL-7402 and HepG2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The drug-resistant BEL-7402/5-FU (5-fluorouracil) and HepG2/ADM (adriamycin) cell lines was purchased from Nanjing Keygen Biotech. Co. (Nanjing, China). The hepatoma cell line H22 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM medium with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C with 5% CO_2. The murine hepatoma cells H22 were treated with doxorubicin for several cycles in vitro to generate drug-resistant H22/5-FU cells. After fourth treatment, the H22 cells acquired chemo-resistant phenotype to 5-FU. To examine the implications of HCC-derived factors on MDSCs, the supernatants of HCC cell culture were collected when cells became 85% confluent and passed through 0.2 mm filter. To block the effects of IL-6, cultured HCC supernatants were pretreated with 5 mg/mL of anti-IL-6 antibody (clone 6708, R & D Systems) for 30 min before addition to MDSCs.

2.2. MDSC generation

MDSCs were generated from bone marrow cells isolated from the tibiae and femurs of C57BL/6 mice. Briefly, after red blood cells were lysed with ammonium chloride, bone marrow-derived cells were stimulated with GM-CSF (40 ng/mL; R & D Systems) and IL-6 (40 ng/mL; R & D Systems) at 37 °C in a 5% CO_2-humidified atmosphere for 4 d. Collected cells were labeled with FITC conjugated anti-CD11b antibody (San Diego, CA).

2.3. RNA extraction and quantitative RT-PCR

Total RNA from MDSCs were extracted by the RNAiso Plus kit (Takara Bio Inc.) according to the manufacturer's instructions and was reverse transcribed to cDNA by the primeScript RT Master kit (Takara Bio Inc.). Quantitative RT-PCR (qRT-CPR) analyses for Arg1, Cox2, Nos2 and Cybb were performed using SYBR Green Master Mix using the 7500 System (AB Applied Biosystems). The specific primers used were shown as follows: Arg1 forward, 5′-GTGGAAACTTGCATGGACAAC-3′, Arg1 reverse: 5′-AATCCTGGCACATCGGGAATC-3′; Cox2 forward, 5′-TAAGTGCGATTGTA CCCGGAC-3′, Cox2 reverse: 5′-TTTGTAGCCATAGTCAGCATTGT-3′; Nos2 forward: 5′-TTCAGTATCACAACCTCAGCAAG-3′; Nos2 reverse, 5′-TGGACCTGCAAGTTAAAATCCC-3′, Cybb forward, 5′-AACGAATTGTAC GTGGGCAGA-3′, Cybb reverse: 5′-GAGGGTTTCCAGCAAACTGAG-3′; Gapdh forward, 5′-GGAGCGAGATCCCTCCAAAAT-3′, Gapdh reverse: 5′-GAAGATGGTGATGGGATTTC-3′; The data were normalized to a Gapdh reference.

2.4. Arginase activity, NO production, and reactive oxygen species production

To analyze arginase activity, cells were washed with DPBS and pellets were frozen until use. After thawing and lysing pellets with the lysis buffer, the arginase activity was measured by the QuantiChrom Arginase Assay Kit (BioAssay Systems, DARG200) according to the manufacturer's instructions. Protein concentration was evaluated with BCA Protein Assay Kit (Pierce Biotechnology) to normalize Arginase activity to total protein concentration. Nitrite quantification was assayed by a standard Greiss reaction. Briefly, equal volumes of indicated culture supernatant (100 μL) were mixed with Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1%N-1-naphthylethylenediamine dihydrochloride) and incubated for 10 min at room temperature. The absorbance at 550 nm was measured using a microplate plate reader (Bio-Rad). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite. The oxidation-sensitive dye DCFDA (Invitrogen) was used to measure ROS production by purified MDSC. Cells were incubated at 37 °C in serum-free RPMI in the presence of DCFDA (3 μM) with or without drug-resistant HCC-derived conditional medium for 30 min. Cells were then washed twice with cold PBS and labeled with anti-CD11b-PE-Cy7 and anti-Gr-1-APC antibodies. After incubation on ice for 15 min, cells were washed with PBS again and analyzed by flow cytometry.

2.5. Measurement of IL-6, VEGF-A, GM-CSF, SDF-1 and MCP-1

The panel of 5 cytokines and chemokines was analyzed in supernatants from HCC cultures (when 90% confluent) using commercially available Kits. All samples were batch run in duplicate and quantified on the basis of a unique standard curve for each index. The assays were carried out according to the manufacturer's instructions. The kits for GM-CSF, VEGF-A and IL6 were purchased from R & D Systems. The kit for SDF-1 and MCP-1 was purchased from eBioscience.

2.6. Measurement of cell viability and caspase-3/7 activity

Cell viability of HCC cell was measured using the CCK-8 Kit (DojinDo, Japan). Briefly, cells were seeded in 96-well plates at densities of 5×10^3 cells per well and treated with 5 mg/mL of anti-IL-6 antibody (clone 6708, R & D Systems) for 24 h. Then the medium was replaced by 100 μL new medium with 10% CCK-8. After incubation for 1 h, the absorbance was measured using an activation wavelength of 450 nm. Cell apoptosis was determined by caspase-3/7 activity, which was measured by a commercial available Kit according to the manufacturer's instructions (Promega).

2.7. Clinical tissue samples and immunohistochemistry

The 20 patients received transarterial chemoembolization in our hospital were enrolled and needle samples were collected in this study. All the process was performed in accordance with guidelines and regulations approved by the Fifth Affiliated Hospital of Wenzhou Medical University. Appropriate informed consent was obtained from each patient. Immunohistochemical analyses were carried out routinely and the intensity of IL-6 (Abcam, ab6672) staining was classified according to a two-level scale: low, weak or partial staining to cytoplasm of cancer cell; high, strong and diffuse staining to cytoplasm. All specimens were evaluated by two independent investigators.

2.8. HCC xenograft model

Animal experiments were approved by the animal care and usage committee of the Fifth Affiliated Hospital of Wenzhou Medical University. The anti-tumor activity of 5-FU and anti-Gr-1 or anti-IL-