International Immunopharmacology 65 (2018) 221–232

Contents lists available at ScienceDirect

International Immunopharmacology

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

Restoration of stemness-high tumor cell-mediated suppression of murine dendritic cell activity and inhibition of tumor growth by low molecular weight oyster polysaccharide☆

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ARTICLE INFO

Keywords:
Colorectal cancer stem cells
Dendritic cells
TGF-β
NF-κB
Oyster polysaccharide

ABSTRACT

Dendritic cells (DCs) play key regulatory roles in tumor immunity: increased activity of DCs infiltrating tumor tissues leads to enhancement of tumor immunity. Functions of DCs are also modulated by tumor cell-derived factors. Here, we investigated the effects of low molecular weight oyster polysaccharide (LMW-OPS) on differentiation and function of bone marrow-derived DCs (BMDCs) exposed to a conditioned medium (CM) obtained from spheres of stemness-high colorectal cancer cell lines CMT93 and CT26. The CM containing a detectable level of TGF-β1 was found to down-regulate the surface expression of major histocompatibility complex class II of BMDCs and to inhibit the potency of BMDCs to stimulate T cells. Those suppressions were partly restored and completely restored by addition of anti-TGF-β1 and LMW-OPS, respectively. Production of IFN-γ during allogeneic T cell responses was inhibited by the CM, whereas production of TGF-β1 was augmented by the CM. The IFN-γ profile was also reversed by addition of LMW-OPS. Nuclear translocation of β-catenin, but not that of NF-κB p65, was induced by TGF-β1. NF-κB p65 nuclear translocation, but not β-catenin nuclear translocation, was induced by LMW-OPS. Intraperitoneal injection of LMW-OPS significantly suppressed tumor growth in syngeneic tumor models using CMT93 and CT26 sphere cells, whereas it had no inhibitory effect on the proliferation of either cell line. The results demonstrated that LMW-OPS relieved stemness-high tumor cell-mediated suppression of BMDC function and indicated the in vivo anti-tumor activity of LMW-OPS in which restimulation of the activity of DCs infiltrating tumor tissues is presumed to be involved.

1. Introduction

T cell-mediated immunity is known to play a major role in anti-tumor immunity. Antigen processing and presentation by dendritic cells (DCs) are required for the activation of naïve T cells [1]. The phenotype and function of tumor-infiltrating DCs (TIDCs) in the tumor microenvironment are altered by tumor cell-derived soluble factors such as interleukin (IL)-6, IL-10 and transforming growth factor (TGF)-β, and impairment of TIDCs can induce T-cell tolerance or energy [2–6]. Therefore, TIDCs are an appropriate target for tumor immunotherapy [7]. In studies on tumor immunotherapy, it has been found that many active ingredients of natural products can induce maturation and activation of DCs [8–12]. Those natural products may be used for anti-tumor therapy by DC activation. We previously found that low molecular weight oyster polysaccharide (LMW-OPS) can induce Th1 response by activating DCs [13], although the anti-tumor activity of LMW-OPS has not yet been investigated.

Many lines of evidence support the theory that tumors contain side populations of cells called cancer stem cells (CSCs) [14,15], which are responsible for self-renewal and resistance to chemotherapeutic agents and radiation therapy [16–18]. Like normal stem cells, homeostasis of CSCs is supported by their microenvironment niche, which contains

☆ Supported by the Shandong Provincial Natural Science Foundation, China (Grant No. ZR2014HM038).
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https://doi.org/10.1016/j.intimp.2018.10.003
Received 5 May 2018; Received in revised form 24 September 2018; Accepted 3 October 2018
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multiple cell types and their secreted factors [18–29]. It is also known that immune cells are involved in the tumor microenvironment. Interaction between immune cells and tumor cells by their secreted factors has often been discussed, and it is known that tumor cell-derived immunosuppressive factors are an important cause of immune tolerance [30–35,2–5]. CSCs also secrete several soluble factors [36], but how CSC-derived factors influence the phenotype and function of DCs is unknown.

Colorectal cancer is the third most common cause of death from malignancies. Many studies have shown that colorectal cancer contains a tumorigenic cell population of CSCs [37–40], which can be isolated in vitro from human tumors in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) by culturing in non-adherent conditions and propagating as spheres. The established mouse colorectal cancer cell lines CMT93 and CT26 contain a CD133- and nucleostemin-positive population of CSCs [41,42].

The aim of this study is twofold. Firstly, we isolated CSC spheres from CMT93 and CT26 cells in vitro and we investigated the influence of CSC-derived soluble factors on DC phenotype and function. Our results showed that CSCs of both cell lines secreted factors that suppress the generation and function of DCs. Secondly, we examined the effects of the DC inducer LMW-OPS on DCs impaired by CSC-secreted factors and on in vivo tumor growth.

2. Materials and methods

2.1. Reagents

Recombinant murine EGF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and bFGF were obtained from R&D (Minneapolis, MN). Anti-mouse monoclonal antibodies for cluster of differentiation (CD)11c, major histocompatibility complex class II (MHC II) and CD86 labeled with fluorescein isothiocyanate (FITC) or R-phycoerythrin (PE) and ELISA assay kits for TGF-β1, interferon (IFN)-γ, IL-6, IL-12, IL-10, prostaglandin E_2 (PGE_2) and vascular endothelial growth factor (VEGF) were obtained from Thermo Fisher Scientific (Waltham, MA). Anti-mouse or anti-rabbit monoclonal antibodies against nuclear factor-κB (NF-κB) p65 (D14E12), β-catenin (E-5), Nanog (5A10), and Sox2 (E-4) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipopolysaccharide (LPS) and [^{3}H] thymidine were from Sigma-Aldrich (St. Louis, MO).

2.2. Mice

All mice, purchased from Shandong University Laboratory Animal Center (Jinan, China), were guaranteed free of particular pathogens in the Laboratory Animal Centre at Jining Medical College. Approval of the animal care protocols was obtained from the Animal Care and Use Committee at Jining Medical College.

2.3. Cell cultures, sphere formation and conditioned medium

The murine colorectal cancer cell lines CMT93 and CT26 were obtained from the Institute of Materia Medica, Chinese Academy of Medical Sciences (Shanghai, China). The cells were maintained as a monolayer in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin G, and 100 μg/mL streptomycin (normal culture medium) at 37 °C in 5% CO2. For tumor sphere formation, 1×10^6 cells were grown in 12-well ultra-low attachment culture plates in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mixture (F-12) supplemented with 10 ng/mL bFGF, 20 ng/mL EGF, 2% B27, 100 IU/mL penicillin G, and 100 μg/mL streptomycin (tumor sphere culture medium) for 8 days [42]. For preparing the conditioned medium derived from the tumor spheres (CM-TS), fresh sphere cells were collected after vigorous pipetting, and the cells (1×10^6 cells/mL) were again grown in 12-well ultra-low attachment culture plates in the tumor sphere culture medium for 48 h. For preparing the conditioned medium of monolayered normal cells (CM-NC), the cells (1×10^6 cells/mL) were grown in 6-well standard culture plates in the normal culture medium for 48 h. Both culture supernatants were collected as conditioned media and stored at −80 °C.

Table 1
The primer sequences for quantitative real-time PCR used in the study.

Gene	Sequence
β-actin	Forward: 5′-CGTTGACATCCGTAAAGACC-3′
	Reverse: 5′-AACAGTCCGCCTAGAAGCAC-3′
Sox2	Forward: 5′-TACATGAACGGCTCGCCCAC-3′
	Reverse: 5′-GCCCTGGAGTGGGAGGAAGA-3′
Oct3/4	Forward: 5′-GAGTTCTGCGGAGGGATGGC-3′
	Reverse: 5′-GGGACTCCTCGGGAGTTGGT-3′
Nanog	Forward: 5′-ACAAGGGCCCTGAGGAGGAG-3′
	Reverse: 5′-GCATCTGCTGGAGGCTGAGG-3′
c-Myc	Forward: 5′-CTTCCTCGGACTCGCTGCTG-3′
	Reverse: 5′-ACCTCTTGGCAGGGGTTTGC-3′
β-catenin	Forward: 5′-ATGCCTTGGGGATGGACCCT-3′
	Reverse: 5′-GGCAGCCCATCCATGAGGTC-3′
Klf4	Forward: 5′-GAGAATCTGCGCCACCTCCC-3′
	Reverse: 5′-CCGAAGGGCTGCTGTACTCG-3′
IL-6	Forward: 5′-GCTACCAAACTGGATATAATCAGGA-3′
	Reverse: 5′-CCAGGTAGCTATGGTACTCCAGAA-3′
IL-12	Forward: 5′-CCAGGTGTCTTAGCCAGTCC-3′
	Reverse: 5′-GCAGTGCAGGAATAATGTTTCA-3′
IL-10	Forward: 5′-CAGAGCCACATGCTCCTAGA-3′
	Reverse: 5′-TGTCCAGCTGGTCCTTTGTT-3′
PTGES2	Forward: 5′-CGTGAGAAGGACTGAGATCAAA-3′
	Reverse: 5′-GAGGAGTCATTGAGCTGTTGC-3′
VEGF	Forward: 5′-GTACCTCCACCATGCCAAGT-3′
	Reverse: 5′-TCATGGGACTTCTGCTCTCC-3′
TGF-β1	Forward: 5′-TGGAGCAACATGTGGAACTC-3′
	Reverse: 5′-GTCAGCAGCCGGTTACCA-3′

PTGES2, prostaglandin E synthase 2.

2.4. Quantitative real-time PCR analysis

Total RNA was extracted using RNAiso Plus (TAKARA Bio Inc., Kusatsu, Japan) and was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was amplified with a SYBR Green PCR kit (Thermo Fisher Scientific) using the ABI Prism 7900 sequence detector (Thermo Fisher Scientific). The relative gene expression levels were determined by normalizing against the levels of β-actin mRNA expression. The primer pairs used in this study are listed in Table 1. Sox2, Oct3/4, Nanog, c-Myc, β-catenin and Klf4 are stem cell marker genes, IL-6, IL-12, IL-10, VEGF and TGF-β1 are immunosuppressive cytokines, PTGES2 is an enzyme to produce immunosuppressive PGE_2, and β-actin was used as a reference gene.

2.5. Generation of bone marrow-derived cultured dendritic cells (BMDCs)

BMDCs were prepared using bone marrow cells obtained from 8 to 10-week-old C57BL/6 male mice as described previously [13]. Briefly, bone marrow cells (5×10^5 cells/mL) were cultured in RPMI-1640 containing 10% FBS, 15 ng/mL GM-CSF, 100 IU/mL penicillin G and 100 μg/mL streptomycin. To investigate the effects of CM-TS, CM-NC, LMW-OPS and LPS on BMDC differentiation, half of the medium was changed every day with CM-TS or CM-NC in the presence or absence of 1 μg/mL anti-TGF-β1 neutralizing antibody starting 1 day after seeding. On day 7, the cultures were treated with or without 0.1 mg/mL or 0.3 mg/mL of LMW-OPS or 1 μg/mL of LPS for 24 h. On day 8, loosely adherent and non-adherent cells were collected and used as BMDCs. The phenotypic characterization of BMDCs was determined by flow cytometry after gating on a $CD11c^+$ population (FacsCanto II, Becton Dickinson, Franklin Lakes, NJ). Mixed lymphocyte reaction (MLR) was

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