Biochemical and Biophysical Research Communications 436 (2013) 53–59

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The Hedgehog inhibitor suppresses the function of monocyte-derived dendritic cells from patients with advanced cancer under hypoxia

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ARTICLE INFO

Article history:
Received 8 May 2013
Available online 22 May 2013

Keywords:
Hedgehog signaling
Cyclopamine
Dendritic cells
Advanced cancer
Hypoxia

ABSTRACT

Immunotherapy using monocyte derived dendritic cells (Mo-DCs) from cancer patients has been developed; however, the Mo-DCs regularly studied have been derived from non-cancer bearing donors or mice, and evaluated in normoxic conditions. In the present study, we investigated the effects of Hedgehog (Hh) inhibitors which are being developed as molecular target drugs for cancer on the functions of Mo-DCs derived from patients with advanced cancer when cultured in a tumor-like hypoxic environment. Mo-DC induction, migration, chemotaxis, phagocytosis, maturation, IL-12 p40 or p70 secretion and the allogeneic lymphocyte stimulation activity of Mo-DCs from patients with advanced cancer were all significantly inhibited by the Hh inhibitor, cyclopamine under hypoxic conditions. Our results suggest that Hh signaling plays an important role in the maintenance and function of Mo-DCs derived from patients with advanced cancer when cultured under hypoxic conditions.

1. Introduction

Hedgehog (Hh) signaling is morphogenically important for embryonic patterning, controlling growth, and cell fate during neonatal development [1–3]. Recently, many studies have revealed that Hh signaling is activated in various types of cancer and that it contributes to the proliferation, invasion and progression of cancer [4–9]. Therefore, the Hh pathway is thought to be a potential therapeutic target in some diseases. Of the Hh inhibitors, Smoothened (Smo) inhibitors are well researched and some are under clinical trial [10,11]. For example, in January 2012, the US Food and Drug Administration approved Smo inhibitor, vismodegib for the therapy of metastatic or unresectable basal cell carcinomas of the skin [12]. Of late, research into combination therapy with chemodrugs and Hh inhibitors has also been reported [13,14]. Combination therapy with Hh inhibitors and immunotherapy using monocyte derived dendritic cells (Mo-DCs) will likely begin in the near future despite the fact that there are few reports investigating the interaction between Hh signaling and Mo-DCs in immunotherapy.

DCs are specialized antigen-presenting cells that initiate the primary T-cell immune response [15]. Re-vaccination with self-derived DCs pulsed with tumor associated antigens (TAAs), tumor specific peptide vaccination, and activated TNK lymphocytes including cytotoxic T lymphocytes are the three standard immunotherapies used today. In immunotherapy using Mo-DCs, donor-derived immature Mo-DCs (imMo-DCs) are injected locally into the tumor and mature Mo-DCs (mMo-DCs) pulsed with TAAs are administrated superficially into the lymph nodes. It has been reported that the oxygen levels are near 5.3% in mixed venous blood, 3.3–7.9% in well-vascularized organs, 1.3% in tumor tissues, and 0.5% in lymphoid organs [16,17]. Thus, DCs function in 0.5–8% O_2 conditions. Therefore, the Mo-DCs used in immunotherapy are harvested and induced under normoxic conditions *ex vivo*, are directly injected into the tumor or lymphoid tissue, and are expected to function well in these hypoxic conditions without an adjustment period [18]. In addition, some authors have shown that Hh signaling plays a pivotal role in DC function [19,20]. However, these results were ascertained using DCs from mice or non-cancerous volunteers and tested under normoxic culture conditions. Importantly, we have shown that the functions of Mo-DCs from patients with advanced cancer were impaired compared with those of Mo-DCs from healthy volunteers [21]. Thus, a better understanding of the functions of Mo-DCs derived from patients with advanced cancer and cultured under hypoxia is essential to improve the effect of immunotherapy. In the present study, we asked if Hh inhibition using Cyclopamine affected the function of Mo-DCs derived from patients with advanced cancer and cultured under hypoxic conditions.

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http://dx.doi.org/10.1016/j.bbrc.2013.05.057

2. Patients and methods

2.1. Patients

Twenty-seven patients enrolled in this study underwent cancer immunotherapy at the Fukuoka General Cancer Clinic (Fukuoka, Japan). The patient characteristics are shown in Supplementary Table 1. Written informed consent was obtained from all individuals.

2.2. Cells and culture conditions

Human peripheral blood mononuclear cells (PBMCs) were collected by apheresis (Haemonetics Co, Stoughton, MA, USA) prior to immunotherapy and stored at −80 °C until used. Thawed PBMCs were cultured in RPMI-1640 (Nipro, Osaka, Japan) supplemented with 0.5% human serum, 100 μg/ml penicillin (Meijiseika, Tokyo, Japan) and 100 μg/ml streptomycin (Meijiseika) (hereafter referred to as RPMI medium). After overnight culture, the non-attached cells were removed and used as lymphocytes. Adherent cells were cultured in RPMI medium supplemented with GM-CSF (100 ng/ml, North China Pharmaceutical Group Corporation-Gene Tech, China) and IL-4 (50 ng/ml, Osteogenetics, Wuerzburg, Germany) for 5–7 days to induce imMo-DC. To induce maturation, 1 μg/ml of LPS (Sigma, St Louis. MO, USA) was added for an additional 2 days. Smo inhibitor, cyclopamin (Certificate of Analysis, North York, Canada), was diluted in 99% ethanol. As hypoxic condition, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigus incubator (Sanyo, Tokyo, Japan).

2.3. Reverse transcription (RT)-PCR

Total RNA was extracted by using High Pure RNA Isolation kit (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's instructions. The sequences of the primers used were: Gli1 forward, 5′-TACATCAACTCCGGCCAATAGG-3′, reverse, 5′-CGGCGGCTGACAGTATAGGCA-3′, Smo forward, 5′-CTGCACACACTCACCTCTAA-3′, reverse, 5′-AAGCTTTCTTGCCTGGCTGA-3′, Shh forward, 5′-ACCATTCTCATCAACCGGGT-3′, reverse, 5′-ATTTGGTAGAGCAGCTGCGA-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-CCACCCATGGCAAATTCCATGGCA-3′, reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. Amplification conditions comprised an initial denaturation step for 2 min at 95 °C followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. RT-PCR products were separated on 2% agarose gels and visualized using ethidium bromide on a Molecular Imager FX (Bio-Rad, Hercules, CA).

2.4. Random migration and chemotaxis assay

The migration of Mo-DCs was assessed by migration through transwell inserts (8.0-μm pore size; BD Biosciences, Heidelberg, Germany). Then 5×10^4 cells were added to the upper chamber and incubated for 16 h under normoxia and hypoxia. After incubation, all cells that had migrated to the lower side were counted under a light microscope (BX50; Olympus, Tokyo, Japan). For chemotaxis assays, 100 ng/ml of recombinant human RANTES (CCL5, Peprotech, Rocky Hill, NJ, USA) or 6Ckine (CCL21, Peprotech) were added to the lower chamber and a filter (8.0-μm pore size) was placed in the well. Then 5×10^4 cells were added to the upper chamber and incubated for 5 h under normoxia and hypoxia. After incubation, all cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus).

2.5. Fluorescence activated cell sorting (FACS) analysis

Mo-DCs were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-CD83 and HLA-ABC mAbs or Phycoerythrin (PE)-conjugated anti- CD86 and HLA-DR mAbs (BD Pharmingen, San Diego, CA, USA). Mouse IgG was used as an isotype control (BD Pharmingen). The fluorescence intensity of the gated Mo-DC population was measured using a FACSCalibur flow cytometer (BD Pharmingen) and analyzed with CellQuest software (BD Pharmingen).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Mo-DCs were cultured with 0.05 KE/ml of streptococcal preparation, OK-432 (Chugai Pharma Co., Tokyo, Japan) for 16 h under normoxia and hypoxia. Then the concentrations of IL-12 p40 and IL-12 p70 in the supernatant were evaluated by ELISA according to the manufacturer's instructions (Biosource, Carlsbad, CA, USA). The detection limit of the assay was 0.5 pg/ml.

2.7. Evaluation of phagocytosis

Fluorescent polystyrene latex microspheres (1.00 μm, Cosmo Bio Co., Ltd., Tokyo, Japan), first coated in serum (opsonization), were added to the RPMI medium for 3 h at 37 °C or 4 °C under normoxia and hypoxia. The Mo-DCs were washed, and the fluorescence intensity in the gated Mo-DC population was measured by FACS analysis.

2.8. Allogeneic T-cell proliferation assay

Lymphocytes were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, Netherlands) in PBS at 37 °C for 10 min. Then lymphocytes were co-cultured with allogeneic Mo-DCs at a 1:7.5, 1:15, or 1:30 Mo-DC/lymphocytes ratio for 5 days under normoxic and hypoxic conditions. Lymphocyte proliferation was assessed by the CFSE dilution method.

2.9. Statistical analysis

An unpaired two-tailed Student's *t*-test was used for statistical analysis. A *p*-value of <0.05 was considered significant.

3. Results

3.1. The Hh inhibitor, cyclopamine, decreased proliferation and migration of Mo-DCs

First, the expressions of Hh signaling related molecules in Mo-DCs were evaluated by RT-PCR. *Gli1* and *Smo* mRNA were detected but *Shh* mRNA was not detected (Fig. 1A). Morphologically, cyclopamine did not affect imMo-DC morphology. The Mo-DCs showed a spindle-like morphology during the LPS treatment; however, cyclopamine-treated Mo-DCs remained round even during LPS treatment (Fig. 1B). Next we investigated whether cyclopamine affected the number of induced Mo-DCs. Cyclopamine significantly affected the number of generated Mo-DCs both under normoxic and hypoxic conditions (Fig. 1C).The ability to migrate is also an important function of Mo-DCs. As such, we evaluated nondirectional migration and chemotaxis in hypoxic conditions. Cyclopamine significantly decreased the capacity of random migration of imMo-DCs (Fig. 1D) and mMo-DCs (Fig. 1E) both under normoxic and hypoxic conditions. Then we used CCL5 and CCL21 as chemotaxic factors for the imMo-DCs and mMo-DCs, respectively.