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Short communication

Hedgehog signaling regulates PDL-1 expression in cancer cells to induce anti-tumor activity by activated lymphocytes

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ABSTRACT

We investigated whether hypoxia-induced activation of Hh signaling contributes to PDL-1 expression in cancer and whether it affects the anti-tumor function of activated lymphocytes. Hypoxia augmented PDL-1 expression and inhibition of Hh signaling reduced PDL-1 expression under hypoxia. When activated lymphocytes were cocultured with cancers treated with a Hh inhibitor, activated lymphocyte cell numbers increased under hypoxia. In contrast, this increase was abrogated when cancer cells were treated with a PDL-1 neutralizing antibody. These results suggest that Hh signaling is one of regulatory pathways of PDL-1 expression under hypoxia and that inhibiting Hh signaling may induce lymphocyte anti-tumor activity.

1. Introduction

Hedgehog (Hh) signaling plays a pivotal role in growth and morphogenic patterning in a wide variety of tissues during embryonic development [1]. Recent studies have revealed that Hh signaling is reactivated in various types of cancers and contributes to cellular invasion, progression, proliferation, and cancer initiation [2–5]. Hh signaling therefore warrants evaluation as a therapeutic target and promising results have been observed *in vitro*. However, good clinical endpoints have not yet been reported in clinical trials using Hh inhibitors [6]. We considered this discrepancy between *in vitro* results and clinical trials and suspect one reason may be the cancer microenvironment and hypoxic conditions. Almost all *in vitro* results were conducted under normoxic conditions, and tumor hypoxia had not been taken into consideration. Indeed, we have already shown that biological functions in cancerous and immune cells, such as activated lymphocytes and dendritic cells (DCs), are modulated differently under hypoxic conditions than under normoxic conditions [7–9]. Notably, it was recently reported that Hh signaling is activated under hypoxic conditions [10,11]. Antibody-mediated blockade of programmed death ligand-1 (PDL-1) as well as programmed death-1 (PD-1) revealed sustained tumor suppression and prolonged stabilization of disease in patients with advanced cancer [12–14]. Regulation of PDL-1 may thus be a promising therapeutic strategy. However, the mechanism (s) regulating PDL-1 expression remains unclear. Recently, it has been shown that hypoxia induces PDL-1 expression in cancer cells through hypoxia inducible factor (HIF)-1α [14]. To assess the potential effectiveness of new cancer therapies, we investigated whether hypoxia-induced activation of Hh signaling contributes to PDL-1 expression in cancer and whether it modulates the anti-tumor function of activated lymphocytes.

2. Materials and methods

2.1. Induction of activated lymphocytes

Three different human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 0.5% human serum, 2000 units/ml penicillin (Meijiseika, Tokyo, Japan), 10 μg/ml streptomycin

Abbreviations: Hh, Hedgehog; MAML3, mastermind-like3; RBPJ, recombination signal binding protein for immunoglobulin-kappa-J region; PD-1, programmed death-1; PDL-1, programmed death ligand-1; Smo, Smoothened; PSK, protein-bound polysaccharide; NKG2D, natural-killer group 2, member D.

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(Meijiseika) and 200 U/ml IL-2 (Primmune) in 2.5 μg/ml anti-CD3 monoclonal antibody (OKT3, JANSSEN PHARMACEUTICAL K.K., Tokyo, Japan)-coated T-75 flask for 7 days. Thereafter the lymphocytes (non-adherent fracture of culture) were transferred to oxygen permeable culture bags and were cultured in RPMI medium with 200 U/ml IL-2 for an additional 7 days. Then, the lymphocytes were collected as activated lymphocytes and were used experimentally.

2.2. Cell culture and reagents

Human pancreatic ductal adenocarcinoma cells lines, Panc-1, gallbladder cancer cell lines, GBd15 and NOZ, and small cell lung cancer cell line, SBC-5 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Life Technologies, Grand Island, NY) and antibiotics (2000 units/ml of penicillin and 10 μg/ml of streptomycin). For normoxic conditions, cells were cultured in 5% CO_2 and 95% air. For hypoxic conditions, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Sanyo, Tokyo, Japan). Cyclopamine (Wako, Osaka, Japan), an Smoothened (Smo) inhibitor was diluted in 99% ethanol. Cancer cells were treated with 10 μM cyclopamine over night to inhibit Hh signaling. To block PDL-1 expressed on cancer cells, 10 μg/ml of anti-human CD274 neutralizing Ab (Biolegend, San Diego, CA, USA) was treated for 30 min. Mouse IgG2a isotype control (eBioscience, San Diego, CA, USA) was used as control.

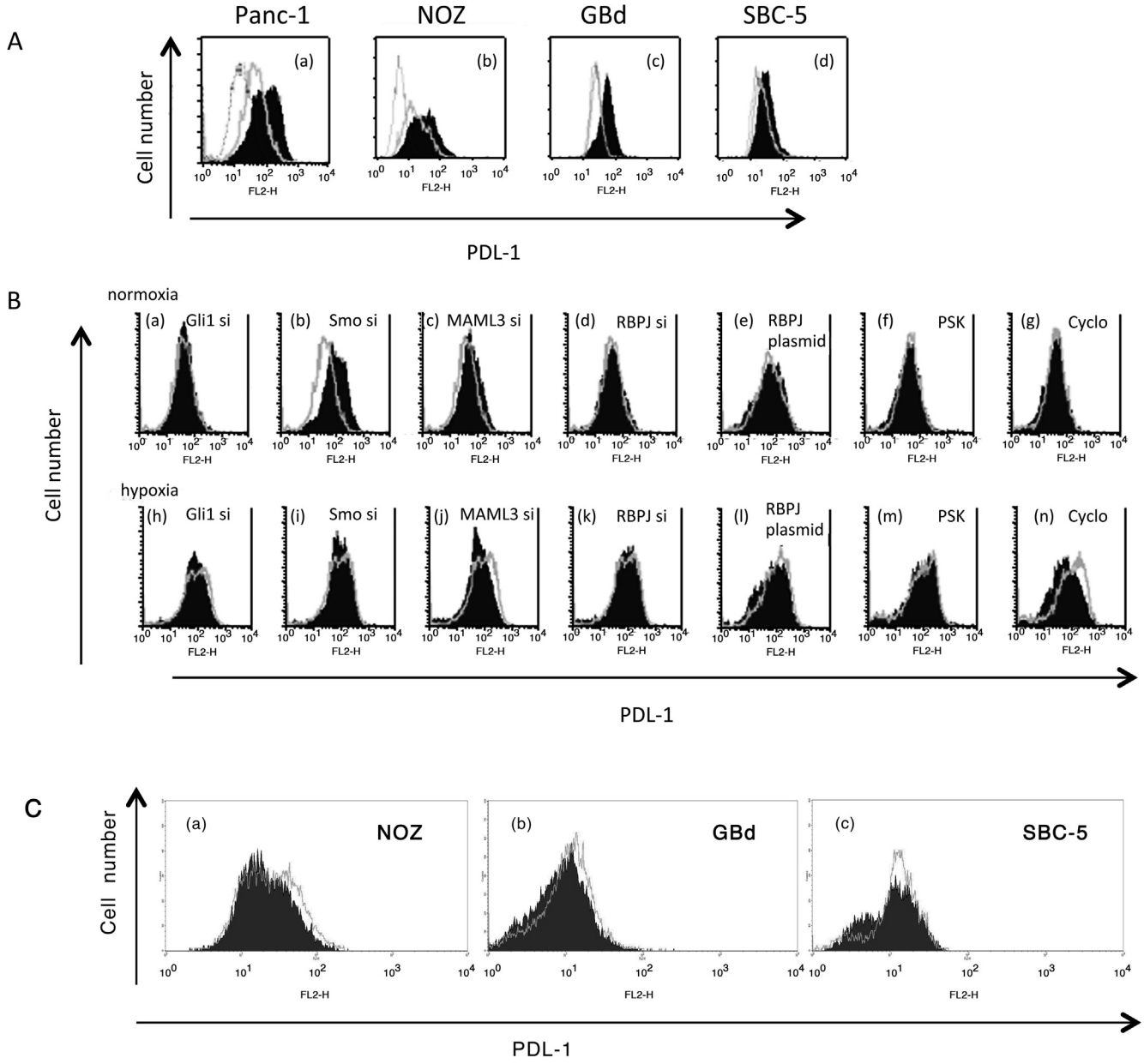

Fig. 1. Hh signaling contributes to PDL-1 expression on cancerous cells under hypoxic conditions. (A) PDL-1 expressions on Panc-1 (a), NOZ (b), GBd (c) and SBC-5 (d) cells under normoxia and hypoxia were investigated by FACS. Dotted line; control IgG, solid line; PDL-1 expression under normoxia, filled hystogram; PDL-1 expression under hypoxia. (B) FACS analyses of PDL-1 expression on Panc-1 cells. Filled histogram shows Gli-si RNA (a, h), Smo-siRNA (b, i), MAML-3 siRNA (c, j), RBPJ siRNA (d, k), and RBPJ plasmid transfected cells (e, l), respectively, and PSK-treated (f, m), and cyclopamine-treated cells (g, n). Vacant lines represent control cells. Cells were cultured under normoxic (a-g) and hypoxic (h-n) conditions for 2 days. (C) Normoxic cancer cells were pretreated with 10 μM cyclopamine for 24 h, then cultured under hypoxic conditions for 2 days. PDL-1 expression on NOZ (a), GBd (b), and SBC-5 (c) cells were examined by FACS. Filled histogram shows cyclopamine-treated cells and vacant histogram shows control cells.