

PD-1 Regulates the Growth of Human Mastocytosis Cells

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ABSTRACT

Background: Programmed death-1 (PD-1) is a marker for human neoplastic T cells. Here, we evaluated whether or not PD-1 was also a marker for human mastocytosis, and explored the role of PD-1 in human mastocytosis cells.

Methods: Immunohistochemical analysis was used to evaluate the expression of PD-1 in clinical samples of human cutaneous mastocytosis. The expression of PD-1 in human mastocytosis cell lines was checked by RT-PCR, western blotting and flow cytometry. We stimulated human mastocytosis cell lines (LAD2 and HMC1.2) with recombinant ligand for PD-1, PD-L1 (rPD-L1), and tested the proliferative activity and the status of signal molecules by Cell Counting Kit-8 and ELISA, respectively.

Results: Ten of 30 human cutaneous mastocytosis cases (33.3%) expressed PD-1 protein. We also found that a human mastocytosis line LAD2 cells expressed PD-1 protein on their surfaces. The administration with rPD-L1 suppressed the stem cell factor-dependent growth of the LAD2 cells. And, rPD-L1 activated SHP-1 and SHP-2 simultaneously, and decreased the phosphorylation of AKT, in LAD2 cells. In contrast, we could not detect the expression of PD-1, and the significant effect of rPD-L1 on the mutated KIT-driven growth of HMC1.2 cells.

Conclusions: PD-1 could be a marker for human cutaneous mastocytosis and regulate the growth of human PD-1-positive mastocytosis cells.

KEY WORDS

inhibitory receptor, KIT, mast cell, mastocytosis, PD-1

INTRODUCTION

Programmed death-1 (PD-1/CD279) is a membrane-bound type receptor, belonging to CD28/CTLA-4 family.¹ PD-1 - deficient mice developed lupus-like autoimmune diseases,² and PD-1 is known to play important roles in the regulation of immune systems. PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273) are shown to be ligands for PD-1.¹ PD-1 transduces inhibitory signals into the cells when interacted with these ligands.¹ The PD-1's inhibitory signals are mainly mediated by Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 and/or SHP-2.³ The expression of PD-1 is observed in both non-neoplastic T and non-neoplastic B cells.¹ Interestingly, only T lymphoproliferative disorders are posi-

tive for PD-1 immunohistochemically, but B lymphoproliferative disorders are negative.⁴⁻⁶ Therefore, PD-1 is utilized to discriminate between T lymphoproliferative disorders and B lymphoproliferative disorders in pathological examination. To our knowledge, there is no report whether or not PD-1 expresses on another hematoproliferative disorder, mastocytosis.

Mastocytosis is occurred by dysregulating proliferation of mast cells.⁶ The activation of KIT (CD117) by its ligand, stem cell factor (SCF, Steel factor/KIT ligand) is critical for the growth, development, and survival of mast cells.⁷ Gain of function mutations in KIT, especially D816V, is shown to cause human mastocytosis.^{8,9} Therefore, inhibition of KIT signals should be therapeutic for this neoplastic disorder.¹⁰ One of the promising methods for this purpose is the

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stimulation of inhibitory receptors on human mastocytosis cells.¹¹ The inhibitory receptors are mostly containing immunoreceptor tyrosine-based inhibitory (ITIM) motifs within their cytosolic domains.¹¹ When the receptors were activated, the non-receptor protein phosphatases, such as Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 or SHP-2, were recruited to the ITIMs and activated followed by reversing the action of tyrosine kinase cascades.¹¹ Some receptors, including CD72, FcγRIIB, Siglecs, mast cell function-associated antigen, signal regulatory protein α, and leukocyte Ig-like receptor B4 (formerly gp49B1), paired Ig-like receptor-B, myeloid-associated immunoglobulin-like receptor I, allergin-1, CD200 receptor, CD300a, and CD84, have been reported their capacities to down-regulate KIT signals on human neoplastic or non-neoplastic cells.¹¹⁻¹⁴ PD-1 is also categorized as an inhibitory receptor, and expected its potential to regulate human mastocytosis cells.

METHODS

PATIENTS

For studies conducted at Kyoto University Hospital and Kyoto City Hospital, skin biopsy samples were obtained from 30 patients diagnosed as cutaneous mastocytosis (Table 1), and 22 patients diagnosed as non-specific dermatitis (non-mastocytosis). We obtained informed consent from patients at each hospital. The methods were approved by the ethical review committee of each hospital.

CELLS

The human LAD2 mastocytosis cell line was cultured in StemPro-34 medium, with supplement, containing L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and recombinant human SCF (100 ng/ml, Peprotech).¹⁵ HMC1.2 (expresses V560G and D816V KIT) human mastocytosis cell lines were grown in IMDM medium supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml).^{9,16}

The 293T and the Jurkat cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), and grown in RPMI1640 medium supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml).

ANTIBODIES AND RECOMBINANT PROTEINS

Anti-human PD-1 antibody (Ab) (mouse monoclonal, clone: J105) was purchased from eBioscience (San Diego, CA, USA). The secondary Abs were peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin (Ig) G antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Recombinant human PD-L1/Fc chimera (rPD-L1) protein was purchased from R&D Systems (Minneapolis, MN, USA). For the stimulation on the cells,

Table 1 Characteristics of patients with cutaneous mastocytosis

Case	Gender	Age	PD-1 expression
1	Female	0	Positive
2	Male	63	Negative
3	Female	30	Negative
4	Male	64	Negative
5	Male	1	Negative
6	Male	39	Negative
7	Male	21	Negative
8	Female	47	Positive
9	Male	0	Positive
10	Female	1	Negative
11	Female	0	Positive
12	Female	0	Negative
13	Female	0	Positive
14	Male	0	Positive
15	Female	1	Negative
16	Male	63	Negative
17	Male	3	Positive
18	Female	46	Negative
19	Female	2	Negative
20	Female	3	Positive
21	Female	0	Positive
22	Male	0	Negative
23	Male	1	Negative
24	Male	2	Negative
25	Male	1	Positive
26	Female	0	Negative
27	Female	1	Negative
28	Male	1	Negative
29	Male	72	Negative
30	Female	1	Negative

rPD-L1 was bound to protein G-sepharose, modified the method of Reference 17.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemistry was executed as previously reported.¹⁸ After deparaffinization with xylene, tissue sections were rehydrated and pretreated with 0.3% hydrogen peroxide for 5 min. After steam heat for 40 min, anti-human PD-1 Ab was added as primary antibody for overnight at 4°C. Staining was performed using the ENVISION kit (DAKO Cytomation, Glostrup, Denmark) by the manufacturer's instructions. We evaluated immunostaining using peripheral T lymphoma cells as positive control for PD-1 staining.^{4,5} Mastocytosis cells in all examined specimens were reacted to anti-PD-1 antibody in all-or-none pattern.

FLOW CYTOMETRY

For the detection of PD-1 expression, LAD2 or HMC1 cells were washed, and fixed by 4% paraformaldehyde

(Sigma-Aldrich, St. Louis, MO, USA). The cells were stained with anti-human PD-1 for overnight at 4°C followed by anti-mouse IgG-FITC for 2 hours at 4°C. The cells were then analyzed using a FACScan flow cytometer.

RT-PCR

Total RNA was extracted from 5×10^5 cells (LAD2, HMC1.2, Jurkat and 293T). Cells were lysed with Trizol (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted using Rneasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized and amplified using SuperScript III One-Step RT-PCR System, according to the manufacturer's instructions (Invitrogen). One μ g of RNA was used for cDNA synthesis. Human PD-1 was amplified using the primers 5'-GAC AACGCCACCTTCACCT-3', and 5'-GCTTGTCGTC TGGTTGCT-3'.¹⁹ cDNA synthesis and PCR amplification were executed using a TaKaRa PCR Thermal Cycler Dice Standard (TaKaRa, Kyoto, Japan) with the following thermal cycle protocol. 50°C for 30 min, 95°C for 2 min followed by 40 cycles of 95°C for 30s, 55°C for 1 min, 72°C for 1 min and a final extension of 10 min at 72°C.

CELL GROWTH ASSAY

The Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used for the evaluation of the growth of LAD2 and HMC1 cells. The cells were re-cultured for 24 hours at 2×10^4 cells/100 μ l of RPMI 1640 + 10% FCS in 96-well plates in the presence or absence of beads-conjugated rPD-L1 or control IgG (10 μ g/ml, each). We added 10 μ l of CCK-8 solution for the last 2 hours and estimated the absorbance of 450 nm, according to the manufacturer's instructions.

ELISA ASSAY FOR SIGNAL MOLECULES

LAD2 were incubated in cytokine-free medium for overnight, and re-cultured with medium containing rSCF (10 ng/ml) in the presence of beads-conjugated rPD-L1 or control IgG (10 μ g/ml, each) for 15 minutes at 37°C. In other experiments, HMC1 were re-cultured without rSCF (10 ng/ml) in the presence of rPD-L1 or control beads for 15 minutes at 37°C. The cells were collected with beads, and re-suspended in lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM Na₃VO₄, 0.5 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, complete protease inhibitor cocktails (Roche, Indianapolis, IN, USA), and 1% NP-40. The samples were assayed for phospho-AKT, phospho-MEK1, phospho-JNK, phospho-p38, phospho-STAT3, phospho-NF- κ B (Cell Signalling Technology, Beverly, MA, USA, respectively), phospho-SHP-1 or phospho-SHP-2 (R&D Systems, respectively) using an ELISA kit, according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Data were expressed as the means $\pm$ SE. A *P* value less than 0.05 indicated statistical significance (Excel: Microsoft, Redmond, WA, USA).

RESULTS

IMMUNOHISTOCHEMICAL ANALYSIS ON HUMAN CUTANEOUS MASTOCYTOSIS

PD-1 is known as human neoplastic T cell marker in clinical pathological field.^{4,5} Here, we tested whether or not PD-1 could also be a marker for human cutaneous mastocytosis samples. We collected the pathological specimens diagnosed as cutaneous mastocytosis, and executed immunohistochemical analysis. Anti-PD-1 was reacted to 10 of 30 cases (33.3%) diagnosed as cutaneous mastocytosis (Table 1, Fig. 1), though PD-1 was undetectable in all-examined non-mastocytosis mast cells, including the samples diagnosed as non-specific dermatitis (Fig. 1).

PD-1 EXPRESSION ON HUMAN CULTURED MASTOCYTOSIS CELLS

We also explored PD-1 expression on human mastocytosis cell lines, LAD2 and HMC1.2. LAD2 cells express normal KIT,¹⁵ and HMC1.2 has gain-of-function type mutation in KIT gene.^{9,16} RT-PCR analysis showed the expression of PD-1 mRNA in LAD2 (Fig. 2A). FACS analysis also revealed the expression of PD-1 in LAD2 (Fig. 2B). We could detect PD-1 mRNA at the very lower level in, but could not PD-1 protein on HMC1.2 (Fig. 2A, B).

THE PD-1'S EFFECT ON THE GROWTH OF HUMAN CULTURED MASTOCYTOSIS CELLS

PD-1 plays inhibitory roles in the growth of human T cells.¹ We tried to determine the PD-1's effect on the growth of LAD2 (PD-1-positive) and HMC1 (PD-1-negative). To stimulate PD-1, we used recombinant PD-L1 (rPD-L1) conjugated with beads.¹⁷ The administration with rPD-L1 was significantly suppressed SCF-induced growth of LAD2 cells (Fig. 3A), but did not mutated KIT-driven growth of HMC1.2 cells (Fig. 3B).

THE PD-1'S EFFECT ON THE SIGNAL MOLECULES OF HUMAN CULTURED MASTOCYTOSIS CELLS

To elucidate the PD-1's inhibitory effect on LAD2, we screened the status of signal molecules of LAD2 stimulated with rPD-L1. The ELISA assay kits were utilized. The stimulation with rPD-L1 for 15 minutes declined SCF-induced phospho-AKT, when compared to the case stimulated with SCF solo (Fig. 4), but did not phospho-MEK1 (surrogate marker for ERK), phospho-JNK, phospho-STAT3, and phospho-NF- κ B (Fig. 4).

SHP-1 and SHP-2 are reported to be involved in the inhibitory effects of PD-1.³ SHP-1 and SHP-2 were ac-

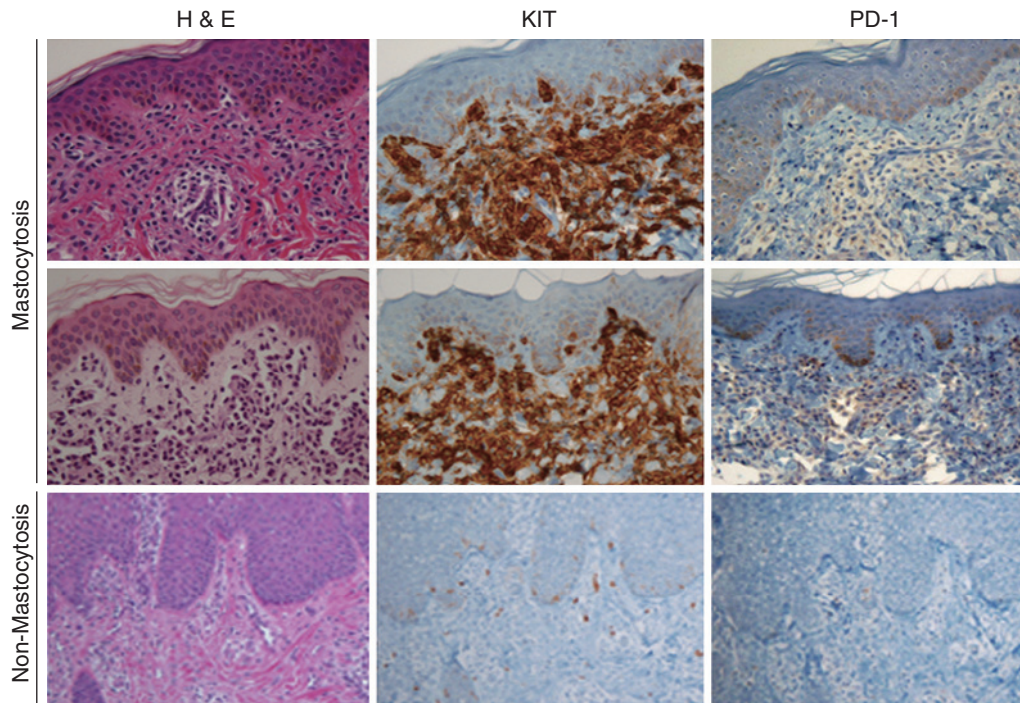


Fig. 1 PD-1 is expressed by human cutaneous mastocytosis cells. Representative cases of immunohistochemical staining for PD-1 (Bars; 50 μ m). Ten of 30 cases with cutaneous mastocytosis were positive for PD-1, immunohistochemically. The all-examined non-mastocytosis cells including the samples diagnosed as non-specific dermatitis were not stained by anti-PD-1 antibody.

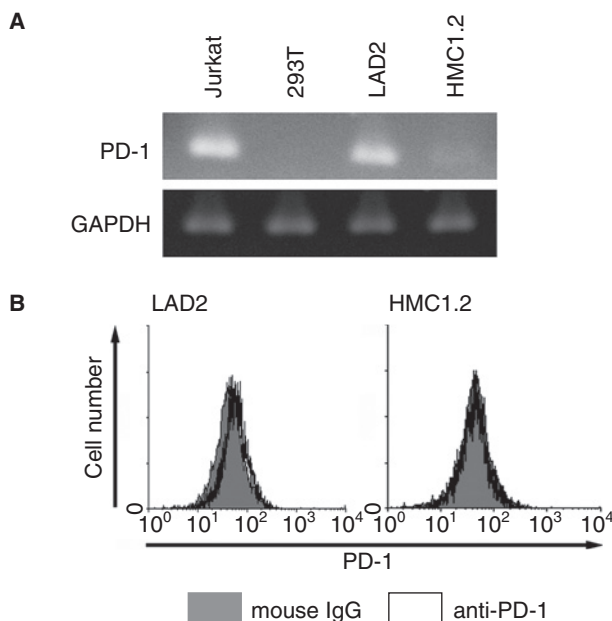


Fig. 2 PD-1 is expressed by human mastocytosis cell lines. (A) RT-PCR and (B) Flow cytometry were performed as described in Methods. The Jurkat cells were used as a positive control, and the 293T cells as a negative control.

tivated by being phosphorylated their C-terminal tails,²⁰ therefore we used an ELISA kit for phospho-SHP-1 and for phospho-SHP-2. The administration with rPD-L1 combined with SCF induced both phospho-SHP-1 and phospho-SHP-2 in LAD2 cells (Fig. 4).

DISCUSSION

Here, we found the expression of PD-1 in some mastocytosis cells included in clinical samples diagnosed as cutaneous mastocytosis and cultured in vitro, and the inhibitory role of this protein on human mastocytosis cells.

Our immunohistochemical analysis detected the expression of PD-1 on human cutaneous mastocytosis, but did not on non-neoplastic mast cells. We detected the very weak expression of PD-1 on human cultured mast cells derived from healthy volunteers²¹ (data not shown), therefore we suspected the PD-1's expression on non-mastocytosis cells in clinical samples too weakly to be detected by the immunohistochemical analysis.

We analyzed the role of PD-1 using human mastocytosis cell lines, and found the PD-1's inhibitory roles in LAD2 expressing PD-1 (Fig. 3A). The PD-1's inhibitory function in T cells is mediated by SHP-1 and/or SHP-2.^{3,4} In LAD2, both SHP-1 and SHP-2 were activated by the stimulation on PD-1 (Fig. 4).

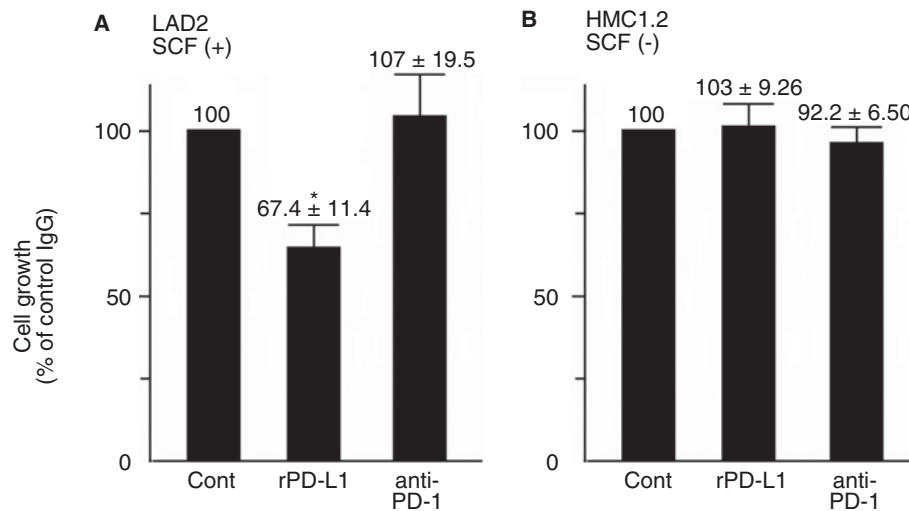


Fig. 3 The stimulation on PD-1 with recombinant PD-L1 suppresses the growth of LAD2 cells, but dose not that of HMC1 cells. The cells were incubated for 24 hours with beads-conjugated control IgG or rPD-L1. The Cell Counting Kit-8 was used for the evaluation of the growth of the cells ($n = 4$), according to the manufacturer's protocol. (A) LAD2. (B) HMC1.2. * $P < 0.05$, when compared with the value of vehicle.

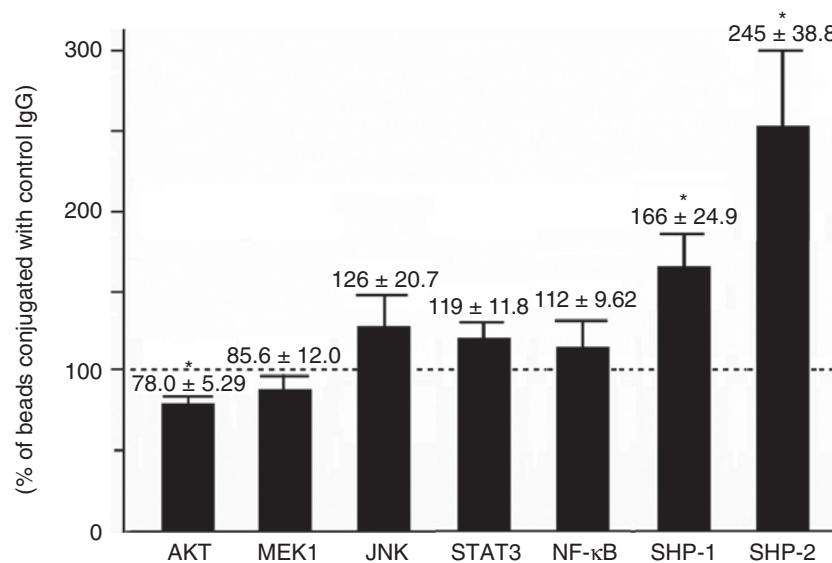


Fig. 4 The administration with rPD-L1 down-regulated the phosphorylation of AKT, and induced the phosphorylations of SHP-1 and SHP-2 in LAD2 cells. After rPD-L1 was administrated for 15 minutes, the cells were collected. The cell lysates were evaluated by the ELISA kit. ELISA for phospho-AKT, phospho-MEK1, phospho-JNK, phospho-STAT3, phospho-NF-κB, phospho-SHP-1 and phospho-SHP-2. * $P < 0.05$, when compared with the value of vehicle.

Signal analysis revealed the decreased phospho-AKT (Fig. 4). PI3K - AKT signals play pivotal role of the growth of human mast cells,²² therefore down-regulated AKT would be sufficient for the suppression of LAD2 growth. SHP-1 activation without SHP-2 activation could not suppress PI3K - AKT signals in human mast cells,¹³ and we thought the dual activa-

tion of SHP-1 and SHP-2 seemed to be necessary for the suppression of activation of AKT in LAD2.

To our knowledge, there is no report on the PD-1's expression on human mast cells, though there is a report on the expression of the ligands for PD-1 in mouse mast cells.²³ PD-L1 or PD-L2 expression on human mastocytosis cells was explored. We could

not detect the expression of PD-L1 or PD-L2 in LAD2 by flow cytometry (data not shown), and could not observe the significant effect of the antibodies which inhibited the PD-1 - PD-L1 interaction on the LAD2's growth (data not shown). HMC1.2 was confirmed its expression of PD-L1 and PD-L2. The growth of HMC 1.2 was not influenced by the administration with anti-PD-1 blocking antibody (data not shown). It would be owing to the deficient expression of PD-1 (Fig. 2A, B). The effect of intracellular PD-1 - PD-L1 interaction could be excluded among LAD2 cells or HMC1.2 cells in our in vitro study (Fig. 3A, B).

Currently, anti-PD-1 antibodies have been tested for the treatment of neoplastic and autoimmune diseases.²⁴⁻²⁶ Our study seems to suggest that such antibodies could affected on human mastocytosis cells. Side effect via mastocytosis cells should be considered when using the anti-PD-1 antibodies.

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