Journal of clinical and experimental hematopathology
Vol. 57 No.1, 21-25, 2017

Original Article

Stat3 inhibitor abrogates the expression of PD-1 ligands on lymphoma cell lines

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Recent studies have indicated the significance of immune checkpoint molecules including programmed death-1 (PD-1), cytotoxic T-lymphocyte associated protein 4, and T-cell immunoglobulin and mucin domain-containing molecule-3 for anti-tumor immune responses. We previously investigated PD-1 ligand 1/2 (PD-L1/2) expression in lymphoma cell lines, and found that PD-L1/2 is expressed on the adult T-cell leukemia/lymphoma (ATL-T) and B-cell lymphoma (SLVL) cell lines. In the present study, we investigated whether the Stat3 inhibitor WP1066 abrogated PD-L1/2 expression in lymphoma cell lines. Incubation with WP1066 inhibited lymphoma cell growth and induced cell apoptosis. PD-L1/2 expression in the ATL-T, SLVL, and human brain malignant lymphoma (HKBML) cell lines was significantly abrogated by WP1066 treatment. These data indicated that a Stat3 inhibitor abrogated PD-L1/2 expression in lymphoma cells. Such an inhibitor is therefore considered to be useful for additional immunotherapy in patients with advanced lymphoma.

Keywords: PD-L1, PD-L2, Stat3, lymphoma

INTRODUCTION

Immuno-checkpoint inhibitors have attracted considerable attention in recent years. The immune escape of tumor cells is closely involved with tumor progression. Such immune escape is caused by T-cell exhaustion and is mediated by inhibitory signals that activate immune checkpoint molecules including programmed death-1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and T cell immunoglobulin and mucin domain-containing-3 (TIM3).[1-3] The PD-1 ligand 1 (PD-L1, also known as B7-H1) is known to be widely expressed by leukocytes including macrophages and tumor cells.[1] PD-L2 (also known as B7-DC) is expressed mainly by dendritic cells and macrophages; however, there are only a few studies that have investigated its expression in tumor cells.[1,3] A significantly high response rate to PD-1 blockade has just been reported in patients with Hodgkin lymphoma,[4] and several clinical trials using immune checkpoint inhibitors are now ongoing.[5]

The expression of PD-L1 has been associated with a poor clinical course in several malignant tumors including lymphoma.[3] Ohshima K *et al*. reported that PD-L1 expression on lymphoma cells was detected in 11% and 7.4% of diffuse large B-cell lymphoma (DLBCL) and adult T-cell leukemia/lymphoma (ATLL) patients respectively, and that the PD-L1-positive groups showed a significantly worse clinical course.[6,7]

We previously studied PD-L1/2 expression in lymphoma tissues, and found that PD-L1/2 was expressed in macrophages in almost all of the cases studied but that it was expressed on lymphoma cells in less than 10% of the cases.[8] In the same study, PD-L1/2 expression in macrophages was found to be dependent on activation of the signal transducer and activator of transcription 3 (Stat3). Although we also showed that the ATL-T (ATLL) and SLVL (B cell lymphoma) cell lines express high levels of PD-L1/2, the involvement of Stat3 activation in PD-L1/2 expression in lymphoma cells has never been tested by both *in vitro* and *in vivo* studies. In the present study, we therefore investigated if Stat3 activation is involved in the regulation of PD-L1/2 expression by lymphoma cell lines.

Received: February 20, 2017. Revised: March 9, 2017. Accepted: March 27, 2017. J-STAGE Advance Published: May 10, 2017.
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MATERIALS AND METHODS

Cell lines

The human ATLL cell line (ATL-T) and the B-cell lymphoma cell line (SLVL) were maintained in RPMI supplemented with 10% fetal bovine serum. The primary central nervous system lymphoma cell line (HKBML) was maintained in DMEM/F12 supplemented with 15% fetal bovine serum. ATL-T was previously established by Prof. Morikawa,[9] and the other cell lines were purchased from the RIKEN Cell Bank (Wako, Japan). PD-L1/2 expression was detected in ATL-T and SLVL cells in our previous study. Mycoplasma testing was performed by using a polymerase chain reaction (PCR) detection kit (Takara Bio Inc., Otsu, Japan).

Cell proliferation and caspase-3 activation assay

For analysis of cell viability, in brief, 1 x 10^4 cells were seeded in a 96-well plate and the cells were then cultured in the presence of the indicated concentration of WP1066 (Santa Cruz Biotech, Dallas, TX, USA) for 3 days. Cell viability was determined using the WST assay (WST-8 cell counting kit; Dojin Chemical, Kumamoto, Japan) according to the manufacturer's protocol. For caspase assay, 2 x 10^5 cells were seeded in a 24-well plate and the cells were then cultured in the presence of WP1066 for 24 h. A caspase-3 activatable fluorescent probe was prepared by conjugating the near infra-red (NIR) fluorescence dye, Cy3 (ex/em, 550/570) and the black hole NIR quencher-2 (BHQ-2, abs., 579 nm) to a Caspase-3 peptide substrate.[10,11] Briefly, Caspase-3 activatable sensor is composed of Cy3 NIR dye (Ex/Em: 550/570 nm) was chemically conjugated N-terminus of the caspase-3 peptide substrate (Gly-Asp-Glu-Val-*Asp-Ala*-Pro-Lys-Gly-Cys, cleavage site: indicate in Italic) and BHQ-2 as Cy3 fluorescence quencher (Absorbance: 579 nm). To confirm the specificity of caspase-3 activatable sensor, 1 ug of Caspase-3 sensors were incubated with 30 nM of recombinant Caspase-3, Caspase-8, Caspase-9, and Caspase-3 with inhibitor (Z-DEVD-FMK) in reaction buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, pH 7.5) for 60 min at 37 °C. Fluorescence signals were acquired with a fluorescence spectrophotometer (Tecan M200 Pro) every 10 min at 37 °C. Under the same environment, sensitivity of the Caspase-3 activatable sensor (1 ug) was tested with different concentrations of recombinant Caspase-3 (60, 30, 15, 7.5, 3.8, 1.9, 1 nM). The cells were treated with this caspase-3 activatable sensor for 2 hours, and fluorescence was subsequently analyzed using the FACSverse (Becton Dickinson, Franklin Lake, NJ, USA) flow cytometer with the FACSuite (Becton Dickinson) software.

Flow cytometry

Cells were detached using cell dissociation buffer (Thermo Fisher Scientific, Waltham, MA), and stained using a PE-labeled anti-PD-L1 antibody and an APC-labeled anti-PD-L2 antibody (BioLegend, San Diego, CA, USA) in Fc receptor blocking solution (BioLegend). Isotype-matched antibodies (BioLegend) were used as controls. The stained cell samples were analyzed using flow cytometry.

Statistics

Student's *t*-test was used for statistical analysis. A *P* value <0.05 was considered to be statistically significant. All data of cell culture studies are representative of at least two independent experiments. All error bars indicate the standard deviation (SD).

RESULTS

The Stat3 inhibitor suppressed lymphoma cell proliferation through induction of cell apoptosis.

We first tested the cytotoxic effect of the Stat3 inhibitor, WP1066, on the ATL-T, SLVL, and HKBML cell lines. These cell lines were cultured with various concentrations of WP1066 for 3 days, following which the total cell number was examined using the WST assay. WP1066 significantly inhibited the growth (viability) of ATL-T and SLVL cells at a concentration of 5 μmol/L and the growth of HKBML cells at a concentration of 2.5 μmol/L compared to control cells (Figure 1). We next tested the effect of WP1066 treatment of these cells on caspase 3 activity to evaluate its effects on cell apoptosis. For this purpose, we used a caspase-3 activatable fluorescent sensor whose specificity for caspase 3 and sensitivity to activated caspase-3, are shown in Figure 2A, 2B, respectively. Incubation of the cells for one day with WP1066 increased caspase 3 activity in a dose dependent manner in all cell lines, as shown in Figure 2C, 2D. WP1066-induced caspase-3 activation was weak at a WP1066 concentration of 20 μmol/L when the cells were

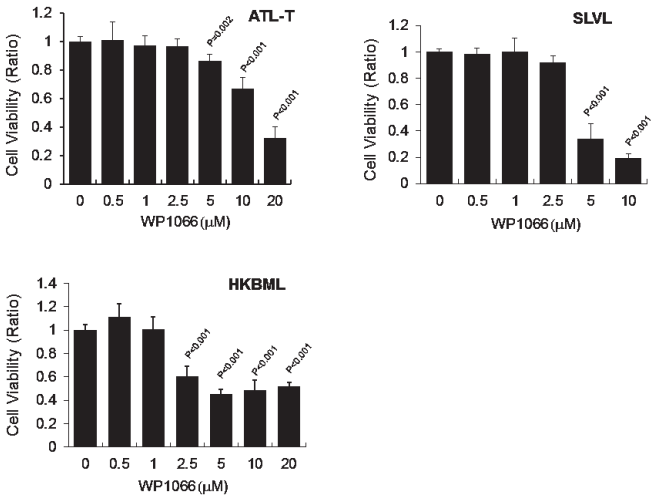

Fig. 1. Anti-lymphoma effect of WP1066. The indicated lymphoma cell lines were cultured with various concentrations of WP1066 for 3 days, following which cell number was evaluated based on the total cell number as determined using a WST assay. DMSO was added to the control well.

incubated with WP1066 for 1 day. WP1066 was therefore used at a concentration of less than 20 μmol/L in the next experiments.

The Stat3 inhibitor down-regulated PD-L1/2 expression in lymphoma cell lines.

We then tested whether WP1066 influenced PD-L1/2 expression in the ATL-T, SLVL, and HKBML cell lines. The cells were cultured with WP1066 for 24 h following which the expression of PD-L1/2 was evaluated using flow cytometry. PD-L1/2 expression was significantly reduced in ATL-T cells by 10 μmol/L WP1066 and in SLVL and HKBML cells by 5 and 10 μmol/L compared to control (Figure 3,4).

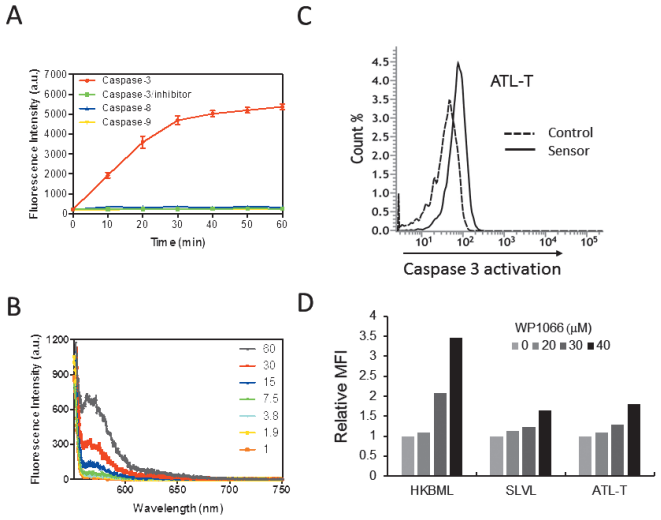

Fig. 2. Caspase-3 activation induced by WP1066. (***A***) Analysis of the specificity of the caspase reporter for caspase-3 activity was performed as described in Materials and Methods. (***B***) Sensitivity of the Caspase-3 activatable sensor was tested with different concentrations of recombinant Caspase-3. (***C***) Histogram of flow cytometric analysis of ATL-T is shown. (***D***) Relative mean fluorescent intensity (MFI) which reflects caspase activation of the sensor is shown.

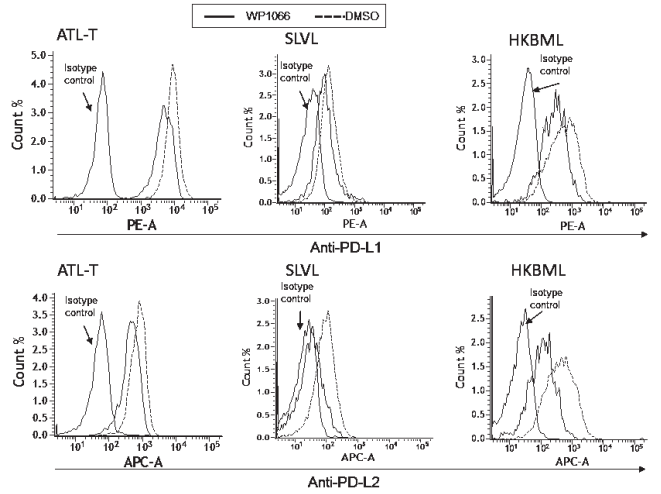

Fig. 3. The effect of WP1066 on PD-L1/2 expression. The ATL-T, SLVL, and HKBML cell lines were incubated with DMSO or WP1066 (10 μM) for 24 h, following which PD-L1/2 expression was evaluated using flow cytometry.

DISCUSSION

In the present study, we demonstrated an anti-lymphoma effect of the Stat3 inhibitor in Figure 1. Stat3 is well established as a transcription factor that is activated by Janus kinase 1/2 (JAK1/2). Phosphorylated Stat3 translocates into the nucleus where it induces the expression of many genes related to tumor progression.[12] It is also well known that activated Stat proteins are observed in lymphoma cells,[13-16] and several Stat3 inhibitors have been found to abrogate lymphoma progression.[17] Just recently, a new generation of antisense oligonucleotide inhibitors of STAT3 has been reported to show antitumor activity in lymphoma and lung cancer patients.[18] Stat3 inhibition significantly abrogated expression of anti-apoptotic molecules such as survivin in lymphoma cells, and has also been suggested to inhibit protumor functions of stromal cells including macrophages.[18] Thus Stat3 inhibition might be a promising approach for anti-lymphoma therapy.

PD-L1 expression has been detected in Hodgkin lymphoma cells, in which its expression is regulated by extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways.[19,20] PD-L1 expression is often observed in anaplastic lymphoma kinase (ALK)-positive T cell lymphoma cells, and its overexpression has been attributed to Stat3 activation induced by chimeric nucleophosmin (NPM)/ALK.[21] In ovarian cancer cells, PD-L1 expression was reported to be up-regulated by IL-27 and IFN-γ via activation of Stat1 and Stat3.[22] However, there have been few studies related to PD-L1/2 expression in aggressive lymphomas. In the present study, we demonstrated that PD-L1/2 expression was significantly reduced by a Stat3 inhibitor, and this finding might be the first report to show a relationship between PD-L1/2 expression and Stat3 in lymphoma.

Stat3 activation is closely linked to immune suppression via its up-regulation of the production of cytokines such as IL-6, IL-10, and TGF-β.[12,23,24] Stat3 is also a key molecule for immune suppression by stromal cells such as tumor associated macrophages (TAMs).[23-25] Stat3 activation in myeloid cells is significantly related to immune suppression.[26,27] We

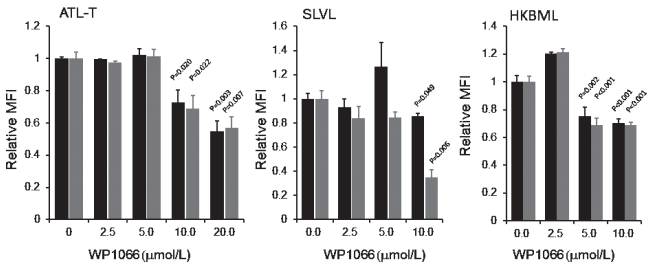

Fig. 4. Summary of the reduction in PD-L1/2 expression by WP1066. The mean fluorescent intensity (MFI) of PD-L1/2 signals obtained in flow cytometric analysis of the indicated cell lines incubated with various concentrations of WP1066 or with DMSO control are summarized (n=3 per group).

previously showed that IL-27/Stat3 signals were closely contributed to PD-L1/2 expression in TAMs.[8] Stat3 activation has been detected in both tumor cells and in TAMs in tumor tissues, and *in vitro* studies have shown that cell-cell interactions between tumor cells and macrophages are closely involved in Stat3 activation in tumor tissues including lymphoma.[28-31] Although the present study indicated the Stat3 inhibitor abrogated the PD-L1/2 expression in lymphoma cells which were positive for PD-L1/2, further studies are necessary to elucidate if Stat3 activation is involved in PD-L1/2 overexpression in lymphoma cells in patients with malignant lymphoma.

In conclusion, we demonstrated that Stat3 activation is related to PD-L1/2 expression in the ATL-T, SLVL, and HKBML cell lines, and that WP1066 significantly abrogated this PD-L1/2 expression. Inhibition of PD-L1/2 expression in lymphoma cells and TAMs might influence anti-lymphoma therapy using a Stat3 inhibitor.

ACKNOWLEDGMENTS

We thank Ms. Ikuko Miyakawa, Ms. Yui Hayashida, and Mr. Takenobu Nakagawa for their technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 16H05162, 16K15503).

CONFLICT OF INTEREST

All authors have no financial competing interests to declare.

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