Myeloma Drug Resistance Induced by Binding of Myeloma B7-H1 (PD-L1) to PD-1

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Abstract

B7 homolog 1 (B7-H1)—expressing myeloma cells not only inhibit myeloma-specific cytotoxic T lymphocytes (CTL), but also confer a proliferative advantage: resistance to antymyeloma chemotherapy. However, it remains unknown whether B7-H1 expressed on myeloma cells induces cellular responses associated with aggressive myeloma behaviors. To address this question, we analyzed the proliferation and drug sensitivity of myeloma cells transfected with B7-H1—specific short-hairpin RNA or treated with programmed cell death (PD)-1–Fc–coupled beads. Knockdown of B7-H1 expression in myeloma cells significantly inhibited cell proliferation and increased apoptosis induced by the chemotherapeutic alkylating agent melphalan, with downregulation of the expression of cell cycle–related genes (CCND3 and CDK6) and antiapoptotic genes (BCL2 and MCL1). B7-H1 molecules thus contributed to myeloma cell-cycle progression and suppression of drug-induced apoptosis. B7-H1—expressing myeloma cells had a higher affinity for PD-1 than for CD80. PD-1–Fc–bead–treated myeloma cells also became resistant to apoptosis that was induced by melphalan and the proteasome inhibitor bortezomib. Apoptosis resistance was associated with the PI3K/AKT pathway. Both myeloma cell drug resistance and antiapoptotic responses occurred through the PI3K/AKT signaling pathway, initiated from “reverse” stimulation of B7-H1 by PD-1. Therefore, B7-H1 itself may function as an oncogenic protein in myeloma cells. The interaction between B7-H1 on myeloma cells and PD-1 molecules not only inhibits tumor-specific CTLs but also induces drug resistance in myeloma cells through the PI3K/AKT signaling pathway. These observations provide mechanistic insights into potential immunotherapeutic benefits of blocking the B7-H1–PD-1 pathway.


Introduction

B7 homolog 1 (B7-H1), also known as programmed death-ligand 1 (PD-L1) or CD274, is an immune checkpoint molecule belonging to the B7 family and is detected on many different tumor cells, such as hepatocellular, breast, pancreatic, colon, and renal cell carcinomas, and hematologic malignancies (1–4). In contrast, the expression of programmed cell death 1 (PD-1), which is a member of the CD28 family of molecules and a B7-H1 receptor, is upregulated on tumor-infiltrating T cells, as well as on other activated T cells (4–8). B7-H1 molecules expressed on tumor cells play a crucial role in tumor evasion from cytotoxic T lymphocyte (CTL)-mediated immune surveillance through PD-1 (9, 10). Tumor-associated B7-H1 delivers an inhibitory signal to tumor-specific CTLs via the B7-H1–PD-1 interaction, resulting in T-cell apoptosis in vitro and in vivo, and the B7-H1–PD-1 interaction attenuates activated tumor-infiltrating T cells through the inhibition of T-cell receptor (TCR) signaling (1, 4, 11, 12). We previously demonstrated that B7-H1—positive myelodysplastic syndrome blasts have a greater ability to induce T-cell apoptosis compared with B7-H1—negative blasts, through the B7-H1–PD-1 pathway (3). B7-H1 molecules can transmit an inhibitory signal to CD80-expressing T cells via the B7-H1–CD80 interaction (13), although the role of that interaction in tumor immunity has not been clarified. The impact of tumor-associated B7-H1 expression on prognosis has been reported in cancer patients. Overall survival rates are significantly lower in patients with high B7-H1 expression compared with those with low B7-H1 expression in some tumors (14–17). Abundant PD-1 on tumor-infiltrating T cells is also associated with poor overall survival (6, 18, 19). Thus, research on B7-H1–PD-1 interactions in the tumor microenvironment is now focused on a new target for immunotherapy in patients with various types of cancer who show high B7-H1 expression on tumor cells. In clinical practice, blockade of the B7-H1–PD-1 pathway using antibodies to PD-1 or to B7-H1 markedly improves survival in some B7-H1—positive patients with non–small cell lung, prostate, and renal cell cancer, and melanoma (20–22).

Multiple myeloma is a malignancy of B cells characterized by an uncontrolled expansion of plasma cells (called myeloma cells) within the bone marrow (23). The treatment of multiple myeloma has made remarkable progress...
following recent approval of immunomodulatory drugs, i.e., thalidomide, lenalidomide, and pomalidomide, and the novel proteasome inhibitor bortezomib, in addition to the standard chemotherapy, although it remains an incurable disease (23–25). B7-H1 expression on plasma cells from patients with multiple myeloma is significantly upregulated compared with cells from patients with monoclonal gammopathy of undetermined significance or healthy volunteers (2, 26) and increases with disease progression (2).

B7-H1 expression on myeloma cells was upregulated by IL6 via STAT3 signaling (2) and IFNγ through a MEK/ERK pathway (26). Expression of B7-H1 on tumor cells correlates with an increased risk of progression to symptomatic myeloma (27). However, it remains unknown whether any signals emanating from B7-H1 in myeloma cells, after interaction with PD-1 or CD80, affect myeloma responses. In this study, we explored cellular responses induced by B7-H1–PD-1 or B7-H1–CD80 interaction in B7-H1–expressing myeloma cells.

Materials and Methods

Cell lines

The human myeloma cell lines were cultured in RPMI1640 medium (Wako Chemical Industries) containing 10% fetal bovine serum (GE Healthcare), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Life Technologies) at 37°C under 5% CO2. B7-H1–transfected KMS28-PE (B7-H1.KMS28-PE) cells were established as described (2). KMS28-PE cells transfected with empty vector (mock.KMS28-PE) were used as a negative control. KMS28-PE cells were kindly provided by Dr. Takemi Otsuki in 2004 (Kawasaki Medical School, Okayama, Japan). The cells were cultured to passages 3 to 5, frozen in the cell-freezing medium TC-protector (DS
Pharma Biomedical), and used within 20 passages in all experiments. To establish the human myeloma cell line MOSTI-1, mononuclear cells were separated from a bone marrow sample of a refractory myeloma patient (obtained in February 2012), after obtaining written informed consent, with Histopaque (Sigma-Aldrich) density centrifugation and cultured in complete medium in 25-cm² tissue culture flasks. After 2 months, the proliferating myeloma cells were isolated as a single clone by limiting dilution.

Reagents
The human PD-1-immunoglobulin (Ig) and CD80–Ig fusion protein were purchased from R&D Systems (catalog No.1086-PD and 140-B1). The blocking antibodies to B7-H1 or CD28 were also from R&D Systems. The antimyeloma agents melphalan and bortezomib were from Sigma-Aldrich. LY294002 (phosphatidylinositol 3-kinase [PI3K]/Akt pathway inhibitor), STAT3 inhibitor V, and pyrrolidine dithiocarbamate (PDTC; NF-kB inhibitor) were from Merck. U0126 (MEK1/2 inhibitor) was from Wako Chemical Industries.

Stable knockdown of B7-H1 expression using short-hairpin RNA
MOSTI-1 cells were transduced with Mission Lentiviral transduction particles containing B7-H1 short hairpin RNA (shRNA; NM_014143.2 clone TRCN0000056915, Sigma-Aldrich) in the presence of 8 μg/mL of hexadimethrine bromide (Sigma-Aldrich) at a multiplicity of infection of 5. MOSTI-1 transduced with nontarget shRNA was used as a control. To establish stable knockdown cell lines, the cells were cultured with complete medium containing 1 μg/mL of puromycin (Sigma-Aldrich) 24 hour after transduction. After 1 week, B7-H1–specific shRNA-transfected cells were isolated as a single clone by limiting dilution.

Flow cytometry
Immunophenotyping was performed as described (3, 28). In brief, after blocking with human gamma Ig (MP Biomedicals), cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies for CD80 and PD-1 (BD Biosciences), and phycocyanin (PE)-conjugated antibody for B7-H1 (eBioscience). Data acquisition was performed in a FACSVerse flow cytometer (BD Biosciences) using FlowJo software (Tree Star). Isotype-matched negative controls for FITC- and PE-conjugated antibody were used.

Cell proliferation, cell cycle, and apoptosis
Cell proliferation was determined in the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay using the Cell Counting Kit-8 (CCK-8; Dojindo). Cells (1 × 10⁴) were seeded in 96-well plates and cultured for 4 to 6 days. The cells were incubated with CCK-8 solution for 4 hours, and then the absorbance of each well was measured at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad). Bromodeoxyuridine (BrdUrd) and cell-cycle analysis was performed with a BrdUrd flow kit (BD Biosciences) and the propidium iodide (PI)-staining method, respectively, as described previously (2). To assess drug-induced apoptosis, cells were exposed to melphalan and bortezomib for 24 hours at concentrations optimal for inducing apoptosis, and then the cells were stained with annexin-FITC (BD Biosciences) and PI (Wako Chemical Industries) for the analysis using flow cytometry (FCM).

mRNA quantification using real-time PCR
Total RNA was extracted from cultured cells using Trizol Reagent (Life Technologies), and then cDNA was synthesized using PrimeScript II reverse transcriptase (Takara Bio Inc.).
mRNA expression of cell cycle (CCNA2, CCNB3, CCND1, CCND2, CCND3, CCNE1, and CCNE2), apoptotic (BAX, BAD, FAS, FASLG, FADD, CASP3, CASP8, and CASP9), and antiapoptotic genes (BCL2, BCL2L1, MCL1, and PIM2) was determined in the quantitative real-time polymerase chain reaction (qPCR) using SYBR Premix Taq II (Tli RNaseH Plus; Takara Bio Inc.) and 7500 fast real-time PCR system (Life Technologies). The relative expression was calculated by the formula $2^{-\Delta \Delta Ct} \times 1,000$. The $\Delta Ct$ value is calculated by subtraction of Ct values (target gene/internal control gene); $\beta$-actin was used as an internal control. Primer sequences are shown in Supplementary Table S1.

Western blotting

Cells were lysed with $2\times$ loading buffer (200 mmol/L Tris-HCl, pH 6.8, 4.0% SDS, 0.004% BPP, and 20% glycerol). After sonication of cell lysates, $1.5 \times 10^5$ lysed cells were electrophoresed on SDS–12.5% and 15% polyacrylamide gel and then transferred to a PVDF membrane. The target protein was detected using the ECL Prime Western Blotting Detection System (GE Healthcare). The first antibodies were: 1,000-fold diluted mouse antibody to cyclin D3, and rabbit antibody to BCL2, MCL1, caspase-3, cleaved caspase-3, phospho-Akt (Ser473), and PI3K p85; 5,000-fold diluted rabbit antibody to Akt (Pan); and 3,000-fold diluted rabbit antibody to $\beta$-actin (Cell Signaling Technology Inc.). The chemiluminescence of targeted protein was detected with image-Quant LAS 4000mini (GE Healthcare) and quantified using ImageJ version 1.46r software (National Institutes of Health, Bethesda, MD).

Preparation of PD-1–Fc or CD80–Fc–coupled beads

M450 Epoxy dynabeads (4 $\times$ 10$^8$; Life Technologies) were coupled with 100 $\mu$g of human PD-1-Ig and CD80-Ig fusion protein according to the manufacturer’s instructions. Human Ig-conjugated dynabeads were used as a negative control.
Statistical analysis
The Student t test and Mann–Whitney U test were used to analyze the two-group comparisons. P values of less than 0.05 were considered statistically significant.

Results
Knockdown of B7-H1 reduced cell proliferation and drug resistance
B7-H1 expression on myeloma cells is associated with biological advantages for the tumor, such as cell proliferation and drug resistance (2), although the mechanisms for these in B7-H1–expressing cells have remained unclear. Therefore, we investigated whether B7-H1 expression on myeloma cells is directly associated with those advantages using the B7-H1 knockdown system. First, we established a novel B7-H1–highly expressing myeloma cell line named MOSTI-1, generated from a refractory myeloma patient with no chromosomal abnormality using G-banding analysis (Supplementary Fig. S1), because most myeloma cell lines expressed lower B7-H1 without stimulation of IL6 or IFNγ (2). MOSTI-1 cells characteristically express extremely high B7-H1 and CD28 but negligible

Figure 4.
Effect of the PD-1–B7-H1 interaction on cell proliferation potential and drug sensitivity in MOSTI-1 cells. Cell-cycle analysis (A) and BrdUrd incorporation (B) in PD-1-Fc bead-treated MOSTI-1 cells were analyzed using FCM after 5 × 10^5 MOSTI-1 cells mixed with 2.5 × 10^6 PD-1-Fc or CD80-Fc beads were seeded in 48-well plates and cultured for 2 days. Sensitivity to melphalan (C) or bortezomib (D) in PD-1-Fc or CD80-Fc bead-treated MOSTI-1 cells. Inhibition of melphalan resistance of PD-1-Fc bead-treated MOSTI-1 cells by anti-B7-H1 (E). The cells were preincubated with 10 μg/mL of monoclonal antibody to B7-H1 or control Ig at 4°C for 30 minutes and then treated with the beads for 24 hours. After incubation with melphalan for another 24 hours, cells with melphalan-induced apoptosis were detected using FCM. Melphalan-induced apoptosis in PD-1-Fc bead-treated mock.KMS28-PE and B7-H1.KMS28-PE cells (F). The data are expressed as mean ± SD of triplicate experiments. *, P < 0.05; **, P < 0.01.
amounts of their receptors and ligands, i.e., PD-1, CD80, and CD86, with mature plasma cell morphology (Fig. 1A and Supplementary Fig. S1). Next, three stable B7-H1–knockdown MOSTI-1 cell lines were established and their growth and drug sensitivity were analyzed. B7-H1 expression on B7-H1–knockdown MOSTI-1 cells was downregulated by 40% to 60% (Fig. 1B), and these cells proliferated more slowly than control cells (Fig. 1C). Consistent with these results, BrdUrd incorporation in B7-H1–knockdown MOSTI-1 cells was significantly lower than control cells (Fig. 1D). When these cells were treated with melphalan, apoptosis was significantly increased in B7-H1–knockdown MOSTI-1 cells compared with control cells (Fig. 1E). These results indicated that B7-H1 expression on myeloma cells is directly associated with their aggressive characteristics, i.e., increased cell proliferation and drug resistance. Although another immune checkpoint pathway, CD28–CD86, with mature plasma cell morphology (Fig. 1A and Supplementary Fig. S1), is previously reported to prevent cell death induced by melphalan (29–31), the blockade of B7-H1 on MOSTI-1 cells using anti-B7-H1 did not affect BrdUrd incorporation and the number of apoptotic cells induced by melphalan (Fig. 1F), suggesting that MOSTI-1 cells expressed no functional receptors for B7-H1.

**Mechanism of aggressive behaviors in B7-H1+ myeloma cells**

To clarify the mechanism by which B7-H1–knockdown MOSTI-1 cells acquired aggressive myeloma behaviors, we examined the expression of cell cycle, apoptotic, and antiapoptotic genes and proteins. The mRNA and protein expression of cyclin D3 (CCND3) in three B7-H1–knockdown MOSTI-1 cell clones was significantly downregulated in comparison with that in control cells (Fig. 2A and B). We also investigated the mRNA of cyclin-dependent kinase 4 (CDK4) and CDK6, which form a functional complex with CCND3 in phase G1 of the cell cycle. CDK6 mRNA was decreased in B7-H11 knockdown MOSTI-1 cells (Fig. 2A), but CDK4 mRNA was the same as that in the controls (data not shown). The mRNA expression of B-cell lymphoma 2 (BCL2) and myeloid cell leukemia 1 (MCL1) was significantly lower in B7-H1–knockdown MOSTI-1 cells than in control cells (Fig. 2C), but the expression of other antiapoptotic and apoptotic genes did not change (data not shown). Protein expression of BCL2 and MCL1 was also decreased by B7-H1 knockdown (Fig. 2B). Cleaved caspase-3 expression was slightly upregulated in B7-H1–knockdown MOSTI-1 cells (Fig. 2B). In contrast, mRNA expression of CCND3, CDK4, CDK6, BCL2, MCL1, and PIM2 was higher in B7-H1–KMS28-PE cells than in mock-KMS28-PE cells (Supplementary Fig. S2). Thus, B7-H1 molecules affected the expression of G1–S phase-specific and antiapoptotic genes and proteins in myeloma cells.

**Binding of B7-H1 expressed on myeloma cells to PD-1 and CD80**

To clarify whether B7-H1–expressing myeloma cells have high affinity for PD-1 or CD80, we investigated the binding affinity of B7-H1 expressed on myeloma cells for PD-1 and CD80. After cultivation of MOSTI-1 cells with dynabeads coupled with either PD-1-Fc or CD80-Fc, MOSTI-1 cells bound more tightly to PD-1-Fc beads than to CD80-Fc beads (Fig. 3A).

**Figure 5.** The reverse signal derived from the PD-1–B7-H1 interaction was associated with the activation of the AKT signaling pathway. PD-1-Fc bead-treated MOSTI-1 cells were cultured with 20 μmol/L of LY294002 (PI3K/AKT pathway inhibitor), 15 μmol/L of U0126 (MEK1/2 inhibitor), 500 nmol/L of PDTC (NF-κB inhibitor) for 2 hours. A and B, after replacing the fresh complete medium, the treated cells were cultured with 15 μmol/L of melphalan (A) or 15 nmol/L of bortezomib (B) for 24 hours and then the apoptotic cells were detected using FCM. C, Western blotting analysis of phosphorylated Akt (p-Akt), Akt, and β-actin in MOSTI-1 or B7-H1.KMS28-PE cells 48 hours after treatment with PD-1-Fc and control Ig beads. D, relative intensity of the p-Akt protein was calculated based on the signals of p-Akt and Akt. Each duplicate experiment was repeated twice, and the data are expressed as the mean ± SD of a representative result. Akt and p-Akt, 60 kDa; β-actin, 42 kDa. *, P < 0.05; **, P < 0.01.
antibody to B7-H1 and then cocultured with PD-1-Fc. The binding of PD-1-Fc to MOSTI-1 cells was inhibited by anti-B7-H1 in a dose-dependent manner (Fig. 3B). Similar to MOSTI-1 cells, PD-1-Fc beads were tightly bound to B7-H1 KMS28-PE but not to mock KMS28-PE cells (Fig. 3A). Mock KMS28-PE cells that lack B7-H1 expression were bound to CD80-Fc beads, because these cells express CD28, one of the receptors for CD80. When these cells were pretreated with antibody to CD28 before cocultivation with CD80-Fc beads, the number of B7-H1 KMS28-PE cells bound to CD80-Fc beads decreased (Fig. 3C). Thus, the binding affinity of B7-H1 expressed on myeloma cells was higher for PD-1 than for CD80.

Figure 6. B7-H1–induced reverse signals in myeloma cells. A, mRNA expression of BCL2, FASLG, and FADD was quantified using real-time PCR in PD-1-Fc– or control Ig bead–treated MOSTI-1 cells. B, Western blotting analysis of PI3K, p-AKT, AKT, BCL2, caspase-3, cleaved caspase-3, and β-actin in melphalan (MEL)– or bortezomib (BOR)–treated MOSTI-1 cells after incubation with PD-1-Fc or control Ig beads. C, relative intensity of the p-Akt protein was calculated based on the signals of p-Akt and Akt. D, relative intensity of the BCL2 and cleaved caspase-3 to β-actin was calculated based on the signals of these proteins, and the data are expressed as mean ± SD of duplicate experiments. Akt and p-Akt, 60 kDa; PI3K, 85 kDa; BCL2, 28 kDa; caspase-3, 35 kDa; cleaved caspase-3, 17 kDa; β-actin, 42 kDa. *, P < 0.05; **, P < 0.01.
Acquired drug resistance in myeloma cells via B7-H1–PD-1 stimulation

To investigate whether the reverse signal resulting from B7-H1 binding to PD-1 or CD80 induced aggressive behavior in B7-H1–expressing myeloma cells, we examined the cell cycle, BrdUrd incorporation, and apoptosis resistance induced by antimonyeloma agents when MOSTI-1 cells were cocultured with beads coated with control Ig, CD80-Fc, or PD-1-Fc. Neither the cell cycle nor BrdUrd incorporation in PD-1-Fc bead–treated MOSTI-1 cells differed significantly from that in control Ig bead–treated MOSTI-1 cells (Fig. 4A and B). However, the percentage of melphanalin-induced apoptotic cells in PD-1-Fc bead–treated MOSTI-1 cells was markedly lower than that in cells treated with either control Ig or CD80-Fc beads (Fig. 4C).

Similarly, PD-1-Fc bead–treated MOSTI-1 cells were more resistant to bortezomib-induced apoptosis compared with control Ig bead–treated cells (Fig. 4D). This drug resistance in PD-1-Fc bead–treated MOSTI-1 cells was prevented by anti-B7-H1 (Fig. 4E).

Although MOSTI-1 cells aggregated when cultured with PD-1-Fc beads (Fig. 3A), the aggregation did not induce melphanalin resistance, because aggregated MOSTI-1 cells treated with CD138 antibody–coupled beads did not acquire resistance to melphanalin-induced apoptosis (Supplementary Fig. S3). Fewer melphanalin-induced apoptotic cells were seen among B7-H1.KMS-28PE cells, compared with mock.KMS28PE cells, and apoptosis of B7-H1.KMS-28PE cells was also inhibited after incubation with PD-1-Fc beads (Fig. 4F). Thus, the binding of PD-1 induced a reverse signal through B7-H1 on myeloma cells that caused resistance to antimyeloma agents, but did not confer a proliferative advantage.

PI3K/AKT pathway critical for reverse signals derived from B7-H1

To clarify which signals derived from B7-H1 are involved in the resistance to antimonyeloma agents, PD-1-Fc bead–treated MOSTI-1 cells were exposed to inhibitors of important cell signaling pathways in myeloma, i.e., PI3K/AKT, ERK, MAPK, STAT3, and NF-κB, before treatment with the agents. B7-H1–induced melphanalin and bortezomib resistance was inhibited by PI3K/AKT, but not by ERK, MAPK, STAT3, or NF-κB inhibitors in PD-1-Fc bead–treated MOSTI-1 cells (Fig. 5A and B). Phosphorylation of Akt (Ser473) in MOSTI-1 and B7-H1.KMS-28PE cells was increased by the binding to PD-1-Fc beads (Fig. 5C and D). Next, we examined the mRNA expression of different antiapoptotic and apoptotic genes in PD-1 Fc and control Ig bead–treated MOSTI-1 cells. BCL2 mRNA was upregulated, while Fas ligand (FASLG) and Fas-associated via death domain (FADD) mRNAs were downregulated in PD-1 bead–treated MOSTI-1 cells (Fig. 6A). After treatment with melphanalin and bortezomib, phosphorylation of Akt was further upregulated in PD-1-Fc bead–treated MOSTI-1 cells (Fig. 6B and C). Moreover, the expression of BCL2 increased and cleaved caspase-3 decreased in PD-1-Fc bead–treated MOSTI-1 cells (Fig. 6B and D). Therefore, resistance to the antimonyeloma agents via the reverse signals from B7-H1 bound to PD-1 was partially due to the activation of the PI3K/AKT pathway and antiapoptotic responses in myeloma cells.

Discussion

Tumor-associated B7-H1 molecules transmit inhibitory signals to tumor-specific CTLs via the B7-H1–PD-1 pathway, which contributes to tumor immune escape (9, 32). However, it had not been confirmed whether the B7-H1–PD-1 interaction transmits reverse signals to tumor cells. Here, we clearly demonstrated that B7-H1 expression on myeloma cells is directly associated with drug resistance and cell proliferation and that the reverse signal through B7-H1, after binding PD-1, activated PI3K/AKT pathway signaling, which is a prosurvival signal and induces chemotheraphy resistance in multiple myeloma.

In this study, the knockdown of B7-H1 expression on MOSTI-1 cells suppressed aggressive myeloma behaviors, including cell growth and drug resistance. Similar to myeloma cells, B7-H1 knockdown in pancreatic cancer cell lines increased susceptibility to chemotherapy and inhibited cell proliferation (33, 34). In a colorectal cancer cell line, silencing of B7-H1 with short interfering RNA (siRNA) inhibited cell growth, migration, and invasion and increased spontaneous apoptosis (14). Thus, B7-H1 molecules not only on myeloma cells but also on cells of various other tumors may be directly associated with aggressive behavior. Wang and colleagues reported that B7-H1 knockdown in pancreatic cancer cells increased the cleavage levels of caspase-3 and poly (ADP-ribose) polymerase 1, an apoptosis-related protein (33). We showed that B7-H1 molecules on myeloma cells directly induced aggressive myeloma behaviors by promoting G1–S phase transition and antiapoptotic responses. Thus, the B7-H1 molecule itself may function as an oncogenic protein.

In tumor immunity, tumor-associated B7-H1 inhibits activation of PD-1–expressing CTls via the B7-H1–PD-1 pathway (32). We showed here that B7-H1–expressing myeloma cells gain resistance to drug-induced apoptosis by binding to PD-1 but not to CD80. Binding to CD80 also did not induce a reverse signal in myeloma cells, which could have been caused by weak binding of B7-H1 to CD80. Consistent with our results, it was reported that B7-H1 molecules have a greater affinity for PD-1 than for CD80 (35). When B7-H1–expressing BxPC-3 pancreatic cancer cells were incubated not only with PD-1 Ig but also with a small synthetic peptide designed to bind to the binding site of the B7-H1–PD-1 complex, drug-induced apoptosis in treated BxPC-3 cells was inhibited (33). Those data indicated that the reverse signal derived from B7-H1 is necessary to bind tightly to the receptor PD-1. The reverse signal derived from B7-H1 binding to PD-1 was associated with the activation of the PI3K/AKT signaling pathway in myeloma cells. The following results lend support to the relationship between the reverse signals and PI3K/AKT pathway: antiapoptotic BCL2 was upregulated in PD-1-Fc bead–treated MOSTI-1 cells, and apoptotic FASLG and FADD were downregulated. BCL2 functions as an activator of PI3K/AKT signaling (36), and, in contrast, FASLG expression is suppressed by the PI3K/AKT pathway (37). Therefore, the reverse signals of the interaction of B7-H1 molecules on myeloma cells with PD-1 further suppress drug-induced apoptosis through upregulation of the antiapoptotic response via activation of the PI3K/AKT pathway.

The PI3K/AKT signaling pathway is a crucial prosurvival signal in many cancers (38, 39). For example, the binding of the CD28 molecule, a costimulatory receptor for T-cell activation on myeloma cells, to its ligand CD80 or CD86 subsequently promoted myeloma survival via the PI3K/AKT signaling pathway (30). CD28 has the Tyr-Met-Asn-Met (YMNM) motif, a binding site of PI3K, in its cytoplasmic domain and induces activation of PI3K/AKT pathway signaling (40). However, the cytoplasmic domain of the B7-H1 molecule has only 30 amino acids and
Reverse Signal Derived from B7-H1 in Myeloma Cells

T cells because B7-H1 is a high affinity ligand for PD-1.

In the bone marrow microenvironment, the interaction between B7-H1 on myeloma cells and PD-1 may be stochastically increased by upregulation of B7-H1 and PD-1 in the tumor microenvironment. Thus, the B7-H1–PD-1 interaction probably induces the reverse signal to myeloma cells in the bone marrow microenvironment.

To prevent escape from the host antitumor immune response, the B7-H1–PD-1 pathway is useful as a target of immunotherapy in some cancers. In a clinical trial, B7-H1–PD-1 blockade with anti–PD-1 antibody CT-011 in patients with small cell lung cancer, advanced melanoma, and renal cell cancer had B7-H1–positive tumors (20, 22), and that the antibody treatment regimen has been approved for use in patients with melanoma in many countries. Rosenblatt and colleagues reported that the cytotoxic function of T cells was inhibited by the interaction with B7-H1–expressing myeloma and that dendritic cells from multiple myeloma patients were upregulated by the anti–PD-1 antibody CT-011 in vitro (43). Other researchers demonstrated that CT-011 enhanced human NK cell function against autologous B7-H1–positive myeloma cells (42). Moreover, low-dose lenalidomide downregulated B7-H1 expression in the myeloma cell line RPMI8226, and combination therapy with CT-011 and lenalidomide showed increased NK cell cytotoxic activity against B7-H1–expressing myeloma cells compared with CT-011 monotherapy in vitro (42). However, in our study, B7-H1 expression on RPMI8226 cells was not downregulated by low- and high-dose immunomodulatory drugs (IMiDs), including lenalidomide and pomalidomide (data not shown). Because lenalidomide enhances T-cell and NK-cell effector functions, combination therapy with anti–PD-1 and lenalidomide may be more effective in the treatment of multiple myeloma. On the other hand, the impact of IMiDs on B7-H1 expression and function is still unclear. Further studies are needed to elucidate that impact.

In conclusion, B7-H1 molecules on myeloma cells are directly associated with aggressive myeloma behaviors, including cell growth and drug resistance. In addition, we demonstrated that B7-H1–expressing myeloma cells treated with PD-1 molecules induced drug resistance in the cells by antiapoptotic responses through the Akt signaling pathway. Therefore, in the bone marrow microenvironment, the interaction between B7-H1 on myeloma cells and PD-1 on tumor-specific CTLs not only inhibits CTL activity via the PD-1 signaling pathway but also induces drug resistance via B7-H1–mediated reverse signals. B7-H1–mediated signals may be associated with disease progression of multiple myeloma, and our findings provide insights into potential immunotherapy targeting the B7-H1–PD-1 pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Ishibashi, H. Tamura, I. Choi

Development of methodology: M. Ishibashi, M. Sunakawa

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Ishibashi, A. Kondo-Onodera, N. Okuyama, Y. Hamada, K. Moriya

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