A Single-Chain Fv Diabody against Human Leukocyte Antigen-A Molecules Specifically Induces Myeloma Cell Death in the Bone Marrow Environment

Etsuko Sekimoto,¹ Shuji Ozaki,¹,² Takashi Ohshima,¹ Hironobu Shibata,¹ Toshihiro Hashimoto,¹ Masahiro Abe,¹ Naoki Kimura,¹ Kunihiro Hattori,¹ Shigeto Kawai,¹ Yasuko Kinoshita,¹ Hisafumi Yamada-Okabe,¹ Masayuki Tsuchiya,¹ and Toshio Matsumoto¹

¹Department of Medicine and Bioregulatory Sciences, The University of Tokushima Graduate School of Health Biosciences; ²Division of Transfusion Medicine, Tokushima University Hospital, Tokushima, Japan; and ³Genome Antibody Research Department, ⁴Pharmaceutical Research Department III, and ⁵Product Planning Department, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan

Abstract
Cross-linked human leukocyte antigen (HLA) class I molecules have been shown to mediate cell death in neoplastic lymphoid cells. However, clinical application of an anti-HLA class I antibody is limited by possible side effects due to widespread expression of HLA class I molecules in normal tissues. To reduce the unwanted Fc-mediated functions of the therapeutic antibody, we have developed a recombinant single-chain Fv diabody (2D7-DB) specific to the α2 domain of HLA-A. Here, we show that 2D7-DB specifically induces multiple myeloma cell death in the bone marrow environment. Both multiple myeloma cell lines and primary multiple myeloma cells expressed HLA-A at higher levels than normal myeloid cells, lymphocytes, or hematopoietic stem cells. 2D7-DB rapidly induced Rho activation and robust actin aggregation that led to caspase-independent death in multiple myeloma cells. This cell death was completely blocked by Rho GTPase inhibitors, suggesting that Rho-induced actin aggregation is crucial for mediating multiple myeloma cell death. Conversely, 2D7-DB neither triggered Rho-mediated actin aggregation nor induced cell death in normal bone marrow cells despite the expression of HLA-A. Treatment with IFNs, melphalan, or bortezomib enhanced multiple myeloma cell death induced by 2D7-DB. Furthermore, administration of 2D7-DB resulted in significant tumor regression in a xenograft model of human multiple myeloma. These results indicate that 2D7-DB acts on multiple myeloma cells differently from other bone marrow cells and thus provide the basis for a novel HLA class I–targeting therapy against multiple myeloma.

Introduction
Multiple myeloma is a B-cell malignancy characterized by the accumulation of neoplastic plasma cells in the bone marrow (1). The incidence of multiple myeloma is increasing in recent years, but this disease remains incurable with conventional and high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation (PBSCT; refs. 2, 3). Because most patients relapse eventually, prevention of disease progression is one of the key clinical issues. Recent studies have shown that interactions between multiple myeloma cells and the bone marrow microenvironment are important for the pathogenesis of multiple myeloma, and much progress has been made in developing molecular-specific agents that target multiple myeloma cells and/or bone marrow stroma cells (4, 5). Clinical studies of new agents, such as thalidomide, lenalidomide, and bortezomib, have shown a significant effect on the outcome of even relapsed or refractory patients with multiple myeloma (6–9). However, the benefits of these agents seem to be limited in terms of response rate and long-term efficacy (10). Therefore, novel therapeutic strategies are needed to further improve prognosis in patients with multiple myeloma.

Human leukocyte antigen (HLA) class I molecules are widely expressed on normal and tumor tissues and play an important role in regulating immune responses. Notably, the level of HLA class I molecules is quite different among cell types, and these molecules mediate distinct intracellular signaling events depending on the activation state or cell type. HLA class I cross-linking with agonistic monoclonal antibodies (mAb) has been shown to promote survival of normal cells, such as lymphocytes, vascular endothelial cells, and smooth muscle cells, by inducing phosphorylation of focal adhesion kinase (FAK), paxillin, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (11–13). On the other hand, either caspase-dependent or caspase-independent cell death has been observed in activated lymphocytes and neoplastic lymphoid cells following ligation by anti-HLA class I mAbs, such as MoAb90, YTH862, CR11-351, RG1, SH7, OK2F3, and W6/32, although these mAbs recognized distinct epitopes on the different α domains of HLA class I molecules (14–18). Early studies have shown that signaling molecules, such as protein tyrosine and serine/threonine kinases, PI3K, and c-Jun NH2-terminal kinase (JNK), are implicated in the process of HLA class I–mediated cell death of neoplastic lymphoid cells (19, 20).

The Rho family of small guanosine triphosphatases (GTPases) can be activated by a variety of signals from growth factors, cytokines, and adhesion molecules and regulates essential biological functions, including reorganization of actin cytoskeleton (21). Recently, HLA class I cross-linking has been shown to trigger activation of Rho proteins and polymerization of actin cytoskeleton that elicits cell survival in endothelial cells (22). In contrast, several studies have shown that ligation of HLA class I and other cell surface molecules markedly induces actin aggregation, which leads to caspase-independent cell death in lymphoid cells (23, 24). Although the precise mechanism for different cell responses between normal
and neoplastic cells remains unclear, these results raise the possibility of therapies that target HLA class I molecules on neoplastic lymphoid cells.

Several studies have shown that multiple myeloma cells express high levels of HLA class I at both the protein and gene expression levels (25–27). Therefore, it might be feasible to develop a HLA class I–targeting therapy for multiple myeloma. However, clinical application of anti-HLA class I antibody is limited by possible side effects due to widespread expression of HLA class I molecules in normal tissues. To reduce the unwanted Fc-mediated function of therapeutic antibody, we have generated a recombinant single-chain Fv diabody (2D7-DB) specific for the α2 domain of HLA-A molecules (28). Our previous studies have shown that this diabody induces cell death of activated lymphocytes and lymphoblastic cell lines through an actin aggregation pathway in a HLA-A expression–dependent manner (29). In this study, we investigated the mechanism of cytotoxicity by 2D7-DB, focusing on multiple myeloma cells and the bone marrow environment. Our data show that 2D7-DB strongly activates Rho proteins and mediates actin aggregation of multiple myeloma cells in a cell type–specific manner. These results provide a basis for HLA class I–targeting therapy in patients with multiple myeloma.

Materials and Methods

Cell lines. U266, ABH-77, and Jurkat cell lines were obtained from the American Type Culture Collection (Manassas, VA); RPMI 8226 and Daudi were from the Health Science Resources Bank (Osaka, Japan). MM.1S and ULMC-2 were kindly kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL) and Dr. Alan Solomon (University of Tennessee, Knoxville, TN), respectively. The multiple myeloma cell lines OPN and TSPC-1 were established in our laboratory (29). The human bone marrow stromal cell line KM102 was a kind gift from Dr. Kenichi Harigaya (Chiba University, Chiba, Japan). These cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Grand Island, NY), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO₂.

Primary cells. The use of patient samples was approved by the Institutional Review Board at the University of Tokushima (Tokushima, Japan), and informed consent was provided according to the Declaration of Helsinki. Diagnosis and clinical staging of multiple myeloma was determined according to Durie-Salmon classification (30). Primary multiple myeloma cells were purified from bone marrow aspirates of specimens of multiple myeloma patients using CD138 microbeads and a magnetic cell sorting system (Miltenyi Biotec, Auburn, CA). PBSC products were obtained from patients undergoing PBSCST as described previously (31).

Anti-HLA class I antibodies. A mouse mAb 2D7 (IgG2b) that recognizes the α2 domain of HLA-A was prepared in our laboratory (32), and a single-chain Fv diabody version (2D7-DB) was constructed from the parent 2D7 mAb as described previously (28). Mouse anti-human HLA class I mAb W6/32 (epitope, α2 and α3 domains) was purchased from NeoMarkers (Fremont, CA), and B9.12.1 (epitope, α1 domain) was purchased from Immunotech (Marseille, France).

Flow cytometry. Expression of HLA class I was analyzed by flow cytometry using FITC-labeled 2D7 IgG mAb or FITC-labeled anti-HLA-ABC common region mAb (Chemicon, San Diego, CA). For the analysis of primary multiple myeloma cells of bone marrow mononuclear cells (BMMC) or hematopoietic stem cells of PBSC products, cells were stained with FITC-labeled 2D7 mAb and phycoerythrin (PE)-labeled anti-CD54 mAb or PE-labeled anti-CD34 mAb (BD Biosciences, San Jose, CA). In some experiments, cells were stimulated with either IFN-α, IFN-γ, or tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN) before analysis of HLA class I expression. The level of HLA class I expression was evaluated as the geometric mean fluorescence intensity (geo MFI) using CellQuest software (BD Biosciences).

Cytotoxicity assay. Multiple myeloma cell lines (2 × 10⁶/mL) or primary multiple myeloma cells (1 × 10⁶/mL) were incubated with anti-HLA class I mAbs for 30 min at 37°C and then cultured with 10 μg/mL F(ab)₂ goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 24 h. Cytotoxic activity of 2D7-DB was examined without secondary goat anti-mouse IgG antibody. Cell viability was measured by a cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Kishida Chemical, Osaka, Japan) or a trypan blue exclusion test. The combination effect of IFN-α, IFN-γ, TNF-α, melphalan (Sigma), or bortezomib (Millennium Pharmaceuticals, Cambridge, MA) was also evaluated.

Hematopoietic stem cell assay. Hematopoietic stem cell assays of PBSC products were done using MethoCult H4434V (Stemcell Technologies, Vancouver, British Columbia, Canada). After 14 days, the colonies were classified and counted as granulocyte-macrophage colony–forming units (CFU-GM) or erythroid burst-forming units (BFU-E).

Western blot analysis. Cells were lysed in lysis buffer (Cell Signaling, Beverly, MA) supplemented with 1 mmol/L phenylmethylsulfon fluoride and protease inhibitor cocktail solution (Sigma). Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in TBS with 0.01% Tween 20 for 1 h at room temperature and incubated for 16 h at 4°C with the following antibodies: cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), phosphorylated ERK1/2, phosphorylated p38 MAPK, phosphorylated JNK, Akt, phosphorylated Akt, phosphorylated p53, and ERK1/2 (Cell Signaling), and FAK (BD Biosciences). After washing, secondary horseradish peroxidase–conjugated antibody was added and membranes were developed using the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Inhibition assay. The mechanism of cytotoxicity was assessed using potential inhibitors, such as broad caspase inhibitor Z-VAD-fmk, MAPK inhibitor PD98059, P38 inhibitor wortmannin, small GTPase inhibitor Cytostatin difficle toxin B (TcDb), actin polymerization inhibitor cytochalasin D (Calbiochem, Cambridge, MA), and microfilament organization inhibitor latrunculin A (Sigma).

Rho activation assay. Activated Rho proteins were measured with a Rho A/Bc activation G-LISA assay kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions.

Immunofluorescence staining of HLA-A and actin filaments. The effect of 2D7-DB on cytoskeletal actin filaments was analyzed by two-color immunofluorescence staining. Cells were incubated with 1 μg/mL of 2D7-DB bearing the FLAG tag for 10 min at 37°C. After stimulation, cytospin specimens were prepared and slides were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed with 0.1 mol/L PBS, and permeabilized in 0.1 mol/L PBS containing 0.1% Triton X-100 and 3% bovine serum albumin for 3 min. The localization of 2D7-DB was detected with 10 μg/mL of FITC-labeled anti-FLAG tag antibody (Sigma), and actin filaments were stained with 5 units/mL rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 60 min at 25°C. These specimens were examined using a TCS NT laser scanning confocal microscope (Leica, Heidelberg, Germany).

In vivo antitumor experiment. To evaluate the in vivo effect of 2D7-DB, a human multiple myeloma xenograft model was established as described previously (33). Severe combined immunodeficient (SCID) mice were injected ip. with 100 μl. anti-asialo GM1 antiserum (Wako, Osaka, Japan) 1 day before tumor inoculation to eradicate residual natural killer cells. Mice were then inoculated i.v. with ARH-77 cells (6 × 10⁵) through the tail vein. Groups of seven to eight mice were tested by i.v. injection of 2D7-DB (1, 5, or 8 mg/kg) or PBS twice daily and monitored for the duration of survival. Serum levels of human M-protein were measured on day 24 by an ELISA.

Results

Expression of HLA class I molecules on multiple myeloma cells. We first examined cell surface expression levels of HLA-A molecules on multiple myeloma cells using 2D7 IgG mAb by flow cytometry. As shown in Fig. 1A, ARH-77 and RPMI 8226 cell lines
expressed high levels of HLA-A. HLA-A expression was also detected on the bone marrow stroma cell line KM102. In contrast, Daudi cells did not express any HLA class I molecules on the cell surface because they do not produce \( \beta_{2}-\)microglobulin (34). High levels of HLA class I expression on multiple myeloma cell lines was also confirmed by using anti-HLA-ABC mAb (data not shown).

We next examined HLA-A expression on BMMCs from multiple myeloma patients. The level of HLA-A expression seemed to be higher in multiple myeloma cells than in normal myeloid cells or lymphocytes (Fig. 1B). As shown in Fig. 1C, the average level of HLA-A expression in 22 patients was significantly higher in multiple myeloma cells (geo MFI, 1,309 ± 1,130 (mean ± SD)) than in myeloid cells (201 ± 219) or lymphocytes (91 ± 44). HLA-A expression levels of CD34+ hematopoietic stem cells were relatively low (109 ± 16; \( n = 3 \)) compared with multiple myeloma cells.

**Effects of HLA class I antibodies on multiple myeloma cells.** Next, we evaluated the cytotoxic activity of anti-HLA class I mAbs on multiple myeloma cells. In the presence of secondary antibody, 2D7 IgG mAb partially mediated cell aggregation and induced cell death of RPMI 8226 cells and primary multiple myeloma cells in a dose-dependent manner (Fig. 2A). Other anti-HLA class I mAbs, such as W6/32 and B9.12.1, also mediated similar cytotoxic effects on multiple myeloma cells (data not shown). In contrast, the single-chain Fv diabody form of 2D7 mAb (2D7-DB) induced cell death more strikingly than 2D7 IgG mAb in both multiple myeloma cell lines and primary multiple myeloma cells (Fig. 2A). This cell aggregation occurred rapidly within 1 h and cell death reached a maximum after 24 h. The morphologic studies revealed that most of the patient multiple myeloma cells aggregated with cytoplasmic vacuolization within 24 h after 2D7-DB treatment (Fig. 2B). The cytotoxic effect of 2D7-DB (1 \( \mu \)g/mL) was further examined in a panel of multiple myeloma cell lines and in six primary multiple myeloma cells. 2D7-DB induced cell death of all multiple myeloma cell lines tested with a range of 37% to 76% (Table 1). Importantly, the cytotoxic activity of 2D7-DB on primary multiple myeloma cells (53–92%) seemed to be higher than on multiple myeloma cell lines. This dosage of 2D7-DB did not induce cell death in Daudi cells, normal lymphocytes (\( n = 3 \)), or normal BMMCs (\( n = 3 \)), indicating that an intensity of >100 geo MFI of HLA-A expression is required for cell death. In addition, cell viability of bone marrow stroma cell line KM102 was not affected by 2D7-DB despite the expression of HLA-A. Therefore, the sensitivity to 2D7-DB might be different among cell types.

We next investigated the effect of 2D7-DB on normal hematopoietic cells. Total BMMCs from multiple myeloma patients were treated with 2D7-DB (1 \( \mu \)g/mL) for 24 h; the remaining cells
were representative of three independent experiments. As shown in Fig. 2C, CD38\(^{-}\) multiple myeloma cells were selectively depleted from the total BMMC fraction of multiple myeloma patients, whereas normal myeloid cells were minimally affected. This observation was confirmed by staining with FITC-labeled anti-HLA-ABC mAb in which only the multiple myeloma cell population overexpressing HLA class I was selectively eliminated (Fig. 2C, arrows). In addition, 2D7-DB (1 \(\mu\)g/mL) did not significantly inhibit the growth of CFU-GM or BFU-E of hematopoietic progenitor cells in PBSC harvests (data not shown).

Interactions between multiple myeloma cells and the bone marrow microenvironment play an important role in growth and survival of multiple myeloma cells, and so we examined the effect of 2D7-DB on multiple myeloma cells in the presence of bone marrow stroma cells. Primary multiple myeloma cells were cultured together with autologous bone marrow stroma cells for 72 h and then treated with 2D7-DB for 24 h. As shown in Fig. 2D, treatment with 2D7-DB effectively eliminated patient multiple myeloma cells that had adhered to the bone marrow stroma cells without affecting the normal stroma cells. This result is consistent with the previous finding of no cytotoxicity in adherent human bone marrow stroma cell line KM102.

**Mechanisms of 2D7-DB-mediated cell death.** To explore the mechanism of cell death induced by 2D7-DB, we examined the effects of several signal transduction inhibitors on the cytotoxic activity of 2D7-DB in multiple myeloma cells. MAPK inhibitor PD98059, broad caspase inhibitor Z-VAD-fmk, and PI3K inhibitor wortmannin had no remarkable inhibitory effects on cell death in RPMI 8226 cells (Fig. 3A). On the other hand, Rho inhibitor TcdB and actin polymerization inhibitors, such as cytochalasin D and latrunculin A, completely prevented cell death induced by 2D7-DB in RPMI 8226 cells (Fig. 3A). We also examined the effect of cytochalasin D and latrunculin A on survival of primary multiple myeloma cells. The addition of 2D7-DB (1 \(\mu\)g/mL) induced a cell death rate of 46 \(\pm\) 2.7\% of primary multiple myeloma cells (patient no. 19). Pretreatment with cytochalasin D or latrunculin A completely inhibited 2D7-DB-mediated cell death of primary multiple myeloma cells (5.7 \(\pm\) 1.2\% and 3.0 \(\pm\) 4.4\%, respectively).

**Figure 2.** Cytotoxic activity of 2D7-DB on multiple myeloma cells. A, effects of 2D7 mAb and 2D7-DB on multiple myeloma (MM) cells. RPMI 8226 cells and purified multiple myeloma cells from patient no. 13 were cultured for 24 h with 2D7 mAb or RPMI 8226 cells and purified multiple myeloma cells from patient no. 13 for 24 h. Camit soil viability was determined by a WST-8 assay or trypan blue exclusion test. Points, mean of triplicate measurements; bars, SD. The statistical difference was determined by a two-factor ANOVA with Scheffe’s F test. *, \(P < 0.05\); **, \(P < 0.01\). B, morphologic findings of multiple myeloma cells after treatment with 2D7-DB. Purified multiple myeloma cells from patient no. 18 were cultured for 24 h in the presence or absence of 2D7-DB (1 \(\mu\)g/mL). Cells were then fixed on slides and stained with Wright-Giemsa. Original magnification, \(\times\)1,000. C, eradication of patient multiple myeloma cells from BMNCs by 2D7-DB. Total BMNCs from patient no. 12 were cultured in the presence or absence of 2D7-DB (1 \(\mu\)g/mL) for 48 h. After cell culture, the remaining cell population was assessed by flow cytometry using FITC-labeled anti-HLA-ABC mAb and PE-labeled anti-CD38 mAb. The values indicate the percentage of multiple myeloma cells (R1) or myeloid cells (R2). Arrows, multiple myeloma cell population expressing high levels of HLA. D, effect of 2D7-DB on multiple myeloma cells adherent to bone marrow stroma cells. Adherent fractions of BMMCs from multiple myeloma patients were cultured in 24-well plates. After growth of stroma cells, patient multiple myeloma cells (1 \(\times\)10\(^6\)/mL) were cocultured in the plates for 72 h and then treated with 2D7-DB (1 \(\mu\)g/mL) for 24 h. Arrows, bone marrow stromal cells adhered to the plates. The small round cells are patient multiple myeloma cells. The numbers of patient multiple myeloma cells markedly decreased in cultures with 2D7-DB. The results are representative of three independent experiments.
**Table 1. Cytotoxic activity of 2D7-DB in multiple myeloma cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cytotoxicity (%)</th>
<th>HLA-A expression (geo MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U266</td>
<td>76</td>
<td>1,610</td>
</tr>
<tr>
<td>OPC</td>
<td>62</td>
<td>2,701</td>
</tr>
<tr>
<td>ARH-77</td>
<td>58</td>
<td>3,944</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>40</td>
<td>721</td>
</tr>
<tr>
<td>TSCP-1</td>
<td>37</td>
<td>1,434</td>
</tr>
<tr>
<td>MM.1S</td>
<td>37</td>
<td>1,320</td>
</tr>
<tr>
<td>Primary multiple myeloma cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>61</td>
<td>851</td>
</tr>
<tr>
<td>No. 5</td>
<td>60</td>
<td>1,392</td>
</tr>
<tr>
<td>No. 13</td>
<td>87</td>
<td>494</td>
</tr>
<tr>
<td>No. 18</td>
<td>92</td>
<td>1,318</td>
</tr>
<tr>
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<td>90</td>
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</tr>
<tr>
<td>No. 27</td>
<td>53</td>
<td>ND</td>
</tr>
<tr>
<td>KM102</td>
<td>0</td>
<td>577</td>
</tr>
<tr>
<td>Duadi</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: Multiple myeloma cell lines and CD138-positive multiple myeloma cells from six patients were cultured with 2D7-DB (1 µg/mL) for 24 h. Cell viability was determined by a WST-8 assay or trypan blue exclusion test. Data represent mean values of experiments done in triplicate.

Abbreviation: ND, not done.

*A human bone marrow–derived stroma cell line that expresses HLA-A.
†Does not express HLA-A and was used as a negative control.

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ARH-77 cells, where 2D7-DB bound to the cell surface HLA-A but actin filaments were still present at the peripheral cytoplasm. In contrast, HLA-A molecules were expressed in a diffuse pattern with focal accumulation in KM102 cells, and actin filaments were distributed throughout the cells (Fig. 3C). Cell death of KM102 was not observed morphologically during the staining process, which is consistent with previous results for bone marrow stroma cells (Table 1; Fig. 2D). These findings suggest that 2D7-DB specifically induces activation of Rho proteins and dynamic actin aggregation in multiple myeloma cells.

To further explore the differences in the effects of 2D7-DB on multiple myeloma cells and on bone marrow stroma cells, we examined the involvement of downstream signaling pathways related to the cross-linking of HLA molecules. As shown in Fig. 3D, 2D7-DB did not cause phosphorylation of FAK, Akt, ERK1/2, p38 MAPK, JNK, or paxillin in RPMI 8226 cells (Fig. 3D; data not shown). In contrast, phosphorylation of FAK, Akt, and ERK2 was detected in KM102 cells after treatment with 2D7-DB (Fig. 3D). These data suggest that 2D7-DB induces a different downstream pathway depending on the cell type. In addition to signaling pathways, we assessed several variables of apoptotic cell death, including phosphatidylserine externalization, caspase activation, and DNA fragmentation. After treatment with 2D7-DB, RPMI 8226 and primary multiple myeloma cells showed a slight increase in Annexin V–positive and propidium iodide–negative cells (5–10%). However, neither activation of caspases nor cleavage of PARP was observed in these cells treated with 2D7-DB (Fig. 3D), and neither was chromosomal DNA fragmentation (data not shown). These results are consistent with previous findings from an inhibition assay using caspase inhibitor Z-VAD-fmk (Fig. 3A) and indicate that 2D7-DB induces multiple myeloma cell death by caspase-independent mechanisms.

**Effects of cytokines and chemotherapeutic agents on 2D7-DB–mediated cytotoxicity.** The cell surface expression of HLA class I molecules can be augmented by stimulation with either IFN-α, IFN-γ, or TNF-α (35), and so the effects of these cytokines on the expression of HLA-A and cytotoxicity by 2D7-DB on multiple myeloma cells and normal lymphocytes were compared. Flow cytometric analysis showed that the expression of HLA-A was up-regulated after stimulation with IFN-α, IFN-γ, or TNF-α in RPMI 8226 cells (Fig. 4A). As a result of the HLA-A up-regulation, the cytotoxic activity of 2D7-DB on RPMI 8226 cells was significantly enhanced after stimulation with IFN-α, IFN-γ, or TNF-α (Fig. 4B). In contrast, HLA-A expression on normal lymphocytes remained at low levels after stimulation with these cytokines compared with multiple myeloma cells, and cell death was not significantly enhanced in these cytokine-stimulated lymphocytes (Fig. 4A and B).

We next assessed whether chemotherapeutic agents, such as melphalan and bortezomib, can enhance the effect of 2D7-DB on multiple myeloma cells. RPMI 8226 cells were first treated with 2D7-DB and then incubated with melphalan or bortezomib. As shown in Fig. 4C, the isobologram analysis showed an additive interaction between 2D7-DB and melphalan or bortezomib. Similar effects were observed when RPMI 8226 cells were first treated with the chemotherapeutic agents followed by 2D7-DB. This additive effect of 2D7-DB did not result in enhancement of PARP cleavage induced by the chemotherapeutic agents (data not shown). These results suggest that 2D7-DB has a distinctive antitumor potency that is different from chemotherapeutic agents against multiple myeloma.

**In vivo antitumor effects of 2D7-DB.** Finally, we evaluated the in vivo efficacy of 2D7-DB in a mouse xenograft model of human multiple myeloma. SCID mice bearing disseminated ARH-77 cells were first treated with 2D7-DB (1 or 5 mg/kg) or PBS twice daily on days 1, 2, and 3. Serum M-protein levels on day 24 were first treated with 2D7-DB (1 or 5 mg/kg) or PBS twice daily on days 1, 2, and 3, serum M-protein levels on day 24 did not significantly decrease in 2D7-DB–treated mice, but two of seven mice survived >180 days in the 5 mg/kg 2D7-DB–treated group (Fig. 5A). When SCID mice were treated with 2D7-DB (8 mg/kg) twice daily on days 1, 2, and 3, serum M-protein levels on day 24 significantly decreased in 2D7-DB–treated mice (20 ± 19 µg/mL) compared with PBS-treated mice (74 ± 66 µg/mL), and survival of these mice was significantly prolonged compared with control mice (Fig. 5B). There was no treatment-related toxicity from 2D7-DB as evaluated by the general condition and the body weight of these mice. These results indicate that 2D7-DB exerts strong antitumor activity without any effector mechanisms in vivo.

**Discussion**

We have shown that multiple myeloma cell lines as well as primary multiple myeloma cells express significantly high levels of HLA-A molecules compared with normal lymphocytes, myeloid cells, and hematopoietic progenitor cells. Moreover, a recombinant single-chain Fv diabody 2D7-DB effectively cross-linked HLA-A on multiple myeloma cells and induced Rho activation and robust actin aggregation that led to caspase-independent cell death. This cytotoxic effect of 2D7-DB on multiple myeloma cells was also observed in an in vivo model of human multiple myeloma. In contrast, because of relatively low HLA-A expression, normal bone marrow cells, such as lymphocytes, myeloid cells, and hematopoietic cells, were not affected by the treatment with 2D7-DB.
More strikingly, 2D7-DB did not induce Rho activation or actin aggregation in bone marrow stroma cells despite expressing a considerable level of HLA-A. Therefore, it seems that there is a sufficient therapeutic window for targeting HLA-A on multiple myeloma cells. These results confirm and reinforce our previous observations that 2D7-DB specifically induces cell death in HLA-A-overexpressing tumor cells (28).

Several mechanisms may explain the increased level of HLA-A expression on multiple myeloma cells. The transcription of HLA class I genes is regulated by two modules: an upstream module consisting of the enhancer A and IFN-stimulated response element and a downstream module containing the W/S, X1, site α, and enhancer B elements (36). Both modules are important for the constitutive and cytokine-induced expression of HLA class I genes. Because the upstream module has binding sites for nuclear factor-κB and IFN regulatory factor family members involved in the pathogenesis of multiple myeloma (37, 38), transcriptional factors may account for the increased level of HLA class I expression in multiple myeloma cells. In addition, multiple myeloma cells are known to strongly express essential parts of the HLA class I complex, such as the transporter associated with antigen presentation and β2-microglobulin (39, 40). The overexpression of these molecules may also contribute to the up-regulation of HLA class I on the cell surface of multiple myeloma cells.

Figure 3. Mechanism of multiple myeloma cell death induced by 2D7-DB. A, effects of various inhibitors on the cytotoxic activity of 2D7-DB against multiple myeloma cells. RPMI 8226 cells were treated with PD98059 (10 μmol/L), Z-VAD-fmk (5 μmol/L), wortmannin (10 μmol/L), TcdB (10 μmol/L), cytochalasin D (1 μmol/L), or latrunculin A (0.1 μmol/L) for 1 h and then cultured with 2D7-DB (1 μg/mL) for 24 h. Cell viability was determined by WST-8 assay. Columns, mean of triplicate measurements; bars, SD. B, activation of Rho proteins by 2D7-DB in RPMI 8226 and KM102 cells. Cells were treated with 2D7-DB (1 μg/mL) for 5 min. Cell lysates were subjected to Rho A,B,C activation assay. C, localization of HLA-A and cytoskeletal actin filaments in ARH-77 and KM102 cells. Cells were incubated with 1 μg/mL of 2D7-DB bearing the FLAG tag for 10 min at 37°C. In some experiments, cells were treated with 0.1 μmol/L latrunculin A (Lat A) for 30 min before the addition of 2D7-DB. Cell specimens were stained with FITC-labeled anti-FLAG tag antibody and rhodamine-phalloidin and examined by confocal laser scanning microscopy. D, RPMI 8226 and KM102 cells were treated with 2D7-DB (1 μg/mL) for the indicated times. Cells were lysed and subjected to Western blotting using specific antibodies. In some experiments, RPMI 8226 cells were treated with 2D7-DB (1 μg/mL) or melphalan (L-PAM; 10 μmol/L) to assess activation of caspase-3 and cleavage of PARP.
Although 2D7-DB did not induce cell death in bone marrow stroma cells, it activated signal transduction pathways, such as FAK, PI3K/Akt, and Ras/MAPK. Several studies have reported the involvement of these signaling pathways in normal endothelial cells after the cross-linking of HLA class I molecules (11–13) while altering aggregation of actin cytoskeleton in activated lymphocytes (19, 20, 23). Similarly, different actin accumulation responses have been observed between attached fibroblasts and lymphoid cells following the cross-linking of HLA-DB; the cytoskeletal actin is tightly organized in filaments along the cell membrane in adherent fibroblasts, whereas the peripheral actin becomes concentrated after the capping of HLA-DB in lymphoid cells (41). These findings support the hypothesis that the signaling pathway through HLA molecules and its role in actin cytoskeleton are different depending on cell types. Indeed, Rho proteins that regulate actin reorganization act differently in normal adherent cells and tumor cells with regard to the balance of signals from surface receptors, including HLA and other adhesion molecules (42). Although we were unable to identify the precise mechanisms that determine the Rho protein activity or the intensity of actin remodeling, it is likely that the cross-linking of HLA-A by 2D7-DB efficiently induces actin aggregation beyond the physiologic levels in HLA-A-overexpressing multiple myeloma cells without mediating survival signaling pathways. Taken together, these findings support an additional mechanism for the therapeutic specificity of 2D7-DB.

Recent studies have shown that Rho family genes are rearranged in multiple myeloma and lymphoma cells, probably leading to their overexpression (43). In addition, Rho proteins are implicated in cell adhesion–mediated drug resistance or regulation of cell growth and morphologic changes along with wingless/int signaling in multiple myeloma cells (44–46). In our experiments, Rho activation by 2D7-DB induced robust actin aggregation that led to cell death of multiple myeloma cells. Although the role of Rho proteins in multiple myeloma cells is not fully understood, it seems that Rho proteins play an important part in the pathogenesis of multiple myeloma cells and may have potential as a target for the treatment of multiple myeloma.

Figure 4. Effects of cytokines and chemotherapeutic agents on 2D7-DB–mediated cytotoxicity. A, effect of IFN-α, IFN-γ, or TNF-α on HLA-A expression of RPMI 8226 cells and normal lymphocytes. Cells were treated with IFN-α (100 units/mL), IFN-γ (100 units/mL), or TNF-α (20 μg/mL) for 48 h and stained with either FITC-labeled control mouse IgG (dotted line) or FITC-labeled 2D7 mAb. Cell surface expression of HLA-A was analyzed by flow cytometry. Bold lines, staining profiles of cytokine-treated cells; solid lines, staining profiles of untreated cells. B, enhancement of 2D7-DB–mediated cell death by cytokines. After treatment with IFN-α, IFN-γ, or TNF-α for 48 h, cells were cultured with 2D7-DB (1 μg/mL) for 24 h. Cytotoxicity was determined by WST-8 assay or trypan blue exclusion test. Columns, mean of triplicate determinants; bars, SD. The statistical significance of the combination effect of 2D7-DB and each cytokine was determined by a two-factor ANOVA with Schefﬂe’s F test, *, P < 0.05; **, P < 0.01. C, isobologram analysis of combination effect of 2D7-DB and melphalan or bortezomib. RPMI 8226 cells were first treated with 2D7-DB for 6 h and incubated with melphalan or bortezomib for 24 h. Each IC50 value was obtained by a WST-8 cell proliferation assay. Line, estimated dose combinations for purely additive interactions.
normal tissues. To avoid side effects related to Fc-mediated effector mechanisms and yet expect potent effects on antigen cross-linking, we have developed a single-chain Fv diabody version of the parent anti-HLA-A IgG mAb. Our results have shown that 2D7-DB directly induces multiple myeloma cell death without the need for host immune effector mechanisms, such as antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. This characteristic of 2D7-DB provides a great advantage for multiple myeloma patients, especially patients with impaired immune functions necessary with mAb therapy (47). However, because 2D7-DB induces activation of FAK, PI3K/Akt, and Ras/MAPK pathways in stroma cells, there is a possibility of side effects by these signals in stroma cells as well as in other tissues. Therefore, we are now investigating the pharmacokinetics and tolerability of 2D7-DB in cynomolgus monkeys that show cross-reactions to 2D7-DB. Our preliminary results indicated that 2D7-DB could be administered safely in a single dose of up to 10 mg/kg with a transient decrease of lymphocytes and neutrophils in the peripheral blood, and this dose of 2D7-DB did not cause any histopathologic changes of the heart, lung, liver, gallbladder, spleen, kidney, stomach, intestine, aorta, thymus, tonsil, lymph node, bone, and bone marrow when examined 2 and 7 days after administration. The structure of 2D7-DB allows the assumption that it has rapid access to multiple myeloma cells and fast clearance from the blood (48), which results in less toxicity to normal tissues.

Several studies have shown that Rho-induced actin aggregates contribute to the enhancement of apoptotic sensitivity by activating caspasases and causing mitochondrial damage in neoplastic cells (49, 50). In this context, the development of specific agonists for an actin-mediated cell death pathway might become an alternative approach for triggering tumor cell death. Because treatment with a single agent is not likely to cure multiple myeloma, combination therapy will be required to enhance cytotoxicity and to overcome drug resistance. In our experiments, treatment of multiple myeloma cells with IFNs up-regulated HLA-A expression and resulted in enhancement of the effect of 2D7-DB. Moreover, treatment of multiple myeloma cells with 2D7-DB enhanced the cytotoxic effects of chemotherapeutic agents, such as melphalan and bortezomib. Therefore, 2D7-DB might provide an important therapeutic potential because of its novel mechanism, which is different from that of chemotherapeutic agents in multiple myeloma.

In conclusion, we have shown that multiple myeloma cells express HLA-A at higher levels than normal lymphocytes, myeloid cells, and hematopoietic progenitor cells. The cross-linking of HLA-A by a new recombinant single-chain Fv diabody 2D7-DB induces effects different for multiple myeloma cells and other bone marrow cells, including stroma cells. These differences make it possible to eliminate multiple myeloma cells without damaging the bone marrow environment. 2D7-DB strongly induces multiple myeloma cell death as a single agent as well as in combination with potential therapeutic drugs, such as IFNs, melphalan, or bortezomib. These results provide the basis for a novel therapeutic approach that targets HLA class I in patients with multiple myeloma.

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Figure 5. Therapeutic efficacy of 2D7-DB in a xenograft model of human multiple myeloma. SCID mice were inoculated i.v. with ARH-77 cells (6 × 10^6/mouse). A, groups of seven SCID mice were treated i.v. with 2D7-DB (1 or 5 mg/kg) or PBS twice daily on days 3, 4, and 5. B, groups of seven SCID mice were treated i.v. with 2D7-DB (8 mg/kg) or PBS twice daily on days 1, 2, and 3. The serum concentration of human IgG was measured on day 24. Columns, mean of seven mice; bars, SD. *, P < 0.05, by one-factor ANOVA. Survival of these SCID mice was analyzed by Kaplan-Meier curve and Wilcoxon test. **, P < 0.05. 6 Unpublished observation.
References

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Etsuko Sekimoto, Shuji Ozaki, Takashi Ohshima, et al.