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Novel Anti-CD70 Antibody Drug Conjugate for the Treatment of Adult T-Cell Leukemia (ATL)

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Abstract. *Background/Aim: Adult T-cell leukemia (ATL) is a hematological malignancy caused by infection with human T-cell leukemia virus type 1 (HTLV-1). Chemotherapy, antibody therapy, and bone marrow transplantation are used to treat this disease, however, median survival time has not been significantly improved. Our aim was to develop and evaluate a novel antibody-drug conjugate (ADC) with regards to cell cytotoxicity and target specificity. Materials and Methods: In this study, we have constructed a novel ADC, which is composed of an anti-CD70 single chain Fv-Fc antibody conjugated with the anticancer agent emtansine using a novel antibody modification method. Cell cytotoxicity and target specificity were assessed using a cell proliferation assay. Results: The anti-CD70 ADC selectively killed HTLV-1-infected cells and ATL cells without affecting other cells. Conclusion: The anti-CD70 ADC offers some chemotherapeutic potential for the treatment of ATL.*

Adult T-cell leukemia (ATL) is a refractory hematological disorder caused by infection with human T-cell leukemia virus type 1 (HTLV-1) (1, 2). A number of HTLV-1 carriers exist in the southern part of Japan, in particular, in Kagoshima and Okinawa areas (3, 4). It is assumed that approximately one million people in Japan and more than 30 million people worldwide are infected with this virus (5). HTLV-1 infection has a long incubation period, and only 5% of the infected individuals will develop ATL during their lifetime (6). However, once developed, its prognosis is extremely poor, and it causes a variety of complications, such as lymphadenopathy, skin rash, diarrhoea, swelling of liver and spleen, and subcutaneous tumor (7, 8). Furthermore, opportunistic infections are major causes of death for ATL patients. There are 4 types of ATL in terms of clinical manifestations: i) acute, ii) lymphoma, iii) smoldering, and iv) chronic ATL (4, 9). Although various therapeutic modalities, including combination chemotherapy, antibody therapy, and bone-marrow transplantation are applied to the acute and lymphoma types of ATL, most of them have serious adverse effects or restrictions of use (10-12). Therefore, it is still mandatory to develop new treatment options for ATL.

Antibody drug conjugates (ADCs) have been recently introduced as part of anticancer therapy (13-17); however, the current methods for producing an accurate number of conjugated drugs in ADCs are difficult to control (18, 19). In addition, anticancer drugs sometimes diminish the antigen-binding capacity of ADCs due to their interaction with the variable region of the antibody. To circumvent this problem, the chemical conjugation by the affinity peptide (CCAP) method using an IgG-binding peptide has been developed (20). We previously reported that CD70, a ligand of the tumor necrosis factor (TNF) receptor superfamily, was highly expressed on the surface of various HTLV-1-carrying T-cell lines and of the primary $CD4^+$ T-cells isolated from acute type ATL patients (21). Such an expression was not observed for uninfected T-cell lines or for primary $CD4^+$ T-cells derived from healthy donors. In general, CD70 is strongly expressed in activated T and B cells and in some hematological malignancies, such as non-Hodgkin lymphoma (22). This molecule is also highly expressed in some solid tumors, such as renal cell carcinoma and glioblastoma (23, 24). In a couple of studies, anti-CD70 ADCs were tested as treatment modalities for patients with

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renal cell carcinoma or non-Hodgkin lymphoma; however, these attempts were not successful (23, 25).

In this study, we generated an anti-CD70 single chain variable fragment (scFv) fused to a crystallizable fragment (Fc), conjugated the anticancer agent emtansine (DM1) to its Fc region using the CCAP method, and created a novel anti-CD70 ADC. When the anti-CD70 ADC was examined for its inhibitory effect on the proliferation and survival of various T-cell lines and primary lymphocytes, it selectively killed HTLV-1-infected cells and ATL cells but not uninfected cells, suggesting that it bears an important chemotherapeutic potential for the treatment of ATL.

Materials and Methods

Cells. The HTLV-1-carrying CD4$^+$ T-cell lines S1T, HuT102, M8166, Su9T01, and MT-2 and the HTLV-1-non-carrying CD4$^+$ T cell lines CEM, Jurkat, and MOLT-4 were used for experiments. S1T and Su9T01 were established from peripheral blood mononuclear cells (PBMCs) of ATL patients, and the HuT102 cell line was derived from a lymph node biopsy tissue of a 28-year-old male having cutaneous T-cell lymphoma (21, 26, 27). MT-2 was established by coculturing umbilical cord blood lymphocytes with MT-1 cells, an HTLV-1-infected T-cell line derived from PBMCs of a 45-year-old female ATL patient (28). M8166 is a subclone of C8166, which was also established by cocultivation of umbilical cord blood lymphocytes with ATL cells (29). S1T, M8166, Su9T01, and MT-2 cells were kindly provided by Dr. Arima (Kagoshima University), while HuT102, CEM, Jurkat, and MOLT-4 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All T-cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (GE Healthcare, USA), 100 U/ml penicillin G (Nacalai Tesque, Kyoto, Japan), and 100 μg/ml streptomycin (Nacalai Tesque). PBMCs were collected from ATL patients and healthy volunteers under informed consent. PBMCs from ATL patients were provided by the Department of Hematology and Rheumatology in Kagoshima University. The experiment using PBMCs was approved by the Kagoshima University Clinical Research Ethics Committee (approval number: 28-138). PBMCs were cultured in RPMI1640 medium supplemented with 20% FBS, antibiotics, and 200 U/ml recombinant human interleukin-2 (IL-2) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Construction and binding capacity of scFv-Fc. A plasmid expressing anti-CD70 scFv-Fc was provided by the Graduate School of Science and Engineering in Kagoshima University, and the scFv-Fc was produced according to the previously described method with slight modifications (30). Briefly, the HEK293 cells were transfected with the plasmid and were cultured for approximately one month. Anti-CD70 scFv-Fc was collected from the culture supernatant by using protein A column (HiTrap™ Protein A HP; Promega, Madison, WI, USA). The molecular weight of the synthesized protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in which 5 μg of the sample was mixed with buffer and loaded into each well of SuperSepTM Ace (Wako, Osaka, Japan). Its target-specific binding was determined by flow cytometry. Briefly, 5×10^5 cells of the CD70-positive T-cell line S1T and the CD70-negative T-cell line Jurkat were incubated with 1 μg of scFv-Fc for 30 min at 4˚C. After incubation, the cells were washed with PBS$^+$ (PBS + 2% FBS), resuspended in PBS$^+$, and incubated with a phycoerythrin (PE)-conjugated anti-human IgG-Fc goat antibody (Rockland, Limerick, PA, USA) for 30 min at 4˚C. After washing with PBS$^+$, 7-amino-actinomycin D (AAD) was added to the cells and was incubated for 10 min at room temperature. After incubation, the cells were washed again and resuspended in 1 ml of PBS$^+$. The cells were analysed using the flow cytometer S3eTM Cell Sorter (Bio-Rad, Richmond, CA, USA).

Drug conjugation to anti-CD70 scFv-Fc. DM1 was purchased from Chemexpress (Shanghai, PR China). All peptides were synthesized by GlyTech, Inc (Kyoto, Japan) (20). Forty μl of the IgG-binding peptide (50 mg/ml in DMSO) and 24 μl of DM1 (50 mM in DMSO) was mixed followed by the addition of 2.6 μl of pyridine. Following incubation for 3 h at 50˚C, 80 μl of DSG (500 mM in acetonitrile) was added and incubated for 3 additional h at 50˚C. The sample was analysed using liquid chromatography-mass spectrometry (LC-MS). The target product was separated by a reverse phase column (InertSustain® C18, GL Sciences, Tokyo, Japan). After removing acetonitrile by evaporation, the product was lyophilised and dissolved in DMSO. Four μl of the constructed peptide (10 mM) was diluted in 1 ml of DMSO, and mixed with 8.8 ml of acetate buffer (10 mM, pH 5.0) and 200 μl of the anti-CD70 scFv-Fc (100 μM). Following incubation for 1 h, the mixture was analysed and fractionated by cation exchange column chromatography using SP825 column (Shodex, Tokyo, Japan). The fractionated solution was desalted and concentrated using a Vivaspin® column (GE Healthcare Life Sciences, Buckinghamshire, UK).

Determination of CD70 expression. Various cells (1×10^6 cells) were suspended in 100 μl of PBS containing 1% BSA and incubated with 20 μl of a PE-conjugated anti-human CD70 IgG3 mouse antibody (Becton Dickinson, Franklin Lakes, NJ, USA) or 5 μl of PE-conjugated IgG3 κ isotype control mouse antibody (Becton Dickinson) for 30 min at 4˚C. The cells were washed and analysed for their fluorescence by a flow cytometer (Bio Rad).

Cell viability assay. The final anti-CD70 ADC product was serially diluted in a 96-well plate. Various cells (1×10^4 cells) were seeded in each well and incubated for 4 days at 37˚C in a 5% CO_2 environment. Following incubation, 10 μl of a viable cell detection reagent (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) was added and cells were further incubated for 2 h. The absorbance was measured using a microplate reader (Bio Rad) at a wavelength of 450 nm and 630 nm. The absorbance measured at 630 nm was subtracted from that at 450 nm to eliminate the effect of nonspecific absorption.

Entry of ADC. Entry of ADC into the target cells was checked using flow cytometry and fluorescent microscopy. S1T and MOLT-4 cells were suspended in PBS (1×10^6 cells/100 μl), and the anti-CD70 scFv-Fc-FITC was added to the suspension. After incubation at 4˚C for 3 h or at 37˚C 1 h, the cells were washed with PBS to remove any unbound anti-CD70 scFv-Fc-FITC. The cells were resuspended in 0.25% trypsin solution to remove any cell surface-bound anti-CD70 scFv-Fc-FITC. After incubation for 10 min, the cells were washed, resuspended in PBS, and analysed using flow cytometry. For microscopic analysis, the cells were cultured in a 24-well plate, and the anti-CD70 scFv-Fc-Alexa Fluor 488 (Thermo Fisher

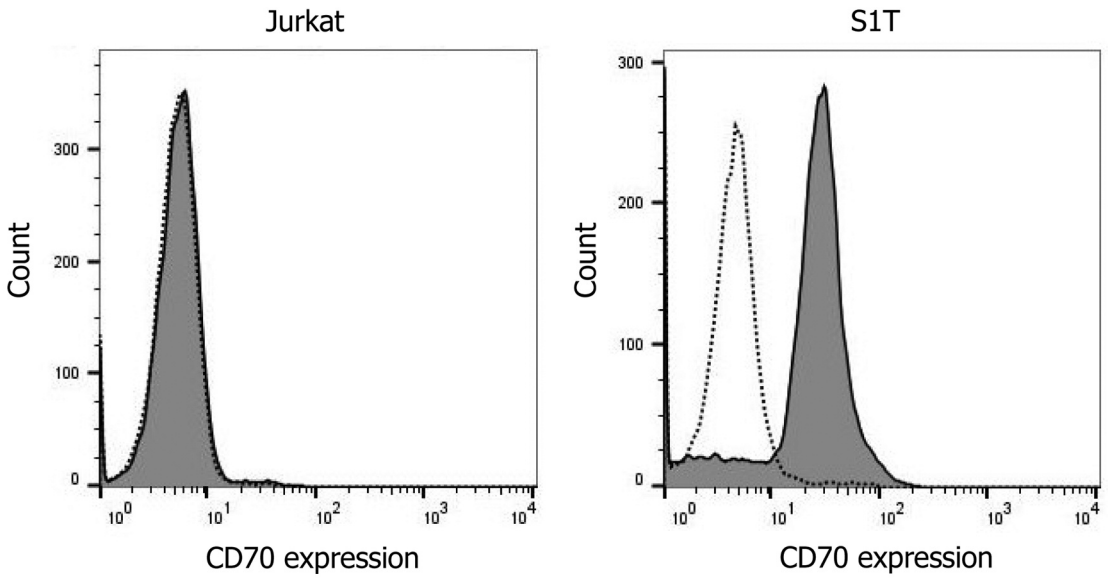

Figure 1. *Cell-binding capacity of anti-CD70 scFv-Fc. The scFv-Fc has target specificity and binds to CD70 expressed in S1T cells. The CD70-negative T-cell line Jurkat and the CD70-positive T-cell line S1T were incubated in the absence (dotted histogram) or presence (solid histogram) of the scFv-Fc.*

Scientific, Waltham, MA, USA) was added to culture medium and incubated for 10 h at 37˚C in a 5% CO_2 environment. Cell fluorescence was observed using a fluorescent microscope (BZ-X700, Keyence, Osaka, Japan).

Effect of CD70 on PBMCs from ATL patients. Primary ATL cells were examined for their surface expression of CD70 and viability. The donors of PBMCs were 1 male and 4 females, and their age ranged between 52 and 69 years. PBMCs were suspended in RPMI1640 (Wako) with 25% FBS (GE Healthcare), 100 U/ml penicillin G (Nacalai Tesque), and 100 μg/ml streptomycin (Nacalai Tesque), 8.3 U/ml interleukin-2 (Milteny Biotec, USA) in the absence or presence of DM1, the anti-CD70 scFv-Fc, or the anti-CD70 ADC. The cells were incubated for 48 h at 37˚C in a 5% CO_2 environment. Following incubation, the cells were washed and resuspended in PBS^+. Ten μl of a PE-conjugated anti-human CD70 mouse antibody (Becton Dickinson) was added. The cells were incubated for 30 min at 4˚C, washed again, stained with Annexin V (Thermo Fisher Scientific), and analysed using a flow cytometer (BD Biosciences, USA).

Results

Preparation of anti-CD70 ADC and its target specificity. The constructed and purified anti-CD70 scFv-Fc was found to specifically bind to S1T cells expressing CD70 (Figure 1). The CCAP method (Figure 2) was used to conjugate DM1 to scFv-Fc. The final product was analysed and showed that two kinds of ADC products were generated: i) an antibody with a single molecule of DM1 (monovalent), and ii) an antibody with two molecules of DM1 (bivalent).

Inhibitory effect of anti-CD70 ADC on cell viability. We initially assessed the expression of CD70 in various T-cell lines and found that the HTLV-1-infected cells (S1T, HuT102, M8166, Su9T01, and MT-2) highly expressed CD70 on their surface. In contrast, uninfected cells (CEM, Jurkat, and MOLT-4) did not express the molecule (Figure 3). When the inhibitory effects of DM1, the anti-CD70 scFv-Fc, and the anti-CD70 ADC (monovalent and bivalent) were examined on these cell lines, the anti-CD70 ADC inhibited with great efficiency the proliferation of HTLV-1-infected cells (Figure 4). Its 50% inhibitory concentration (IC_{50}) ranged between 1.0 and 10.7 nM (Table I). Interestingly, MT-2 cells appeared to be resistant to DM1. The anti-CD70 ADC did not affect the proliferation or viability of CEM, Jurkat, and MOLT-4 cells (IC_{50}s>50 nM). DM-1 inhibited both proliferation and viability in all of the cell lines, except for MT-2, and the anti-CD70 scFv-Fc itself had no activity. These results indicate that the anti-CD70 ADC is a potent and selective inhibitor of HTLV-1-infected cells.

Entry of ADC. When the entry of fluorescently-labeled anti-CD70 scFv-Fc into CD70-expressing cells was analysed by fluorescent microscopy, only S1T cells showed a positive green signal (Figure 5A). Flow cytometry analysis also showed that this scFv-Fc was internalized by S1T cells despite of incubation temperature or trypsinization but not by MOLT-4 (Figure 5C-5E).

Inhibitory effect of anti-CD70 ADC on primary ATL cells. It seems important to confirm that the anti-CD70 ADC is also inhibitory for CD70-expressing primary ATL cells. Figure 6 shows that DM1 killed 76% of PBMCs from a healthy donor and 55% of PBMCs from an ATL patient. This indicates that

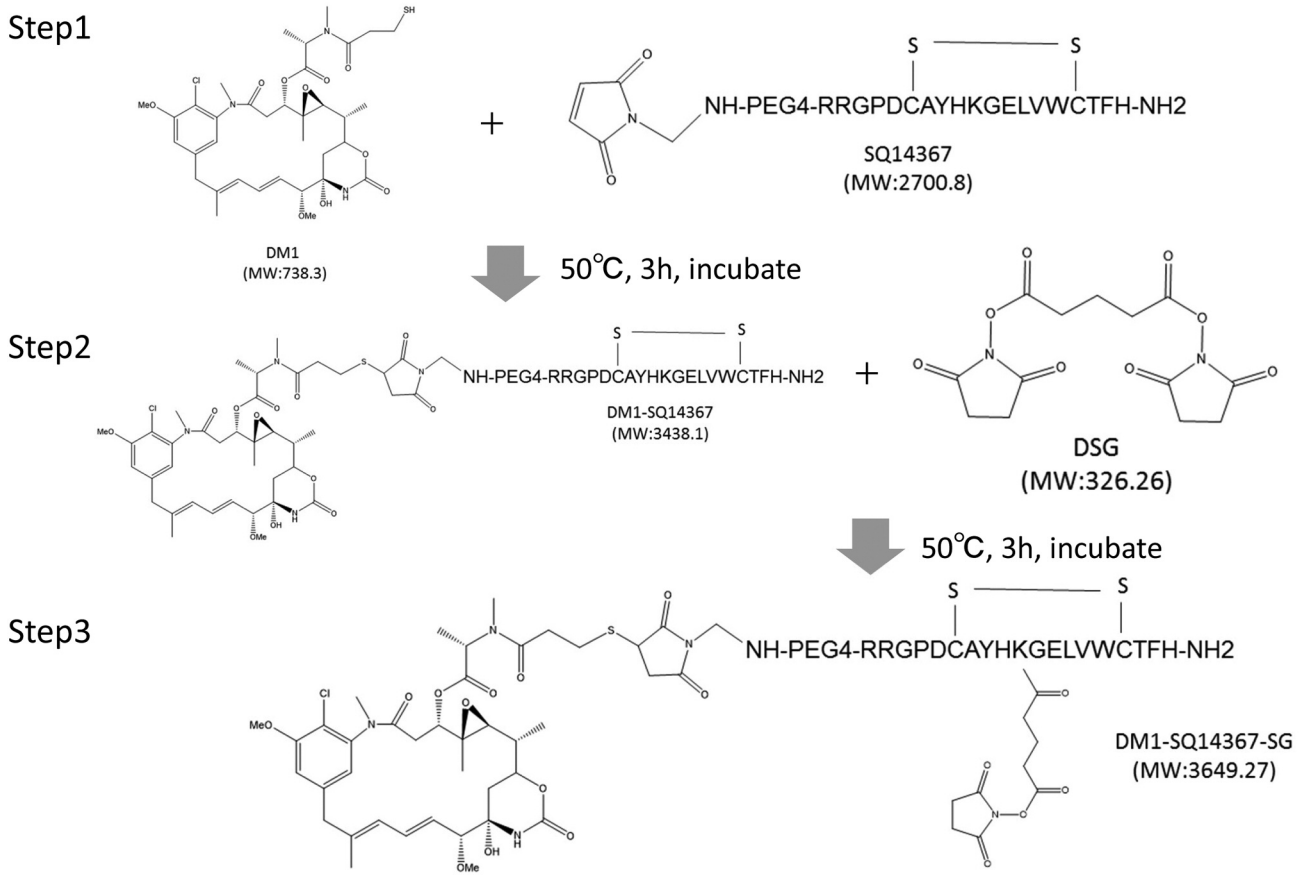

Figure 2. *Conjugation of DM1 with peptide using CCAP method. The DM1-conjugated peptide was synthesized step by step by using the CCAP method. DM1: Emtansine; CCAP: chemical conjugation by affinity peptide; DSG: disuccinimidyl glutarate.*

PBMCs from the healthy donor are more sensitive to DM1. On the other hand, 49% of PBMCs from the healthy donor and 56% of PBMCs from the ATL patient died, when DM1 was conjugated to scFv-Fc. These results suggest that the ADC can selectively kill PBMCs from ATL patients (Figure 6).

Discussion

Various treatment options for ATL are currently in use, but the median survival time of the acute and lymphoma types of ATL has not improved (31). Many anticancer drugs have serious side effects, so that most of ATL patients are not able to tolerate conventional anticancer chemotherapeutic regimens (32, 33). Various treatment modalities to reduce the side effects of anticancer chemotherapy have being developed. The ADC Kadcyla® (trastuzumab-emtansine) is widely used for treating certain types of human epidermal growth factor receptor 2-positive ($Her2^+$) early breast cancer or $Her2^+$ metastatic breast cancer (34). This ADC is a conjugate of DM1 to an anti-Her2 antibody, with an average ratio of drug to antibody (DAR) at 3.5 (35). The conjugated DM1 is released from the antibody by lysosomal degradation following internalization by cells, where it binds to tubulin and inhibits its polymerization to exert cytoxicity (34).

The peptide that specifically binds to human IgG1 has been isolated from random peptide libraries (36). Previous studies have demonstrated that this peptide is bound to the Fc site of an antibody with an affinity of 10 nM (20). Since the CCAP method can specifically modify the Fc site of the antibody, it does not interfere with the binding capacity of the antibody itself. It has been previously reported that the CCAP method can be used to create an ADC against breast cancer cells (20). Kadcyla has an average of 3.5 anticancer drugs bound to one antibody molecule, whereas the DAR of our anti-HER2 ADC ranged between 1 and 2. Nevertheless, its potency was comparable to that of Kadcyla against breast cancer cells (SK-BR3), indicating that the CCAP method is an innovative antibody modification technique (20).

We have previously demonstrated that CD70 is specifically expressed on the surface of HTLV-1-infected cells at high levels (21). Based on this finding, we attempted to prepare the scFv-Fc that specifically bound to CD70. DM1 used alone

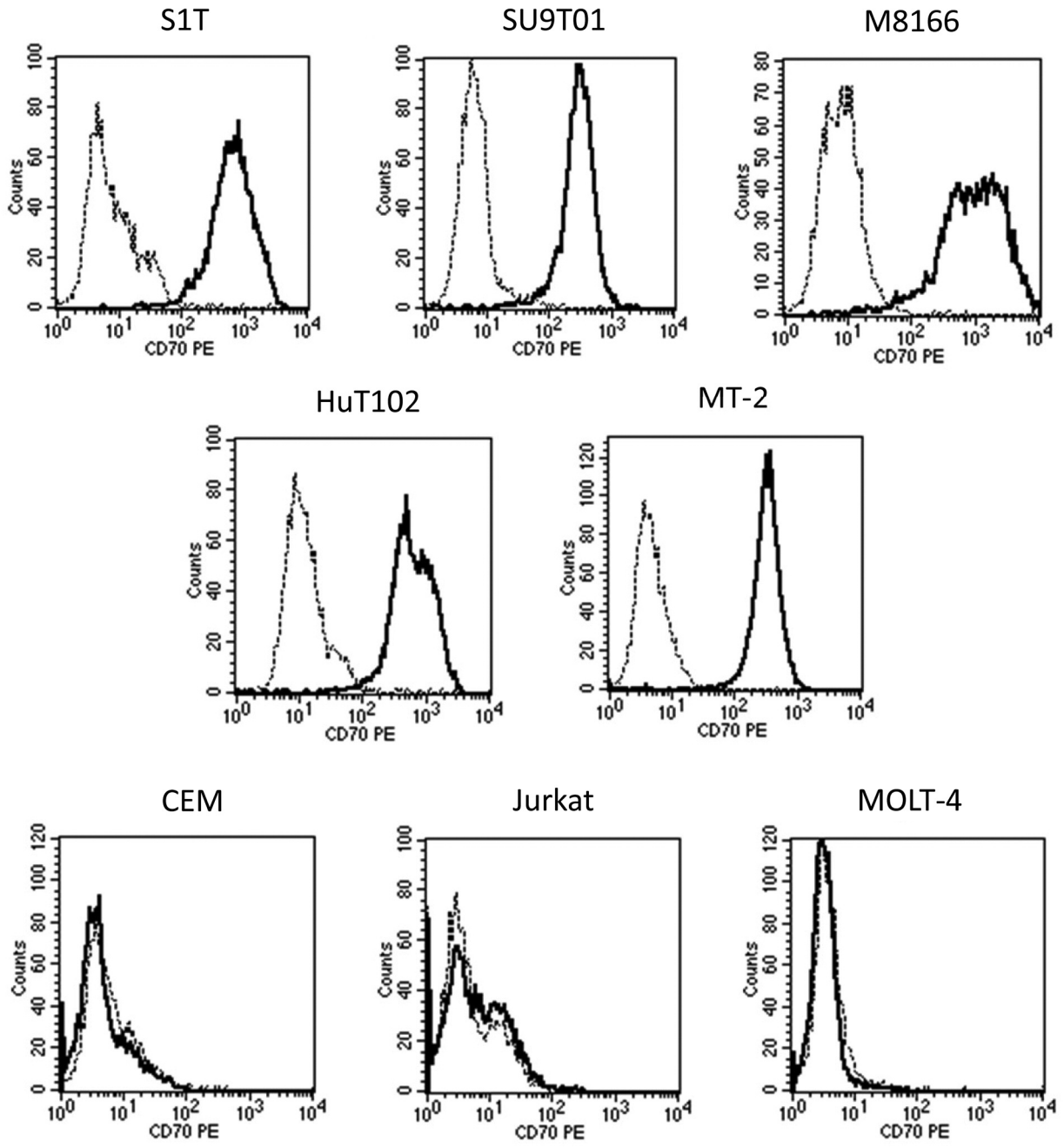

Figure 3. *Expression of CD70 in ATL and non-ATL cells. Among the T-cell lines examined, S1T, Su9T01, M8166, Hut102, and MT-2 are infected with HTLV-1, while CEM, Jurkat, and MOLT-4 are uninfected. Black lines and dotted lines indicate the cells stained with a PE-conjugated anti-human CD70 mouse antibody and an isotype control antibody, respectively.*

was strongly cytotoxic in all cell lines, except for MT-2 cells. However, when conjugated to an anti-CD70 antibody, it exhibited toxic effects specifically in target cells. Significant difference in the cytotoxicity profiles of the anti-CD70 ADC was also observed between PBMCs from a healthy donor and those from an ATL patient, which is an important finding with regards to the approach towards reducing side effects of anticancer drugs *in vivo*. Furthermore, internalization of the ADC was detected after its binding to the surface of target cells. These results suggest that, like Kadcyla, the anti-CD70 ADC may release DM1 in the target cells and exhibit cytotoxicity through lysosomal degradation.

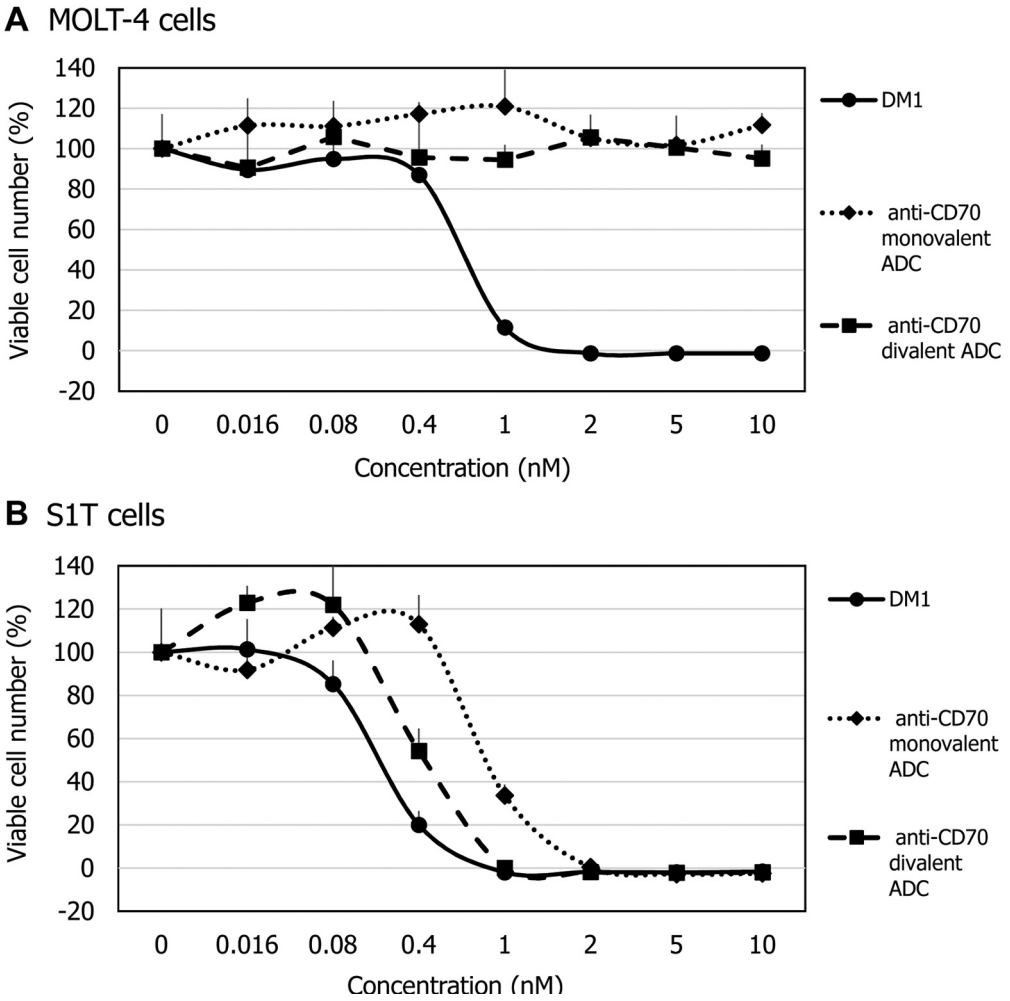

Figure 4. *Inhibitory effect of DM1, anti-CD70 monovalent ADC, and divalent ADC on cell proliferation. The ADC inhibited the proliferation of S1T but not of MOLT-4, while DM1 inhibited the proliferation of both cell lines. (A) MOLT-4 cells and (B) S1T cells incubated with (●) DM1, (♦) anti-CD70 monovalent ADC, or (■) divalent ADC at various concentrations for 4 days.*

In addition to CD70, some molecules have been reported to be expressed on the surface of ATL cells, including i) interleukin 2 receptor (CD25), ii) CC chemokine receptor 4 (CCR4), iii) caveolin 1 (CAV1), iv) prostaglandin D2 synthase (PGDS), and v) cell adhesion molecule 1 (CADM1) (37-40). However, ADCs targeting these molecules have not been developed. On the other hand, an ADC targeting CD70 is currently on the market for research use to study Non-Hodgkin lymphoma (NHL). This ADC uses monomethyl auristatin F (MMAF) as an anticancer agent conjugated to the antibody (41). Its chemotherapeutic potential against ATL still remains to be demonstrated.

In this study, the constructed anti-CD70 ADC showed selective killing of PBMCs from an ATL patient, suggesting that it offers a chemotherapeutic potential as a novel and selective anti-ATL agent. Additional studies with a larger

Table I. *Inhibitory effect of DM1, Anti-CD70 scFv-Fc, and anti-CD70 ADCs on various T-cell lines.*

Cells	IC_{50} (nM)			
	DM1	Anti-CD70 scFv-Fc	Monovalent ADC	Bivalent ADC
S1T	0.3±0.2	>50	1.8±1.0	1.0±0.4
HuT192	1.3±0.5	>50	1.9±1.0	1.2±0.7
M8166	0.20±0.05	>50	10.7±3.5	7.3±2.2
Su9T01	0.12±0.05	>50	8.2±5.2	7.0±5.5
MT-2	>50	>50	>50	>50
MOLT-4	0.5±0.2	>50	>50	>50
Jurkat	0.6±0.2	>50	>50	>50
CEM	1.0±0.2	>50	>50	>50

ADC: Antibody-drug conjugates; scFv-Fc: single chain variable fragment fused to a crystallizable fragment; IC_{50}: 50% inhibitory concentration. All data represent means±SD for three separate experiments.

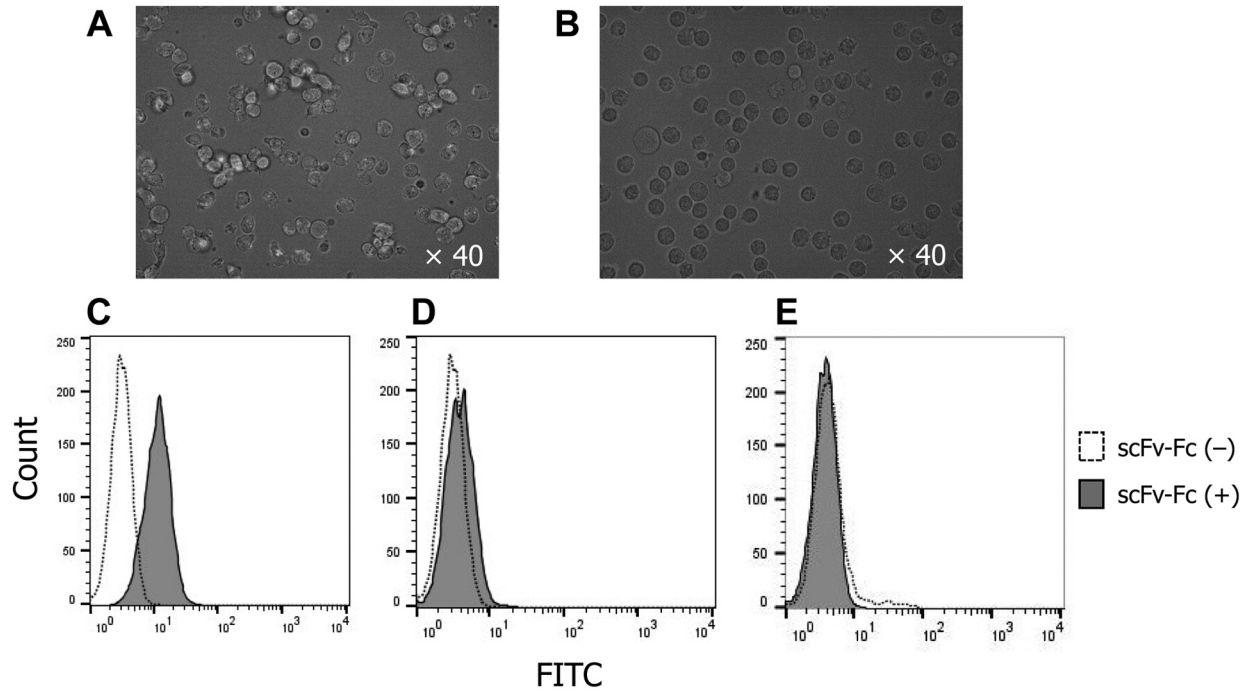

Figure 5. *Internalization of fluorescently-labeled anti-CD70 scFv-Fc. Microscopic images of (A) S1T and (B) MOLT-4 after incubation with a fluorescently-labeled scFv-Fc. Flow cytometric analysis for S1T cells incubated at (C) 37˚C for 1 h or (D) 4˚C for 3 h, followed by trypsinization at room temperature for 10 min. (E) MOLT-4 cells incubated at 37˚C for 1 h followed by trypsinization.*

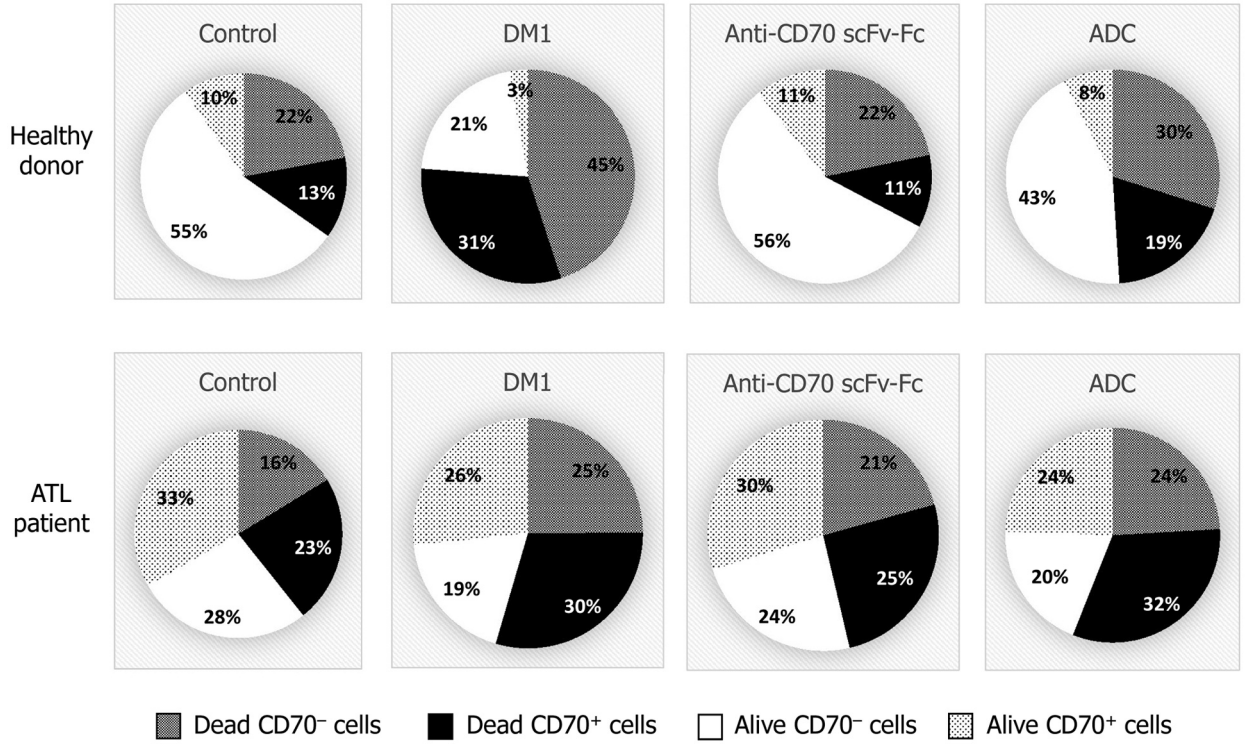

Figure 6. *Surface expression of CD70 and inhibitory effect of DM1, anti-CD70 scFv-Fc, and anti-CD70 ADC on PBMCs from a healthy donor and an ATL patient. The ADC selectively killed PBMCs from the ATL patient but not those from the healthy donor.*

number of PBMCs from different patients are warranted for establishing this potential.

Conflicts of Interest

MB, YI, and RY are inventors of the patent application on anti-CD70 ADC. The other co-authors have no other conflicts of interest.

Authors' Contributions

RY, SH, IW, carried out the experiments of constructing ADCs. SK, MT, YI, and MB developed the theory and designed experiments. MO, YI and MB provided idea of whole study. MY, KI and YI provided samples and advised for this research. RY and MB wrote the article.

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