A new disulfide-linked dimer of a single-chain antibody fragment against human CD47 induces apoptosis in lymphoid malignant cells via the hypoxia inducible factor-1α pathway

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CD47 belongs to the immunoglobulin superfamily and is associated with β-integrins. Recently it was reported that CD47 ligation rapidly induces apoptosis in B-chronic lymphocytic leukemia (CLL) cells. Chronic lymphocytic leukemia is still an incurable hematological malignancy even with the novel therapeutic agents; therefore, new and effective agents for the treatment of CLL in clinical settings are urgently needed. We generated a murine monoclonal antibody against an extracellular domain of human CD47 (designated MABL). Subsequently, we created a disulfide-stabilized dimer of a single-chain antibody fragment of MABL (S-S diabody) to get rid of the adverse effect of MABL such as hemagglutination. In this study, we analyzed the effects of this new antibody on cellular proliferation, and the molecular mechanism of CD47-mediated apoptosis in human lymphoid malignant cells. Treatment with S-S diabody alone induced apoptosis of CD47-positive primary B-CLL and leukemic cells (MOLT-4 and JOK-1). In addition, administration of S-S diabody significantly prolonged the survival of severe combined immunodeficiency (SCID) mice inoculated with JOK-1 cells. In gene expression profiling of the S-S diabody-treated MOLT-4 cells, hypoxia inducible factor (HIF)-1α downstream genes (RTP801 and BNP3) were upregulated, and the mRNA expression levels of HIF-1α, RTP801 and BNP3 were increased. Knockdown of HIF-1α by siRNA repressed S-S diabody-induced apoptosis in MOLT4 cells. In conclusion, CD47 will be a molecular target for the treatment of lymphoid malignancies, and S-S diabody might have potential as a novel therapeutic agent for B-CLL. (Cancer Sci 2011; 102: 1208–1215)

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D47, an integrin-associated protein (IAP), is a member of the immunoglobulin (Ig) superfamily with a single IgV-like domain at its N-terminus, a highly hydrophobic stretch with five membrane-spanning segments and an alternatively spliced cytoplasmic C-terminus. It is expressed as a cell surface antigen in many different types of cells in almost all tissues, except for erythrocytes of Rh-null individuals.1–4 CD47 was reported to be identical to a cancer antigen, OV-3, which is upregulated on erythrocytes of Rh-null individuals.5–7

Recently it was reported that the ligation of CD47 rapidly induces cell death in T-cells and chronic lymphocytic leukemic B cell chronic lymphocytic leukemic (B-CLL) in a caspase-independent manner.8,9 B cell chronic lymphocytic leukemic is the most common hematological malignancy in Western countries.10 However, B-CLL is still considered an incurable disease; thus, new and effective agents for the treatment of CLL in clinical settings are urgently needed.9,10–13

In this report, we created a disulfide-stabilized dimer of scFv of MABL, designated S-S diabody, and found that it strongly induced apoptosis of CD47-positive lymphoid leukemia cells as well as apoptosis of fresh samples from patients with B-CLL in vitro and in vivo.

Materials and Methods

Reagents. A whole IgG antibody against CD47, designated MABL, was established as previously reported.6 Disulfide-stabilized dimer of scFv of MABL (designated S-S diabody) was generated according to the method described previously.22 Variable regions of heavy and light chains (VH and VL) were linked by a flexible linker Gly3Ser, and two cysteines were introduced at position 44 in the framework region 2 (FR2) of VH and at position 100 in the FR4 of VL (amino acid numbering is based on the system of Kabat et al.).23 Cobalt chloride (CoCl2) and HIF-1α inhibitor, echinomycin, were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

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Cells and cell culture. MOLT-4 and JOK-1 lymphocytic leukemia cell lines were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan) and the Fujisaki Cell Center of Hayashibara Biochemical Laboratory (Okayama, Japan), respectively. Peripheral blood samples from patients with B-CLL and from two normal volunteers were obtained and validated according to the Human Protection Committee guidelines at Keio University School of Medicine (Tokyo, Japan) with written informed consent. Mononuclear cells were separated by Lymphoprep (Nycomed Pharma As, Oslo, Norway). CD34+ umbilical cord blood (UCB) cells were purchased from Allcells LLC. (Emeryville, CA, USA). Cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Equitech-Bio Inc., Kerrville, TX, USA), 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO₂. Cell morphology was evaluated by cytospin slide preparation with Giemsa staining, and cell viability was assessed by trypan blue dye exclusion.

Transelectron microscopy. Cells were fixed with 2.5% glutaraldehyde for 60 min at 4°C, then washed and fixed with 1% osmium tetroxide for 60 min at 4°C. The samples were then dehydrated with a graded ethanol series and embedded in Epon (Taab Laboratories, Aldermaston, UK). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and then examined with a JEM1200EX transmission electron microscope (JEOL, Tokyo, Japan).

Colony-forming assay. Colony formation assays using CD34+ UCB cells were performed using MethoCult GF H4034 (StemCell Technologies, Vancouver, BC, Canada). One thousand cells per well of CD34+ UCB cells were seeded, and the number of colonies was counted after culturing the cells for 14 days in the presence or absence of 10 μg/mL of S-S diabody at 37°C.

Assays for apoptosis. Apoptotic cells were quantified by Annexin V-FITC and propidium iodide (PI) double staining using a staining kit purchased from Pharmingen (San Diego, CA, USA). The percentage of living cells was quantified by Annexin V-FITC and PI double staining, which was carried out using a staining kit (Pharmingen).

Cell lysate preparation and immunoblotting. Cells were collected by centrifugation, and the pellets were then resuspended in a lysis buffer (1% NP40, 1 mM phenylmethylsulfonyl fluoride, 40 mM Tris–HCl [pH 8.0] and 150 mM NaCl) at 4°C for 15 min. Nuclear extracts were collected using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Mitochondria and cytosol were fractionated using the Mitochondria/Cytosol Fractionation kit (BioVision Inc., Mountain View, CA, USA). Protein concentrations were determined using a detergent-compatible protein assay system (Bio-Rad Laboratories, Richmond, CA, USA). Cell lysates (20 μg of protein/lane) were fractionated in 12.5% SDS-polyacrylamide gels before being transferred to the membrane (Immobilon-P membrane; Millipore, Bedford, MA, USA). Antibody binding was detected using an enhanced chemiluminescence kit with hyper-enhanced chemiluminescence film (Amersham, Buckinghamshire, UK). β-actin was used as an indicator of equality of lane loading. The antibodies used in the present study were: caspases-3, -8, -9 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); cytochrome c (Cell Signaling Technology, Beverly, MA, USA); HIF-1α (Nippon Becton Dickinson Ltd., Tokyo, Japan); BNIP3 clone ANa40; RTP801 (Sigma-Aldrich, Tokyo, Japan); and horseradish peroxidase-linked anti-mouse IgG (GE Healthcare, Tokyo, Japan) for mouse monoclonal antibodies and anti-rabbit IgG (Invitrogen). Expression of activated caspase-3 was also determined by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (PCR) and real-time PCR. The ELISA was performed using a TransAM kit (Active Motif) according to the manufacturer’s instructions. The PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 50°C and 1.5 min at 72°C. Primer sequences for HIF-1α responsive RTP801 were sense 5′-ATGCCCTAGCTTTGGGACCG-3′ and antisense 5′-TCAAACACTCTTATGAGCA-3′; and BNIP3 were sense 5′-CTCTGGGTAAGAATCGACTC-3′ and antisense 5′-ACGCTCTGTTTCCTCATTGCTG-3′. The PCR products were analyzed on 2% agarose gel. To normalize the amount of RNA we used the amplification of the human β-actin gene as a control. Real-time PCR was performed using each primer set as TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), reagents as TaqMan Universal PCR Master Mix (Applied Biosystems) and systems as ABI Prism 7700 (Applied Biosystems).

siRNA experiments. MOLT-4 cells were transfected with 100 nM Stealth Select RNAi for HIF-1α or 100 nM non-targeting siRNA (Invitrogen). For transfections, Amaxa nucleofection technology was used (Amaxa, Cologne, Germany). MOLT-4 leukemia cells were resuspended in the nucleofector L solution (Amaxa), and were electroporated using the C-005 protocol. Briefly, 2 × 10⁶ cells were resuspended in aliquots of 100 μL with 100 nM siRNA and were transferred to a cuvette and electroporated with the Amaxa Nucleofector device. Cells were immediately transferred into wells containing 37°C pre-warmed culture medium in 24-well plates. Two days after electroporation, the gene expression levels were analyzed by real-time PCR as described above. The cells were divided into two groups, with or without S-S diabody, and viability was assessed using Annexin V-FITC (Pharmingen) 24 h later.

Antitumor activity of S-S diabody in vivo. The antitumor activity of S-S diabody was examined in SCID mice transplanted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenously injected with JOK-1 cells (5 × 10⁶ cells/mouse). The SCID mice were planted with JOK-1 cells. Five-week-old male SCID mice (CLEA Japan Inc., Tokyo, Japan) were intravenous...
carrying JOK-1 cells were subcutaneously administered vehicle (20 mM sodium acetate [pH 6.0], 0.02% Tween-20, 0.15 M NaCl) or the S-S diabody at a dose of 30 mg/kg twice a day (seven mice per group) on days 1–5 post-tumor transplantation. The animals used in the experiments were treated in accordance with the guidelines established by Chugai Pharmaceuticals (Kamakura, Japan) on the ethical care, handling and termination of animals.

**Statistical analysis.** Statistical analysis was performed using SAS SYSTEM version 6.12 (SAS Institute, Cary, NC, USA). The Kaplan–Meier method was used to compare survival between the S-S diabody-treated group and the vehicle control group. Differences with a P-value <0.05 between the two groups were considered significant.

**Results**

**Induction of apoptosis in leukemia cells and primary samples from patients with B-CLL.** We previously confirmed that human leukemia cell lines, MOLT-4 and JOK-1, express CD47 antigen.(6,21) S-S diabody (1 µg/mL) induced apoptosis in JOK-1 cells (Fig. 1a) and MOLT-4 cells in a dose-dependent manner (Fig. 1b), and also did so in fresh leukemia cells from patients with B-CLL (Fig. 1c). Interestingly, a low dose (<1 µg/mL) of S-S diabody induced apoptosis in MOLT-4 cells and fresh samples from patients with B-CLL (Fig. 1b,c).

**Transelectron microscopy examination.** Transelectron microscopy examination of fresh leukemic cells from patients with B-CLL treated with 1 µg/mL S-S diabody for 6 h showed a chromatin condensation pattern consistent with apoptosis (Fig. 1d,e). Interestingly, cell–cell adhesion was also seen in both MOLT-4 and fresh B-CLL leukemic cells; this is not typical in apoptosis (Fig. 1f).

**Effect of the S-S diabody on colony formation and apoptosis of CD34+ UCB cells and HUVEC.** Next we examined whether S-S diabody induces apoptosis against normal cells. To address this question, CD47-expressed both CD34+ hematopoietic stem/progenitor cells derived from UCB and HUVEC cells were cultured with S-S diabody. S-S diabody (10 µg/mL) did not significantly affect either the numbers of colonies of CFU-GEMM, CFU-GM, BFU-E derived from CD34+ UCB cells or the percentages of alive (Annexin-V-negative, PI-positive) cells of CD34+ UCB and HUVEC (Fig. 2a–c). In addition, we examined the effects of S-S diabody on normal peripheral blood mononuclear cells because normal leukocytes also express surface CD47 antigen. Treatment of S-S diabody for 12 h did not induce apoptosis of normal mononuclear cells (Fig. S1). These results clearly indicate that apoptosis caused by S-S diabody was rather specific to cancer cells.

**Assays for caspase activity and MMP.** It has been reported that apoptosis induced by the CD47 pathway is caspase independent with decreased MMP activity.(14,15) Therefore, we treated MOLT-4 cells with 1 µg/mL S-S diabody for 24 h and found that S-S diabody did not induce caspases-3, -8 and -9 activities in MOLT-4 cells using ELISA and western blotting (Fig. 3a,b). We then investigated MMP in MOLT-4 cells, and found that...
S-S diabody decreased MMP (Fig. 3c). Furthermore, the decrease of MMP was followed by the release of cytochrome c from mitochondria to cytosol in a time-dependent manner (Fig. 3d).

**DNA microarray analysis.** We used microarray technology to identify gene expression changes associated with CD47 ligation. The analysis detected 12 genes (13 probe sets) including RTP801, EGR-1 and BNIP3, with more than twofold upregulation and 114 genes (126 probe sets) with <0.5-fold downregulation (Fig. 4). Recent investigations have suggested that RTP801 and BNIP3 induce apoptosis via the HIF-1α pathway, (25–29) and EGR1 was reported to induce apoptosis without HIF-1α in a hypoxic condition. (30) Therefore, we examined the expression of these genes to address the molecular mechanisms of S-S diabody-induced apoptosis.

**Expression of HIF-1α-related genes and proteins in CD47-positive MOLT4 cells.** We next examined the expression of various molecules in the HIF-1α pathway, including HIF-1α, RTP801 and BNIP3, which were found to be upregulated in the gene expression profiling. HIF-1α binding activity to HIF-1α responsive element was enhanced by the treatment of S-S diabody (Fig. 5a). The expression of HIF-1α in MOLT-4 cells was induced by 6 h but not by 1 h of treatment of 1 μg/mL S-S diabody (Fig. 5a–c). S-S diabody induced expression of HIF-1α in both total cellular proteins and nuclear extract of MOLT-4 cells (Fig. 5b,c). We then examined the expression of downstream molecules of HIF-1α such as RTP801 and BNIP3, which were found to be upregulated in the gene expression profiling. HIF-1α binding activity to HIF-1α responsive element was enhanced by the treatment of S-S diabody (Fig. 5a). The expression of HIF-1α in MOLT-4 cells was induced by 6 h but not by 1 h of treatment of 1 μg/mL S-S diabody (Fig. 5a–c). S-S diabody induced expression of HIF-1α in both total cellular proteins and nuclear extract of MOLT-4 cells (Fig. 5b,c). We then examined the expression of downstream molecules of HIF-1α such as RTP801 and BNIP3 using real-time PCR. Treatment with 1 μg/mL of S-S diabody upregulated the expression of RTP801 and BNIP3 mRNA in a time-dependent manner (Fig. 5d,e). BNIP3 and RTP801 proteins were at a detectable level in MOLT-4 cells after treatment with CoCl2. The level of BNIP3 protein slightly increased in cells treated with S-S diabody for 24 h (Fig. S2).

**Knockdown of HIF-1α expression by siRNA.** To further specify the molecular mechanisms of S-S diabody-induced apoptosis in MOLT-4 cells, we used siRNA to downregulate the HIF-1α pathway. It has been reported that CoCl2 stabilizes HIF-1α and induces HIF-1α responsive genes with kinetics similar to that of hypoxia. (31) Therefore, a hypoxia mimic agent, CoCl2, induced the expression of HIF-1α mRNA in MOLT-4 cells (Fig. 6a). In contrast, treatment with HIF-1α-specific siRNA for 48 h downregulated HIF-1α expression in MOLT-4 cells (Fig. 6a). After an additional 24 h of treatment with 1 μg/mL S-S diabody, downregulation of HIF-1α completely repressed S-S diabody-induced apoptosis in MOLT-4 cells (Fig. 6b). Therefore, we concluded that S-S diabody induced apoptosis of CD47-positive leukemia cells mediated through the HIF-1α pathway.

**In vivo anti-tumor effect of S-S diabody.** The anti-tumor effects of S-S diabody in vivo were assessed using JOK-1 cells (5 × 10⁶ cells/mouse) transplanted intravenously into SCID mice that were given S-S diabody or vehicle as a control. In the control vehicle-treated mice, tumor growth was detectable at the injection site. In contrast, S-S diabody treatment prevented tumor development and significantly prolonged median survival.

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**Fig. 2.** (a) Colony-forming assays of CD34+ UCB cells treated with S-S diabody. CD34+ UCB cells (1 × 10⁶ cells/well) were seeded, and the number of colonies (CFU-GEMM, CFU-GM and BFU-E) was counted after culturing the cells for 14 days in the presence or absence of 10 μg/mL S-S diabody at 37°C. Results of two representative experiments are shown. (b,c) Effect of S-S diabody against CD34+ UCB cells and HUVEC. Left panel: expression of CD47 in CD34+ UCB cells (b) and HUVEC (c). Right panel: ability of S-S diabody to induce apoptosis against CD34+ UCB cells (b) and HUVEC (c) was evaluated.
compared with the control mice (median survival: S-S diabody-treated mice versus vehicle-treated mice, 21 days vs 30 days, \(P < 0.0005\)) (Fig. 7).

Discussion

CD47 is ubiquitously expressed and known to modulate several cellular processes on hematopoietic cells, including phagocytosis, cytokine production, T-cell responsiveness, and leukocyte adhesion and transendothelial migration \textit{in vitro} \((33-35)\). Mateo \textit{et al.} \((14)\) have reported that ligation of anti-CD47 antibody to B-CLL cells induced caspase-independent cell death. Subsequently, the same group found that caspase-independent apoptosis induced by CD47 ligation in B-CLL cells is sufficient to trigger a signal for phagocytosis in human dendritic cells. \((15)\) They showed that CD47-induced apoptosis is mediated by cytoskeletal reorganization linked to the Cdc42/WASP protein (WASP) signaling pathway. \((15)\) More recently, it has been reported that CD47 ligation induces caspase-independent programmed cell death via pharmacological modulation of F-actin in CLL cells. \((36)\)

In the present study, we generated a new monoclonal antibody against the extracellular domain of human CD47 (designated MABL), and further created a disulfide-stabilized dimer of a single-chain antibody fragment of MABL (designated S-S diabody).

Fig. 3. Effects of S-S diabody on caspase activation and mitochondrial transmembrane potential (MMP).
(a) MOLT-4 cells were cultured with 1 \(\mu\)g/mL of S-S diabody for 24 h and analyzed for the activation of caspase-3 by flow cytometry. (b) Western blot analysis of caspase-3 and caspase-9 proteins. Total cellular proteins (20 \(\mu\)g per lane) were separated on 12.5% SDS-polyacrylamide gels and transferred to the membrane. Protein levels were detected using antibodies against caspase-3 and caspase-9. \(\beta\)-actin was used to confirm that equal amounts of protein were in each lane. (c) Flow-cytometric analysis of MMP using DioC6 intensity. (d) Western blot analysis of cytochrome c proteins. Cell fractionation of mitochondria and cytosol was performed using the Mitochondria/Cytosol Fractionation Kit (Bio-Vision).

Fig. 4. Gene expression profiles of S-S diabody-induced apoptosis. The gene expression profiles of MOLT-4 cells treated with MABL-1 and goat anti-mouse IgG as the crosslinker for 0, 0.5, 1, 3 and 6 h were examined using Affymetrix Human Genome U133A and B arrays. Signals representing the levels of mRNA at 0 h were calculated as 1.0. Twelve genes (13 probe sets) including Hif-1\(\alpha\), RTP801, EGR-1 and BNIP3, whose expression was upregulated more than twofold, are shown.
We previously demonstrated that the anti-CD47-specific antibody MABL and the MABL F(ab’)2 antibody, but not the MABL scFv-15 dimer, caused hemagglutination. This observation resembled the finding that, in the scFv dimer of anti-D monoclonal antibody D10, the specific hemagglutination of D-positive red blood cells by D10 is used as the general method to determine Rh blood type. In this report, the hemagglutination activity of the D10 scFv dimer was lower than that exhibited by the parental D10 IgG or cross-linked with an anti-peptide tag. These results suggest that each binding region of the scFv dimer is an insufficient area to access two red blood cells, and the scFv dimer might not have enough avidity to overcome the minus charge on the red blood cell surface. Therefore, it cannot draw one cell toward the other. However, S-S diabody-induced apoptosis of MABL scFv-15 dimer are still insufficient as a single agent, so we established a disulfide-stabilized dimer of a single-chain antibody fragment of MABL, designated S-S diabody. As we have shown in this report, S-S diabody alone induced apoptosis in CD47-positive lymphoid leukemia cells as well as in fresh samples from patients with B-CLL, but not in CD34+ UCB cells and HUVEC. Previous reports have suggested that CD47 expression and CD47-induced cell death do not correlate with the expression of CD47 antigen. Therefore, S-S diabody could be a specific antibody for lymphoid leukemia cells, with the exception of normally mononucleated cells such as normal T cells.

S-S diabody-induced apoptosis was caspase-independent, concurrent with the opening of the mitochondrial permeability transition pore, in accordance with Mateo et al. and Barbier et al. A recent molecular and biological approach suggests that CD47 ligation induces dynamin-related protein 1 (Drp1) translocation from cytosol to mitochondria, where it evokes massive reactive oxygen species (ROS) generation leading to execution of the cell death pathway. Electron micrographs of B-CLL cells treated with S-S diabody showed cell shrinkage, chromatin condensation and cell–cell adhesion. Cell shrinkage and chromatin condensation are typical morphological changes of classical apoptosis; however, cell–cell adhesion is not relevant to apoptosis. This might be a unique morphological change in S-S diabody-treated B-CLL cells. CD47 interacted with SIRPα, and this interaction can mediate...
of HIF-1α repressed S-S diabody-induced apoptosis. A specific HIF-1 inhibitor, echinomycin, induced apoptosis of MOLT-4 cells as previously reported in myeloma cells treated with this specific HIF-1 inhibitor.\(^{123}\) In the presence of echinomycin, S-S diabody did not inhibit the induction of apoptosis in MOLT-4 cells. Therefore, our data shows that the HIF-1α pathway is related to S-S diabody-induced apoptosis. In addition, in the search for the downstream pathway of HIF-1α, upregulation of RTP801 and BNIP3 was seen after S-S diabody treatment. These genes were reported to be upregulated in accordance with HIF-1α-induced apoptosis.\(^{25–29}\) However, only a slight increase in BNIP3 and RTP801 proteins were detectable in S-S diabody-treated MOLT4 cells by western blotting. A more sensitive assay is necessary to detect the quantities of BNIP3 and RTP801 proteins. Therefore, we can assume that the HIF-1α pathway might be the main route of CD47-mediated apoptosis in lymphoid leukemia.

In conclusion, CD47 is potentially a putative molecular target of hematopoietic lymphoid malignancies. We have also reported the efficacy of bivalent MABL scFvs against another B cell malignancy, multiple myeloma, in vitro and in vivo with abundant effects.\(^{46}\) suggesting that S-S diabody could be applied accurately and safely to B cell malignancies including B-CLL and multiple myeloma. Thus, we propose that our newly established antibodies against CD47 might have potential as novel therapeutic agents for incurable lymphoid malignancies, including B-CLL.

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Disclosure Statements

The authors have no potential conflicts of interest.

References


Fig. 7. Antitumor activity of the S-S diabody in mice transplanted with JOK-1. Male SCID mice were intravenously injected with JOK-1 cells (5 x 10⁶ cells/mouse). The mice carrying JOK-1 cells were subcutaneously administered vehicle or S-S diabody at a dose of 30 mg/kg twice a day (seven mice/group) on days 1–5 post-tumor transplantation. Survival of the mice was monitored daily. Survival was significantly prolonged in mice that received the S-S diabody (*P < 0.0005, Kaplan–Meier).
Sagawa et al.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effects of CD47 S-S diabody in normal peripheral blood mononuclear cells.

Fig. S2. Expression of BNIP3 and RTP801 proteins in CD47 S-S diabody-treated MOLT-4 cells.

Fig. S3. Apoptosis induced by CD47 S-S diabody in the presence of echinomycin in MOLT-4 cells.

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