

Antitumor activity of a monoclonal antibody against CD47 in xenograft models of human leukemia

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Abstract. The ligation of CD47 induces the apoptosis of leukemic cells in a caspase-independent manner. We generated a monoclonal antibody against CD47 (mAb-MABL) that possibly induced apoptosis from the ligation of CD47 in CCRF-CEM and JOK-1 cells *in vitro*. To confirm whether the ligation of CD47 caused cell death *in vivo*, we examined the antitumor activity of F(ab')₂ of mAb-MABL in two xenograft models: The acute lymphoblastic leukemia (CCRF-CEM) and the B-cell chronic lymphocytic leukemia (JOK-1) cell line. Furthermore, in order to clarify the apoptotic activity selective for the tumor cells, we examined F(ab')₂ of mAb-MABL apoptotic effects on CD34⁺ hematopoietic progenitor/stem and human endothelial cells. Male SCID mice were intravenously injected with CCRF-CEM (5x10⁶ cells/mouse) or JOK-1 cells (5x10⁶ cells/mouse) and intraperitoneally with JOK-1 cells (2x10⁷ cells/mice). After the implantation of the cells, the mice were intravenously administered the vehicle or the F(ab')₂ fragment of mAb-MABL at several doses and the length of survival was measured. F(ab')₂ of mAb-MABL markedly prolonged the survival of mice transplanted with CCRF-CEM and JOK-1. Significantly, 40% of the mice intraperitoneally injected with JOK-1 cells became tumor-free when administered F(ab')₂ of mAb-MABL, whereas even a high dose of fludarabine only slightly prolonged the median survival time. On the contrary, F(ab')₂ of mAb-MABL showed no apoptotic effect on CD34⁺ hematopoietic progenitor/stem or human endothelial cells. Thus, monoclonal antibodies that cause cell death from the ligation of CD47 could be novel therapeutic agents for incurable leukemia after further optimization such as humanization or making single chain diabodies.

Introduction

Apoptosis is a new strategy for the eradication of malignant cells. Members of the tumor necrosis factor (TNF) receptor superfamily, such as the TNF receptor, Fas, and TNF-related apoptosis ligands (TRAIL) (1-3) transduce the death signal in hematopoietic cells. In addition to the TNF receptor superfamily, the ligation of cell surface molecules by monoclonal antibodies induces apoptosis in these cells. Such cell surface molecules include CD43 expressed in hematopoietic progenitor cells (4,5), CD20 in B cells and B cell lymphoma (6), CD47, CD99, and the major histocompatibility complex (MHC) class I in T cells (7-9).

CD47 is expressed on the surface of a wide variety of cells such as hematopoietic cells, keratinocytes, and the brain (10). CD47 is associated with $\alpha v \beta 3$ integrin and is implicated in the modulation of integrin functions, such as cell adhesion, phagocytosis, and cellular migration events (11-14). Certain studies have demonstrated that the ligation of CD47 induces the apoptosis of T- and B-cell chronic lymphocytic leukemia (B-CLL) in a caspase-independent manner, and that in T-cells it leads to cytoskeleton reorganization which involves the Cdc-42/WAS protein-signaling pathway (15). Furthermore, CD47 physically interacts with BNIP3 and, upon certain signals, it induces the migration of BNIP3 to the mitochondria in order to trigger apoptosis (16,17). In addition to the blood cells, cell death induced by the CD47 ligand (thrombospondin) and the anti-CD47 monoclonal antibody has been demonstrated in breast tumor cells, monocytes, dendritic cells, and fibroblasts (18-20).

B-CLL is the most common adult hematological malignancy in western countries and is incurable even by new chemotherapeutic agents such as fludarabine and 2-chlorodeoxyadenosine (21-23). Some cytokines, such as interleukin-4 and interferon- γ and even stromal cells, protect the B-CLL cells from apoptosis and augment the survival of malignant cells *in vivo* (24).

In an attempt to generate therapeutic agents for leukemia, we generated a monoclonal antibody against CD47 (mAb-MABL), which induces apoptosis against leukemic cells. The F(ab')₂ fragment of mAb-MABL which caused the apoptosis of leukemic cells possibly by inducing the ligation of CD47, showed antitumor activity *in vivo* in the acute lymphoblastic leukemia (ALL) (CCRF-CEM) (25) and B-CLL (JOK-1) cell line (26,27) xenograft models. Thus, anti-CD47 monoclonal

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antibodies could be developed as antitumor agents after further optimization such as humanization or making single chain diabodies (28,29).

Materials and methods

Generation of a monoclonal antibody against CD47. L1210 cells expressing human CD47 (hCD47-L1210) were generated by the transfection of the human CD47 cDNA into mouse L1210 cells (ATCC, CCL-219) and the subsequent selection with G418. DBA/2 mice (Charles River Japan, Yokohama, Japan,) were injected intraperitoneally with 5×10^6 hCD47-L1210 cells. A boost injection was carried out at 4-week intervals. At the last interval, the animals were injected with 10^8 hCD47-L1210 cells. The splenocytes of the mice immunized with hCD47-L1210 were fused with P3-U1 mouse myeloma cells with polyethylene glycol (Nacalai Tesque Inc., Kyoto, Japan). Hybridomas were selected by a medium containing hypoxanthine-aminopterin-thymidine (HAT) (GIBCO BRL, Rockville, MD, USA) as described previously (30,31). Hybridomas were tested i) for their activity to bind to human CD47 by flow cytometry using hCD47-L1210 cells, and ii) for their ability to induce apoptosis in hCD47-L1210 cells. A hybridoma clone that produces a monoclonal antibody with a high affinity to human CD47 and strong activity to induce apoptosis in hCD47-L1210 cells was selected, and the antibody was designated as mAb-MABL.

Cells and FACS analyses. The expression of CD47 was examined using the following cells: CCRF-CEM (the human ALL cell line) (25), JOK-1 (the human B-CLL cell line) (26,27), CD34⁺ hematopoietic progenitor/stem and HUVEC cells. JOK-1 cells that express the B-cell marker CD19 (26,27) were derived from a patient with hairy cell leukemia which was classified as CLL based on the French-American-British criteria. The JOK-1 cells were kindly provided by the Fujisaki Cell Center of the Hayashibara Institute of Biochemical Science (Okayama, Japan). These cells were cultured at 37°C in RPMI-1640 medium with 10% fetal bovine serum (FBS). For immunoanalysis, the cells were incubated for 1 h at 4°C with 5 µg/ml mAb-MABL or isotype-matched control mouse antibody (mouse IgG1). After washing, the cells were incubated for 1 h with FITC-conjugated anti-mouse IgG goat antibody (Becton-Dickinson Co., Franklin Lakes, NJ, USA). The amounts of CD47 on the surface of these cells were examined using FACSCalibur (Becton-Dickinson Co.). We also examined the expression of CD47 on CD34⁺ hematopoietic progenitor/stem cells. Human cord blood cells were stained with anti-CD34-PE, MABL together with anti-mouse IgG-FITC, and fluorescences were analyzed with a FACSCalibur. Cord blood cells and HUVEC were purchased from ALLCells Ltd. Liability Company and Cambrex Corporation, respectively.

Analyses of apoptosis. The apoptosis of hCD47-L1210, CCRF-CEM, JOK-1, CD34⁺ hematopoietic progenitor/stem and HUVEC cells by mAb-MABL was examined with Annexin-V-FLUOS (Roche Diagnostics, Basel, Switzerland) (32). The hCD47-L1210 cells were seeded at a density of

4×10^3 cells/ml in 2 ml IMDM (10% FBS, Moregate, USA) containing mouse IgG (10 µg/ml) or mAb-MABL (10 µg/ml) for 72 h at 37°C under 5% CO₂. The CCRF-CEM and JOK-1 cells were seeded in a 96-well plate (round bottom) at a density of 10^5 cells/100 µl and cultured in RPMI-1640 (10% FBS, HyClone, Logan, UT, USA) containing 1 µg/ml of the F(ab')₂ fragment of mAb-MABL or 1 µg/ml of the F(ab')₂ fragment of the control mouse IgG1 (Cappel, PA, USA) for 24 or 48 h at 37°C under 5% CO₂. Human cord blood cells and HUVEC were seeded in a 96-well plate at a density of 6×10^4 cells/100 µl and cultured in RPMI-1640 (10% FBS) containing 10 µg/ml of the F(ab')₂ fragment of mAb-MABL or 10 µg/ml of the F(ab')₂ fragment of the control mouse IgG1. The cells were then centrifuged, stained with the Annexin V-FITC Apoptosis Detection Kit I (Pharmingen, San Diego, CA, USA), and analyzed using FACSCalibur.

Examination of antitumor activities. The antitumor activity of the F(ab')₂ fragment of mAb-MABL was examined with SCID mice implanted with CCRF-CEM or JOK-1 cells. The F(ab')₂ fragment of mAb-MABL was generated by digesting the mAb-MABL with pepsin (Sigma Chemical Co., St Louis, MO, USA) and the subsequent removal of whole IgG by protein A column chromatography. Male SCID mice were intravenously injected with CCRF-CEM (5×10^6 cells/mouse) or JOK-1 cells (5×10^6 cells/mouse).

SCID mice carrying CCRF-CEM (derived from ALL) were intravenously administered the vehicle (PBS) or the F(ab')₂ fragment of mAb-MABL at a dose of 0, 50, and 200 µg/mouse twice a day (7 mice/each group) on days 3, 4, and 5 post tumor implantation.

SCID mice carrying JOK-1 cells (derived from B-CLL) were intravenously administered the vehicle (PBS) or the F(ab')₂ fragment of mAb-MABL at a dose of 1 or 5 mg/kg twice a day (7 mice/each group) on days 3, 4, and 5 post tumor implantation. Fludarabine was also administered intravenously at 135 mg/kg twice a day for 10 days (from day 3 to 7 and from day 10 to 14) after tumor implantation. In separate experiments, mice were intraperitoneally injected with JOK-1 cells (2×10^7 cells/mice), and they were intravenously administered the vehicle (PBS) or the F(ab')₂ fragment of mAb-MABL at 1 mg/kg twice a day (7 mice/each group) on the first 5 days after the tumor implantation. In this study, fludarabine was administered intraperitoneally at a dose of 200 mg/kg twice a day for 10 days (from day 1 to 5 and from day 15 to 19) after tumor implantation.

Seven- or eight-week-old male SCID mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and kept under specific pathogen-free conditions during the experimental period. The animals used for the experiments were treated in accordance with the guidelines established by Chugai Pharmaceuticals on the ethical care, handling, and termination of animals.

Statistical analysis. Statistical analysis was performed using the SAS system version 6.12. The Wilcoxon test was used to compare the survival between the mAb-MABL-treated group and the vehicle control group. Differences with a p-value <0.05 between the two groups were considered significant.

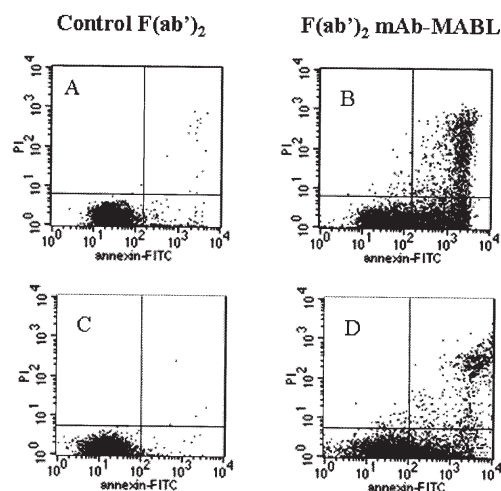


Figure 1. Induction of apoptosis by $F(ab')_2$ of mAb-MABL in CCRF-CEM and JOK-1 cells. The CCRF-CEM (A and B) and JOK-1 (C and D) cells were seeded in a 96-well plate (round bottom) at a density of 10^5 cells/ $100 \mu\text{l}$ and cultured in a medium containing $1 \mu\text{g/ml}$ $F(ab')_2$ of the control mouse IgG1 (A and C) or $1 \mu\text{g/ml}$ $F(ab')_2$ of mAb-MABL (B and D) for 24 or 48 h. The cells were then stained with Annexin V-FITC and PI and analyzed by FACS.

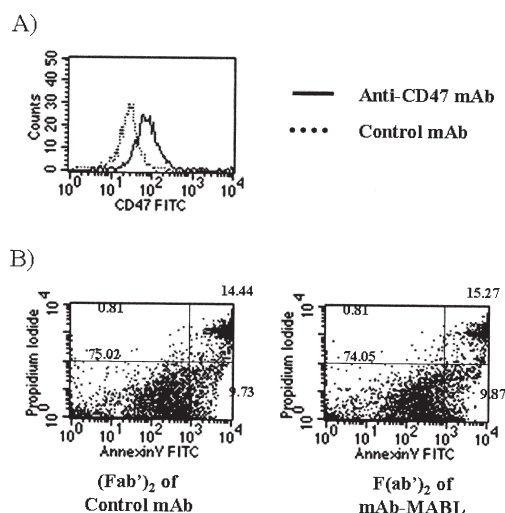


Figure 3. The expression of CD47 (A) and cell death by $F(ab')_2$ of mAb-MABL (B) in HUVEC cells. (A) Cells were stained with mAb-MABL (anti-CD47) together with anti-mouse IgG-FITC, and fluorescences were analyzed with a FACSCalibur. (B) Cells (6×10^4) per well of HUVEC on a 96-well plate were cultured in the presence and absence of $10 \mu\text{g/ml}$ $F(ab')_2$ fragment of mAb-MABL or $10 \mu\text{g/ml}$ $F(ab')_2$ fragment of the control mouse IgG1. After incubation at 37°C for 24 h, the cells were stained with Annexin V-FITC. The numbers indicate the percentages of cells in each fraction.

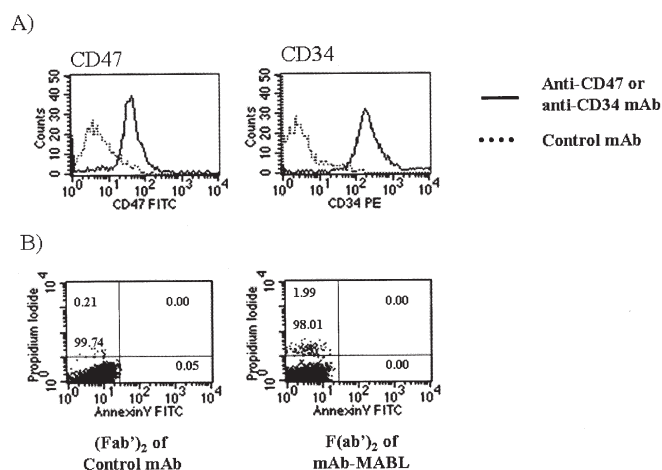


Figure 2. The expression of CD47 and CD34 (A) and cell death by $F(ab')_2$ of mAb-MABL (B) in hematopoietic stem/progenitor cells. (A) Cord blood cells were stained with anti-CD34-PE, mAb-MABL (anti-CD47) together with anti-mouse IgG-FITC, and fluorescences were analyzed with a FACSCalibur. (B) Cells (6×10^4) per well of cord blood cells on a 96-well plate were cultured in the presence and absence of $10 \mu\text{g/ml}$ of the $F(ab')_2$ fragment of mAb-MABL or $10 \mu\text{g/ml}$ of the $F(ab')_2$ fragment of the control mouse IgG1. After incubation at 37°C for 24 h, the cells were stained with Annexin V-FITC. The numbers indicate the percentages of cells in each fraction.

Results

FACS analysis revealed that the monoclonal antibody against CD47 designated as mAb-MABL, bound to CD47-L1210, which expressed the human CD47, but not to the vector-transfected L1210 cells (not shown). In addition, mAb-MABL induced apoptosis in the hCD47-L1210 cells but not in the vector-transfected L1210 cells as judged by the Annexin-V assay (not shown). The induction of apoptosis was also

observed in other leukemic cells. CCRF-CEM and JOK-1 cells expressed CD47 antigens and mAb-MABL induced apoptosis in both cells lines within 48 h, as judged by the Annexin-V-FLUOS assay (Fig. 1). We also examined whether $F(ab')_2$ of mAb-MABL induced cell death against the CD34⁺ hematopoietic stem/progenitor cells and HUVEC. Although both CD34⁺ cord blood cells and HUVEC expressed certain amounts of CD47 on the cell surface, $F(ab')_2$ of mAb-MABL did not induce cell death against these cells. The percentages of cell death of CD34⁺ cord blood cells and HUVEC which occurred in the presence of $F(ab')_2$ of mAb-MABL were almost the same as those in the presence of $F(ab')_2$ of the control mouse IgG1 (Figs. 2 and 3).

The ability of mAb-MABL to induce the ligation of CD47 and subsequent apoptosis prompted us to examine the antitumor activities of the antibody *in vivo*. To eliminate the antitumor effects mediated by effectors *in vivo*, the antitumor activity was examined by administrating the $F(ab')_2$ fragment of mAb-MABL into SCID mice implanted with CCRF-CEM or JOK-1 cells. The $F(ab')_2$ fragment of mAb-MABL sustained the ability to induce apoptosis in CCRF-CEM and JOK-1 cells *in vitro*, and apoptosis by $F(ab')_2$ of mAb-MABL was mediated by CD47 whereas apoptosis by $F(ab')_2$ of the control mouse IgG1 was not (Fig. 1).

SCID mice implanted with CCRF-CEM derived from ALL died within 49 days; their mean survival time was 31 days. Administration of $F(ab')_2$ of mAb-MABL twice a day on days 3, 4, and 5 post tumor implantation greatly prolonged the mean survival time. In addition, four out of seven (57%) mice became tumor-free when they were given $50 \mu\text{g/mouse}$ of the $F(ab')_2$ fragment of mAb-MABL, and all the mice became tumor-free when administrated $200 \mu\text{g/mouse}$ of the $F(ab')_2$ fragment (Fig. 4).

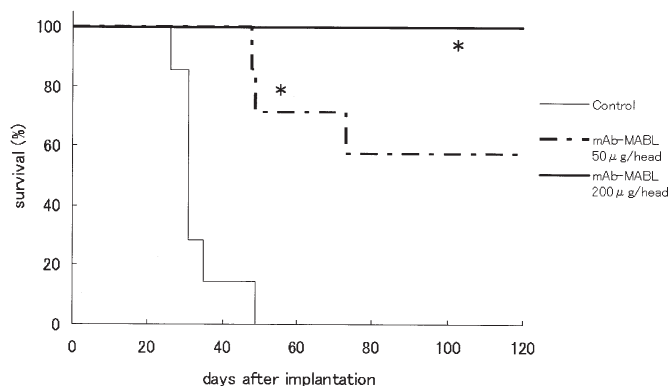


Figure 4. Antitumor activity of the F(ab')₂ fragment of mAb-MABL in mice implanted with CCRF-CEM. Male SCID mice were intravenously injected with 5×10^6 cells/mouse of CCRF-CEM cells. Thereafter, the mice were intravenously administered PBS (control) or the F(ab')₂ fragment of mAb-MABL at doses of 0, 50, and 200 μ g/mouse twice a day (7 mice/each group) on days 3, 4, and 5 post tumor implantation. The survival of the mice was monitored daily. * $P < 0.05$ compared with the vehicle administered group (Wilcoxon test).

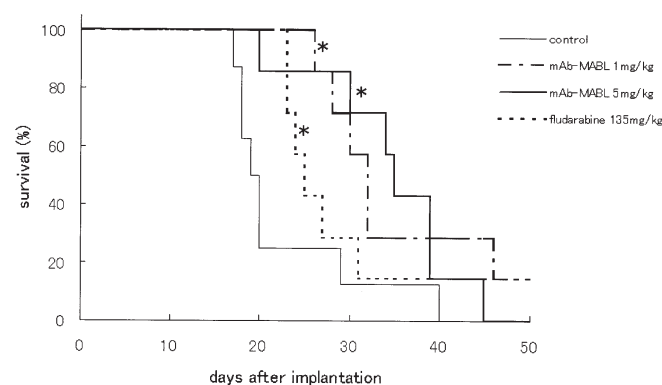


Figure 5. Antitumor activity of the F(ab')₂ fragment of mAb-MABL in mice transplanted with JOK-1. Male SCID mice were intravenously injected with 5×10^6 cells/mouse of JOK-1 cells. Thereafter, the mice were intravenously administered PBS (control), F(ab')₂ fragment of mAb-MABL at a dose of 1 or 5 mg/kg twice a day (7 mice/each group) on days 3, 4, and 5 post tumor transplantation, or fludarabine at 135 mg/kg twice a day for 10 days (from day 3 to day 7 and from day 10 to day 14) post tumor transplantation. Survival of the mice was monitored daily. * $P < 0.05$ compared with the vehicle administered group (Wilcoxon test).

Administration of the F(ab')₂ fragment of mAb-MABL improved the median survival time in SCID mice intravenously injected with JOK-1 cells originating from B-CLL. The median survival time was 19.5 days in mice given only the vehicle, and 32 and 35 days in mice administered 1 mg/kg and 5 mg/kg of the F(ab')₂ fragment, respectively (Fig. 5). Prolongation of survival by the F(ab')₂ fragment was more pronounced than by fludarabine. When administered intravenously at 135 mg/kg twice a day for 10 days, fludarabine prolonged the median survival time to 25 days, which was prominently shorter than that achieved by the F(ab')₂ fragment of mAb-MABL (Fig. 5).

We also examined the antitumor activity of the F(ab')₂ fragment of mAb-MABL in mice intraperitoneally injected

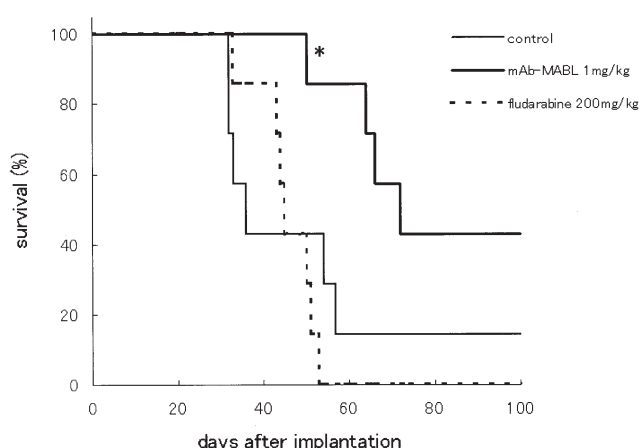


Figure 6. Antitumor activity of the F(ab')₂ fragment of mAb-MABL in mice intraperitoneally injected with JOK-1. Male SCID mice were intraperitoneally injected with 2×10^7 cells/mouse of JOK-1 cells. Thereafter, the mice were intravenously administered PBS (control) or the F(ab')₂ fragment of mAb-MABL at 1 mg/kg twice a day (7 mice/each group) on the first 5 days post tumor implantation. Fludarabine was also administered intraperitoneally at a dose of 200 mg/kg twice a day for 10 days (from day 1 to 5 and from day 15 to 19) post tumor implantation. The survival of the mice was monitored daily. * $P < 0.05$ compared with the vehicle administered group (Wilcoxon test).

with JOK-1 cells and compared it to that of fludarabine. Intraperitoneal administration of 200 mg/kg fludarabine twice a day for 10 days prolonged the survival time. The median survival time was 45 days for mice given fludarabine, whereas it was 36 days in the vehicle-treated mice. At 200 mg/kg twice a day for 10 days, fludarabine caused severe toxicity as judged by body weight loss. Prolongation of survival was more pronounced when mice were administered the F(ab')₂ fragment of mAb-MABL. The median survival time increased to 72 days, and 3 out of 7 mice became tumor-free when given 1 mg/kg of the F(ab')₂ fragment of mAb-MABL (Fig. 6).

Discussion

The monoclonal antibody against CD47, mAb-MABL, caused apoptosis in leukemic cell lines possibly by inducing the ligation of CD47, which raises the possibility that the ligation of CD47 leads to antitumor activity *in vivo*. To address this possibility, we examined the antitumor activity of the F(ab')₂ fragment of mAb-MABL in human leukemia xenograft models. In these models representing ALL and B-CLL, the F(ab')₂ fragment of mAb-MABL markedly prolonged survival time. In addition, the ligation of CD47 and the subsequent apoptosis of the cells is sufficient to elicit the antitumor activity, as the F(ab')₂ fragment of mAb-MABL does not induce antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cell cytotoxicity (CDC). Thus, the ligation of CD47 and the subsequent induction of apoptosis is a feasible approach to the treatment of leukemic cells.

The mAb-MABL was efficacious against B-CLL cells, Mateo *et al* (15) reported that the ligation of CD47 induced apoptosis, even when the tumor cells were co-cultured with

stromal cells. Mice injected intravenously with JOK-1 cells developed paraplegia by day 18, which is an indication of tumor growth in the bone marrow resulting in the suppression of the spinal cord, and died at a median survival time of 19.5 days. Administration of the F(ab')₂ fragment of mAb-MABL significantly attenuated the development of paraplegia and prolonged the median survival time. Furthermore, the prolongation of the mean survival time by the F(ab')₂ fragment of mAb-MABL was much more profound than by fludarabine.

SCID mice intraperitoneally injected with JOK-1 also developed lymphadenopathy, splenomegaly, with the appearance of white nodules in the liver, indicative of tumor cell growth in these organs. Intravenous administration of the F(ab')₂ fragment of mAb-MABL greatly improved the median survival time, and some mice became free of tumor cells. In contrast, fludarabine only slightly prolonged the median survival time even at the dose of 200 mg/kg twice daily for 10 days, which caused severe toxicity. Together, these results support the possibility that the F(ab')₂ fragment of mAb-MABL kills leukemic cells growing in the bone marrow, lymph nodules, liver, spleen, and in the milieu where leukemic cells are thought to be protected from apoptosis thereby becoming resistant to conventional chemotherapy.

Furthermore, F(ab')₂ of mAb-MABL did not induce cell death against the CD34⁺ cord blood cells or HUVEC, although they expressed certain amounts of CD47 on the cell surface. The reason why these cells did not die by the ligation of CD47 is not clear, but the data indicate that the signaling from CD47 is a valuable therapeutic target for the therapy of leukemia.

In conclusion, the F(ab')₂ fragment of mAb-MABL bound to CD47 and induced apoptosis of leukemic cells possibly by the ligation of CD47. In addition, the F(ab')₂ fragment was efficacious against the ALL and B-CLL xenografts without affecting the CD34⁺ hematopoietic progenitor/stem cells or HUVEC. Thus, mAb-MABL could be developed as a potential therapeutic agent for incurable leukemia after further optimization such as humanization or making single chain diabodies (28,29).

Acknowledgements

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