Therapeutic effect of an anti-Fas ligand mAb on lethal graft-versus-host disease

Keiko Miwa1, Hideo Hashimoto1,2, Takehiro Yatomi3, Norio Nakamura3, Shigekazu Nagata1,4 and Takashi Suda1,5

1Department of Molecular Biology, Osaka Bioscience Institute, Osaka 565-0874, Japan
2Department of Orthopaedic Surgery, Osaka University Medical School, Osaka 565-0871, Japan
3Biosciences Research Laboratory, Mochida Pharmaceutical Co., Ltd, Tokyo 115-0043, Japan
4Department of Genetics, Osaka University Medical School, Osaka 565-0871, Japan
5Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan

Keywords: apoptosis, bone marrow transfer, cytotoxic T lymphocyte, disease model

Abstract

Several anti-Fas ligand (FasL) inhibitory mAb (FLIM) were raised and characterized in this study. One, FLIM58, showed more potent neutralizing activity than Fas-Fc, the previously established artificial neutralizing agent for FasL. Several murine models of acute graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation have been used to show that both FasL and perforin, the major effector molecules of cytotoxic T lymphocytes, are involved in this disease. In our GVHD model, FasL rather than perforin was associated with lethality. Administration of FLIM58 or Fas-Fc reduced the weight loss and mortality caused by GVHD, although other signs of GVHD, such as skin lesions, lymphoid hypoplasia and mononuclear cell infiltration in the liver, did not improve significantly. FLIM58 was more effective than Fas-Fc in reducing mortality. Our results demonstrated that neutralizing agents for FasL are therapeutic for lethal GVHD.

Introduction

The cell killing activity of cytotoxic T lymphocytes (CTL) has been attributed to the two major molecular mechanisms, the perforin–granzyme system and the Fas–Fas ligand (FasL) system (1–3). We previously demonstrated that CTL-induced fulminant hepatitis in animal models can be prevented by administration of Fas-Fc, the artificial soluble Fas that can neutralize FasL activity (4). Thus, we have suggested that exaggeration of FasL-mediated cytotoxicity may play a pathological role in diseases involving tissue injury and that neutralizing agents may have therapeutic value for this type of disease.

Bone marrow transfer is a common remedy for a relapsed acute lymphoblastic leukemia or aplastic anemia. The major obstacle of this treatment is severe or even lethal graft-versus-host disease (GVHD). Immunosuppressive drugs have been used to control this serious side effect; however, such drugs cause another side effect, i.e. bacterial, fungal and viral infections. The donor CTL are the major mediators of GVHD (5). Accordingly, use of autologous bone marrow cells or elimination of T cells from non-autologous bone marrow cells prevents GVHD, but is associated with an increased incidence of leukemia relapse (6). Transplantation of bone marrow cells from mice carrying homozygously the lpr gene, a loss-of-function mutation of the Fas gene, into wild-type mice results in lethal GVHD (7). We previously discovered that lpr T cells express high levels of FasL and suggested that FasL is involved in this lpr-GVHD (8). Involvement of FasL as well as perforin in the mortality of acute GVHD induced by allogeneic bone marrow transplantation has been demonstrated using murine models (9,10). Selective suppression of one of these two cytotoxic mechanisms might reduce the severity of GVHD without significantly increasing the risk factors mentioned above. For this study, we raised several neutralizing mAb to mouse FasL. Some of these antibodies showed stronger neutralizing activity than Fas-Fc. We then investigated the possible therapeutic value of an anti-FasL mAb in acute lethal GVHD.
Establishment of stable transformants expressing chimeric proteins made from mouse CD40 ligand (CD40L) and FasL

The expression plasmid pEF-CD40LFL carrying a cDNA encoding a chimeric protein consisting of the cytoplasmic and transmembrane regions and a part of the extracellular region of mouse CD40L (amino acids 1–79) and the tumor necrosis factor (TNF) homologous region of mouse FasL (amino acids 132–279) was generated as described previously (11). pEF-CD40LFL is similar to pEF-CD40L, except that the TNF homologous region of mouse FasL was replaced with almost the entire extracellular region of mouse FasL (amino acids 101–279) carrying the gld mutation. These plasmids were linearized by ApaLI and transfected into the WR19L mouse lymphoma cell line by electroporation as described previously (12) to generate stable transformants, W40LFL and W40LGX1 respectively.

Establishment of FasL inhibitory mAb (FLIM)

A peptide (CPSTPSEKKEPRSVAH) corresponding to the amino acids 133–147 of mouse FasL was custom-synthesized and conjugated to BSA by Multiple Peptide Systems (San Diego, CA). Female Armenian hamsters were immunized with 1 × 10⁶ W40LFL cells with complete Freund’s adjuvant s.c. at the tail base, followed by injection of 0.2 µg BSA-conjugated peptide in incomplete Freund’s adjuvant 4 times at 1 month intervals. Finally 0.1 µg BSA-conjugated peptide in incomplete Freund’s adjuvant was injected into the foot pads 1 month after the last tail base immunization. Alternatively, female Armenian hamsters were immunized with 1 × 10⁷ W40LFL cells with complete Freund’s adjuvant s.c. at the tail base, followed by injection of 2 × 10⁶ W40LFL cells with incomplete Freund’s adjuvant at the tail base and 5 × 10⁶ W40LFL cells into the foot pads at one month intervals. Three days after the foot pad immunization, popliteal lymph node cells from the immunized hamster were isolated and fused with P3U1 mouse myeloma cells. Hybridomas were selected in HAT medium. Hybridoma supernatants were screened for neutralizing activity against recombinant soluble mouse FasL using the assay described below. Several hybridomas were cloned and expanded, and mAb were purified as described previously (13).

mFas-Fc and hFas-Fc

mFas-Fc, a chimeric protein consisting of the extracellular region of mouse Fas and the Fc portion of human IgG1, was prepared as described previously (14). hFas-Fc is a chimeric protein containing the extracellular region of human Fas in place of mouse Fas and was prepared like mFas-Fc.

Flow cytometry

FITC-conjugated mAb against CD4 (RM4-5) and CD8 (53–6.7) were purchased from PharMingen (San Diego, CA). FITC-conjugated goat anti-hamster IgG was purchased from Cappel (Durham, NC). FLIM58 were biotinylated by NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer’s protocol. Spleen cells (5 × 10⁶ cells/well) were cultured for 2 days in 1 ml of RPMI medium (Nikkken BioMedical, Kyoto, Japan) supplemented with 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 µM L-glutamine, 100 µg/ml streptomycin, 10 µg/ml penicillin (Meiji Seika, Tokyo Japan), 100 µg/ml streptomycin (Meiji Seika) and 10 ng/ml recombinant human IL-2 in a 24-well plate that had been coated with 10 µg/ml anti-CD3 mAb (2C11). The cells were expanded and passaged in medium containing IL-2 (without anti-CD3 mAb) for 4 days. The cells were harvested and dead cells were removed using Histopaque 1083 (Sigma, St Louis, MO). Viable cells (4 × 10⁵ cells/well) were cultured in 100 µl of medium containing IL-2 and 10 µg/ml of either mFas-Fc or hFas-Fc for 7 days. Viable cells were reactivated with anti-CD3 mAb for 4 h. Cells were then stained with biotinylated FLIM58 (shaded) or a control hamster antibody (dotted lines), followed by phycoerythrin-conjugated avidin and with either FITC-conjugated mAb against CD4 (RM4-5) and CD8 (53–6.7) or anti-Fc receptor mAb (2.4G2; PharMingen) in 100 µl of medium containing IL-2 and 10 µg/ml anti-CD3 mAb (2C11). The cells were expanded and passaged in medium containing IL-2 (without anti-CD3 mAb) for 4 days. The cells were harvested and dead cells were removed using Histopaque 1083 (Sigma, St Louis, MO). Viable cells (4 × 10⁵ cells/well) were further cultured in 100 µl of medium containing IL-2 and 10 µg/ml of FasL-Fc in a 96-well plate coated with anti-CD3 mAb for 4.5 h and then analyzed for FasL expression by flow cytometry.

Aliquots of 1–10⁴ cells in round-bottomed 96-well plates were washed once in staining solution (PBS containing 2% FCS and 0.02% sodium azide) and incubated with 0.25 µg of anti-FcγRII/III receptor mAb (2.4G2; PharMingen) in 10 µl of staining solution for 5 min on ice. Optimal doses of staining antibodies in 40 µl of staining solution were then added and cells were further incubated for 30 min on ice. After washing twice, where appropriate, the cells were incubated with FITC-conjugated goat anti-hamster IgG or phycoerythrin-conjugated avidin for 30 min on ice. Cells were then stained briefly with 5 µg/ml of propidium iodide and washed 3 times.
Therapeutic effect of an anti-FasL mAb on lethal GVHD

Fig. 2. Neutralizing activity of anti-FasL mAb and Fas-Fc to soluble and membrane-bound FasL. (A) WX1 (5 U; recombinant soluble mouse FasL) was preincubated with the indicated concentrations of FLIM4 (Δ), FLIM58 (○) or mFas-Fc (●) for 15 min at 37°C and then incubated with W4 cells overnight. The viability of the W4 cells was determined as described in Methods. (B) A3.4C6 T cell hybridomas activated by phorbol myristate acetate and ionomycin were preincubated with the indicated concentrations of FLIM4 (Δ), FLIM58 (○) or mFas-Fc (●) for 15 min at 37°C and then incubated with the 51Cr-labeled W4 cells for 5 h. Percent of specific lysis was determined as described in Methods.

with staining buffer. Three-color flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA) equipped with a 488 nm argon laser and data was analyzed using CellQuest software (Becton Dickinson). Dead cells stained with propidium iodide were excluded from analyses.

In vitro neutralization assay

The neutralizing activity of mFas-Fc and FLIM was examined against recombinant soluble FasL and membrane-bound FasL expressed on the CD4+ T cell clone, A3.4C6 (15) (kindly provided by Dr Shoichi Ozaki, Second Division, Department of Internal Medicine, Kyoto University Faculty of Medicine). Then 5 U of WX1, the recombinant soluble mouse FasL (16), in 10 µl culture medium (10% FCS/RPMI supplemented with 100 U/ml benzyl-penicillin potassium, and 100 µg/ml streptomycin sulfate) and 50 µl of various concentrations of mAb or Fas-Fc were mixed and preincubated for 15 min at 37°C in flat-bottomed 96-well plates. W4 cells (8×10⁴) which express mouse Fas, in 40 µl medium, were then added to the mixture (total 100 µl/well) and cultured for 14 h. The viability of cells was determined using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt and 1-methoxy-5-methylphenazinium methylsulfate (Dojin Laboratories, Kumamoto, Japan) as described previously (16). One unit of recombinant soluble mouse FasL was defined previously (16). Neutralizing activity against membrane FasL was assessed as follows. A3.4C6 cells were activated by 10 ng/ml phorbol myristate acetate and 500 ng/ml ionomycin for 4 h. The activated A3.4C6 cells (1×10⁵ cells) in 50 µl of medium were mixed with 50 µl of various concentrations of mAb or Fas-Fc in round-bottomed 96-well plates and preincubated for 15 min at 37°C. 51Cr-labeled W4 cells (1×10⁴ cells) in 100 µl medium were then added to the mixture (total 200 µl/well) and spun down by brief centrifugation. After another 5 h incubation, the plates were spun and 100 µl aliquots of the supernatant fractions were assayed for radioactivity using a γ-counter. The spontaneous release of 51Cr was determined by incubating the target cells with medium alone, whereas the maximum release was determined by adding Triton X-100 to the final concentration of 0.1%. Spontaneous 51Cr release was ~10% of the maximum release. The percentage of specific lysis was calculated as follows: percent specific lysis = (experimental 51Cr release – spontaneous 51Cr release) / (maximum 51Cr release – spontaneous 51Cr release)×100.

Experimental model for lethal GVHD

One day after lethal irradiation (1200 rad), (C57BL/6×DBA/2)F₁ (BDF₁) mice were injected with a mixture of 2×10⁷ bone marrow cells and 2×10⁷ spleen cells from BDF₁ (n = 6, □), B6 (n = 7, ○), B6-gld/gld (n = 8, ▲) or B6-perforin⁻/⁻ mice (n = 8, ▼) was injected together into lethally irradiated BDF₁ mice i.v. Percentages of survivors (upper panel) and the average body weight of the survivors of each group (lower panel) at indicated days after the transplantation are plotted.
Therapeutic effect of an anti-FasL mAb on lethal GVHD plantation at intervals of 3 days. All mice were purchased as specific pathogen-free grade from SLC (Shizuoka, Japan) and kept in the specific pathogen-free animal facility of the Osaka Bioscience Institute. In most of experiments, female mice aged 10-12 weeks old were used as recipients and donors.

Histological analysis of liver sections
Freshly isolated livers were cut into several blocks, fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned (6 µm) and stained with hematoxylin & eosin.

Results and discussion
Characterization of FLIM
Armenian hamsters were immunized either with the BSA-conjugated peptide corresponding to amino acids 133-147 of mouse FasL or with the W40LFL cell line expressing the CD40LFL chimeric protein whose C-terminal portion consists of the TNF-homologous region of mouse FasL. Several hybridoma lines producing mAb capable of neutralizing the cytotoxicity of recombinant mouse FasL [WX1 (16)] were established. mAb derived from peptide-immunized animals (represented by FLIM4) and those derived from W40LFL-immunized animals (represented by FLIM58) exhibited distinctive characteristics. FLIM4 specifically recognized mouse FasL but not human FasL expressed on 1A12 cells (13), whereas FLIM58 weakly cross-reacted with human FasL. Previously established anti-human FasL mAb 4H9 (13) weakly cross-reacted with mouse FasL. We also investigated whether FLIM could distinguish between wild-type FasL and FasL carrying the gld point mutation (Phe273 → Leu) which results in a loss of function (17). In spite of the cross-reactivity of FLIM58 between the two species, it did not bind to the W40LFLX1 cells which express the chimeric protein carrying the gld mutation in its FasL sequence (Fig. 1Ac), suggesting that FLIM58 recognized an epitope close to or including Phe273 of FasL. Alternatively, FLIM58 might recognize a three-dimensional structure of the FasL trimer. In contrast, FLIM4 equally recognized both W40LFL and W40LFX1.

It has been shown that Fas and FasL mediate the apoptosis of previously activated T cells upon secondary activation by TCR-mediated stimuli (18-20). Consistent with this fact, FasL expression was detected using FLIM58 on both CD4 and CD8 T cells upon secondary activation by an anti-CD3 mAb.

Fig. 4. Therapeutic effect of FLIM58 and Fas-Fc on lethal GVHD. (A and B) Donor cells from BDF1 (n = 7, □) or B6 mice (●, ○) were injected into irradiated BDF1 mice as described in the legend to Fig. 3. Mice that received B6 cells were each administered 0.5 mg of FLIM58 (n = 14, ●) or normal hamster IgG (n = 11, ○) s.c. from day 0 to 42 at intervals of 3 days. Percentages of survivors (A) and the average body weight of the survivors of each group (B) at the indicated days after the transplantation are plotted. (C) B6 donor cells were injected into irradiated BDF1 mice. Aliquots of 0.5 mg of normal hamster IgG (○), FLIM58 (●), or hFas-Fc (▲), or 0.1 mg of FLIM58 (□) or hFas-Fc (△) were administered to the recipient mice (n = 5 mice/group) as described above. Percentages of survivors of each group at the indicated days after the transplantation are plotted.
Therapeutic effect of an anti-FasL mAb on lethal GVHD

Fig. 5. Histological analysis of liver sections. Donor cells from BDF1, B6, B6-gld/gld or B6-perforin$^{-/-}$ mice were injected into irradiated BDF1 mice as described in the legend to the Fig. 3. Mice that received B6 cells were each administered 0.5 mg of FLIM58 or normal hamster IgG s.c. from day 0 to 24 at intervals of 3 days. Mice were sacrificed 27 days after the transplantation, and liver sections were stained with hematoxylin & eosin.

We previously discovered that expression of FasL mRNA is up-regulated in lpr and gld mice (8). Consistently, FasL expression on lpr T cells was significantly higher compared with wild-type T cells (Fig. 1Bb and e). The lack of reactivity of FLIM58 to gld FasL was confirmed because gld T cells were not stained by FLIM58 under the same conditions (Fig. 1Bc and f).

Inhibitory activity of FLIM to the cytotoxicity of FasL

Fas-Fc, an artificial soluble Fas, has been successfully used to neutralize FasL activity in vivo and in vitro (4,14). Therefore, the neutralizing activity of FLIM4, FILM58 and Fas-Fc against the Fas-dependent cytotoxicity of WX1 and a CD4$^+$ T cell line (A3.4C6) was compared using the Fas-expressing transformant W4 as the target. The cytotoxicity of A3.4C6 against W4 cells is entirely mediated by FasL (15). In a representative experiment shown in Fig. 2(A), FLIM4, FILM58 and mFas-Fc showed similar ED$_{50}$s (doses for 50% inhibition of cytotoxicity, ~100 ng/ml) in the neutralization assay against 5 U of WX1. In this assay, batches of FLIM58 and mFas-Fc have shown ranges of ED$_{50}$ of 10–100 and 100–1000 ng/ml respectively. In general, higher doses of neutralizing reagents were required to inhibit the cytotoxicity of A3.4C6 cells than those required to neutralize WX1. Remarkably, FLIM4 did not inhibit the cytotoxicity of A3.4C6 at all. These results indicate that the cytotoxicity of FasL on CTL is more difficult to be neutralized than that of soluble FasL. This is noteworthy especially when one seeks a therapeutic mAb against FasL, because membrane-bound FasL possesses more potent cytotoxicity compared with soluble FasL (21, 22). Despite that the same preparations of reagents were used in the experiments shown in Fig. 2(A and B), the neutralizing activity of FLIM58 to the cytotoxicity of A3.4C6 was ~7-fold stronger than that of mFas-Fc (ED$_{50}$ of FLIM58, 1800 ng/ml; mFas-Fc, 12000 ng/ml).
Involvement of FasL and perforin in the lethal GVHD

To induce acute lethal GVHD, lethally irradiated BDF1 mice were injected with a mixture of spleen and bone marrow cells from semiallogeneic B6 mice. As shown in Fig. 3, irradiated BDF1 mice receiving syngeneic donor cells were completely rescued, whereas those receiving B6 cells were killed within 30 days with severe weight loss. In contrast, cells from B6-gld/gld mice, which lack the functional FasL gene, rescued host mice for >80 days, although weight loss within the first 7 days was as severe as in mice receiving B6 cells and the mice never completely recovered their original weight. The used B6-gld/gld mice (10–12 weeks old) did not show spleen or lymph node enlargement. The proportions of normal B220+ Thy-1+ T cells in spleen cells of 12-week-old B6-gld/gld mice were not reduced compared to those of wild-type mice (gld, 29.4 ± 1.9%; wild, 26.0 ± 2.8%), although abnormal B220+ Thy-1+ T cells were significantly increased (gld, 12.3 ± 4.2%; wild, 3.2 ± 0.9%). Therefore, the lack of lethality of gld donor cells was not due to the reduced number of functional T cells. To confirm this notion, we performed a similar experiment to Fig. 3 using 6-week-old B6-gld/gld mice as donors in which a significant increase of B220+ Thy-1+ cells was not observed, and obtained a consistent result. Host mice receiving perforin−/− cells survived twice as long as those receiving wild-type cells, but they eventually lost as much body weight as the B6 cell hosts and died. These results support the idea that both perforin and FasL are involved in the GVHD. Braun et al. (9) and Baker et al. (10) have investigated roles of perforin and Fas ligand using gld and perforin−/− mice, and concluded that both perforin and FasL are involved in the GVHD. Our results are basically consistent with these reports. However, in these previous reports, gld donor cells, as compared with wild-type cells, only prolonged survival time, and eventually failed to rescue recipient mice. This discrepancy is not surprising when considering substantial differences of experimental conditions including host and donor mouse strain combinations among our and their reports. The severity of GVHD depends on the strain combination. It may also affect the involvement of FasL in lethality. In previous reports, recipients and donors were fully allogenic. Therefore, host-versus-graft responses by residual radio-resistant T cells of host origin may have affected their results. In the report by Braun et al., bone marrow cells were not replaced by donor cells. Thus, in their system, host bone marrow cells were targets of donor T cells. If bone marrow cells are highly susceptible to perforin-mediated cytoxicity, this could be a reason why gld donor cells killed host mice in their system.

Suppression of lethal GVHD by administration of FLIM58 and Fas-Fc

In our experimental model, FasL rather than perforin played an essential role in the lethality of GVHD. Taking advantage of this fact, we then investigated whether FLIM58 and Fas-Fc could prevent GVHD. In the experiments shown in Fig. 4, lethally irradiated BDF1 mice were given B6 donor cells, and either normal hamster IgG, FLIM58 or hFas-Fc was administered s.c. once every 3 days from day 0 to 42 after the transplantation. The FLIM58 and hFas-Fc used in these experiments showed ED50s of ~10 and 200 ng/ml respectively in the neutralizing assay against WX1 (data not shown). Most of the host mice that received B6 cells and 0.5 mg/dose of FLIM58 survived as long as they received the antibody. Although half of these mice died between day 50 and 60 (Fig. 4A and C), the other half survived >80 days. Doses of 0.5 mg of hFas-Fc gave levels of protective effect similar to that achieved by 0.5 mg of FLIM58. However, 0.1 mg/dose of hFas-Fc protected only 40% of recipient mice at day 40, while the same amount of FLIM58 protected all mice during the same period, indicating that FLIM58 has stronger neutralizing activity than Fas-Fc does, not only in vitro but also in vivo. Reduction of body weight was partly but not completely suppressed by FLIM58 (Fig. 4B). In contrast, administration of control antibody showed no effect on survival and weight loss.

Other signs of GVHD

To investigate how FLIM58 protects animals from the lethality of GVHD, general signs of GVHD were examined. Baker et al. (10) reported that the symptoms observed in recipient mice of cells from wild-type or perforin-deficient allogeneic mice, such as skin lesions, lymphoid hypoplasia with marked elimination of B cells and perivascular mononuclear cell infiltration in the liver, were absent or markedly reduced when the donor cells were replaced with those from gld mice. Consistent with this, we observed a reduction in skin lesions when gld mice were used as the donor. However, administration of FLIM58 failed to reduce skin lesions, indicating that the effect of FLIM58 is limited compared with the complete loss of functional FasL (data not shown). In our experimental model, mononuclear cell infiltration in the liver and splenic hypoplasia were observed when donor cells of B6 background (but not BDF1) mice were used, irrespective of FasL and perforin genotypes, and antibody treatments (Fig. 5 and data not shown). In spite of the massive mononuclear cell infiltration in the liver, the serum level of glutamate-pyruvate transaminase was not significantly increased in any case (data not shown), suggesting that damage to the hepatocytes was not prominent in this GVHD model. We previously demonstrated that hepatocytes are highly sensitive to FasL-mediated cytotoxicity of CTL (4). Therefore, mononuclear cells infiltrating the liver might not express FasL. Closer examination is required to determine the action points of FLIM58 at the organ and tissue levels.

In conclusion, our study supports the idea that FasL is one of the important mediators of lethal GVHD, and indicates that neutralizing agents for FasL, such as neutralizing antibodies or Fas-Fc, could be useful in treating patients with this disease. FLIM58 is useful for investigating the physiological and pathological roles of FasL, and the therapeutic value of FasL-neutralizing agents.

Acknowledgements

We thank Ms R. Hayashi for secretarial assistance. This study was supported in part by Special Coordination Funds of Science and Technology Agency of the Japanese Government, by grants-in-aid from the Ministry of Education, Science and Culture of Japan, and by The Mochida Memorial Foundation for Medical and Pharmaceutical Research.
Therapeutic effect of an anti-FasL mAb on lethal GVHD

Abbreviations
CD40L CD40 ligand
CTL cytotoxic T lymphocyte
FLIM FasL inhibitory mAb
FasL Fas ligand
GVHD graft-versus-host disease
TNF tumor necrosis factor

References