A novel murine anti-human Fas mAb which mitigates lymphadenopathy without hepatotoxicity

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Keywords: anti-Fas mAb, apoptosis, dual function, hepatotoxicity, lymphadenopathy, rheumatoid arthritis

Abstract

Defects in Fas-mediated apoptosis are implicated in autoimmune diseases including rheumatoid arthritis (RA). Although induction of Fas-mediated apoptosis could have therapeutic effects on these diseases, it might cause deleterious effects in liver as Fas ligand or an agonistic anti-murine Fas antibody Jo2 causes severe hepatic injury in mice. We report here on the interesting characteristics of the newly obtained anti-Fas mAb, HFE7A, which cross-reacts with the Fas molecules of various species ranging from human to mouse and mitigates autoimmune symptoms without hepatotoxicity in mice. The administration of HFE7A to mice induced apoptosis in the thymocytes, although administration of HFE7A to mice or to marmosets did not induce any sign of hepatitis. The effect of HFE7A on liver is different from that of anti-murine Fas antibody Jo2, which causes acute and lethal hepatic injury to mice. Administration of HFE7A reduced lymphadenopathy and abnormal T cells in MRL-\textit{gld/gld} mice. HFE7A induced apoptosis in synovial cells prepared from RA patients. Surprisingly, HFE7A protected mice from fulminant hepatitis induced by Jo2. Therefore, HFE7A is a potential therapeutic antibody not only for autoimmune diseases including RA but also for fulminant hepatitis.

Introduction

Apoptosis, or programmed cell death, plays an important role in many biological processes, including embryogenesis, development of the immune system, elimination of virus-infected cells and maintenance of tissue homeostasis (1–3). The Fas antigen/Apo-1/CD95 (Fas) molecule is a type I cell-surface receptor belonging to the tumor necrosis factor (TNF) receptor/nerve growth factor (NGF) receptor superfamily. It can transduce an apoptotic death signal in cells when stimulated by agonistic anti-Fas antibodies or the Fas ligand (FasL) (4–8). FasL is a type II membrane protein belonging to the TNF family (7), and is expressed mainly in activated T lymphoid-myeloid lineage cells, in the eye, in reproductive organs and in some tumors (9–11).

The expression of the Fas and FasL protein was analyzed in detail on T and B lineage cells, including thymocytes and peripheral lymphocytes, and it has revealed that the Fas–FasL system plays an important role in maintaining the immune system (12–16). Mice with Fas-defective lymphoproliferation (\textit{lpr}) and FasL-defective generalized lymphoproliferative disease (\textit{gld}) mutations develop massive lymphadenopathy (17,18) and autoimmune diseases, which indicates that the Fas–FasL system plays an important role in the elimination of autoreactive lymphoid cells. Administration of anti-murine Fas antibody RK-8 to FasL-deficient MRL-\textit{gld/gld} mice reduced the autoimmune symptoms, including those of lymphadenopathy, nephritis, arthritis and vasculitis (19). Intra-articular administration of RK-8 to the HTLV-I tax transgenic mice, which spontaneously develop arthritis and are considered to be one of the most suitable models for human rheumatoid arthritis (RA), was effective in improving paw swelling in these mice (20). Fas expression was detected on fibroblast-like synoviocytes. It was reported that anti-
human Fas antibody CH-11 induces apoptosis in synoviocytes and infiltrating mononuclear cells from RA patients in vitro or in an in vivo RA model in which human RA tissue is grafted onto SCID mice (21–23). These facts suggest that anti-Fas antibody is a candidate for use as a therapeutic agent for autoimmune diseases including RA. On the other hand, administration of Jo2, another hamster anti-murine Fas mAb, caused fulminant hepatitis or hemorrhage and death in mice (24). The use of anti-Fas antibody as a therapeutic could cause adverse effects in the liver. RK-8 caused only slight hepatotoxicity even though it induces apoptosis in premature neutrophils (25), indicating that the apoptosis-inducing patterns of various anti-Fas mAb in discrete cell types can differ from one to another (26). On this basis we tried to obtain an anti-human Fas antibody which has apoptosis-inducing activity on autoreactive lymphocytes or RA synovial cells with less toxicity.

We obtained a novel mouse anti-Fas mAb, HFE7A, which reacts with both human and murine Fas. Although administration of HFE7A induced apoptosis in thymocytes, it did not show any sign of hepatotoxicity in mice. HFE7A reduced the lymphadenopathy that had developed in MRL-\textit{gld}/\textit{gld} mice. Moreover, the administration of HFE7A to mice blocked the injury of the liver induced by Jo2. In addition, HFE7A induced apoptosis in synovial cells from RA patients in vitro. Exploitation of humanized HFE7A may provide useful therapeutics for the treatment of autoimmune diseases such as RA and fulminant hepatitis.

Methods

Cells, culture condition and animals

Murine T cell lymphoma WR19L, its transformant WR19L12a expressing human Fas, murine T cell lymphoma L5178Y and its transformant L5178YA1 expressing murine Fas were maintained at 37°C under 5% CO2 in RPMI 1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 \( \mu \)g/ml), 10% FBS and 50 \( \mu \)M 2-mercaptoethanol. Synovial cells in RA were prepared as follows: synovial tissue obtained from an affected region of a patient with RA was cut into small pieces with scissors. The fat was removed and collagenase was then added to make a final concentration of 5 mg/ml and the mixture was incubated at 37°C for 90 min. The resulting incubated cells then served as the synovial cells for the remainder of the experiment. BALB/c and MRL-\textit{gld}/\textit{gld} mice were obtained from Charles River Japan (Tokyo, Japan) equipped with a 488nm argon laser. Thymocytes of MRL-\textit{gld}/\textit{gld} mice injected with HFE7A (5\( \times \)10\textsuperscript{5} cells/samples) were stained with FITC-conjugated goat IgG fraction against mouse IgG (whole molecule) (Organon Teknika, Durham, NC) or FITC-conjugated goat anti-mouse IgM antibodies (Biosource International, Camarillo, CA) on ice for 1 h. After washing with the staining buffer, the cells were stained with 20 \( \mu \)g/ml of FITC-conjugated goat IgG fraction against mouse IgG (whole molecule) (PharMingen) and PE-conjugated anti-B220 mAb (PharMingen). The cells were analyzed for Thy1.2 or B220 expression by flow cytometry. Data were analyzed using the Cyto ACE system program version 3.06 (Jasco). Dead cells stained with propidium iodide were excluded from analyses.

Preparation of recombinant human Fas protein

The plasmid pFAS-AIC2, an expression vector for a fusion proteins of the extracellular region of human Fas antigen (amino acids –16 to 150) and the extracellular region of the mouse IL-3 receptor (amino acids 3 to 423) under the control of the SR-\( \alpha \) promoter were introduced into COS-1 cells by electroporation. The cells were cultured in the DMEM containing 10% of FBS for 24 h. Subsequently, the culture supernatant was removed and the cells were suspended in serum-free DMEM. The cells were further cultured at 37°C for 48 h. The culture supernatant was collected. Human Fas–IL-3 receptor fusion protein was partially purified using ion-exchange chromatography.

Preparation of mAb

Fas-deficient mice were immunized with partially purified human Fas antigen and the splenocytes from the immunized mice were fused with mouse myeloma NS1 using standard cell fusion techniques. Hybridomas were screened by flow cytometry for the production of antibodies that bind to WR19L12a expressing human Fas or L5178YA1 expressing murine Fas, but not to the parental WR19L cells or L5178Y cells. One hybridoma line, producing a mAb reacting with both human and murine Fas, was cloned 5 times by limiting dilution and designed as was HFE7A. The hybridoma-producing HFE7A (2\( \times \)10\textsuperscript{5} cells) were cultured in 1000 ml of serum-free ASF104 medium (Ajinomoto, Tokyo, Japan) for 3 days at 37°C. The anti-Fas antibody, HFE7A, was purified using HiTrap protein G column (PharMingen, San Diego, CA) as described by the manufacturer. The IgG fraction was dialyzed in PBS. The purified anti-Fas mAb was analyzed by SDS–PAGE and the purity was indicated to be >90%. The isotype of HFE7A was determined by using a mAb typing kit (Pierce, Rockford, IL).

Flow cytometry

Peripheral lymphocytes derived from human, chimpanzee, Japanese monkey, crab-eating monkey and marmoset were cultured in RPMI 1640 medium containing 5 \( \mu \)g/ml of phytohemagglutinin-P (Sigma, St Louis, MO) at 37°C for 24 h. The cells were washed and cultured in RPMI 1640 medium containing 10 U/ml of IL-2 at 37°C for a further 72 h. Activated T cells derived from human, chimpanzee, Japanese monkey, crab-eating monkey and marmoset, and thymocytes of BALB/c mice (1\( \times \)10\textsuperscript{6} cells/sample) were washed with a staining buffer (PBS containing 5% FBS and 0.04% NaN\textsubscript{3}) and incubated with 20 \( \mu \)g/ml of HFE7A or CH-11 (MBL, Nagoya, Japan) for 1 h on ice. After washing with the staining buffer, the cells were stained with 20 \( \mu \)g/ml of FITC-conjugated goat IgG fraction against mouse IgG (whole molecule) (Organon Teknika, Durham, NC) or FITC-conjugated goat anti-mouse IgM antibodies (Biosource International, Camarillo, CA) on ice for 1 h. After washing with the staining buffer, samples were analyzed on a flow cytometer, Cyto ACE-150 (Jasco, Tokyo, Japan) equipped with a 488nm argon laser. Thymocytes of MRL-\textit{gld}/\textit{gld} mice injected with HFE7A (5\( \times \)10\textsuperscript{5} cells/samples) were stained with FITC-conjugated anti-Thy1.2 mAb (PharMingen) and PE-conjugated anti-B220 mAb (PharMingen). The cells were analyzed for Thy1.2 or B220 expression by flow cytometry. Data were analyzed using the Cyto ACE system program version 3.06 (Jasco). Dead cells stained with propidium iodide were excluded from analyses.

Cytotoxicity assay

Various concentrations of HFE7A were prepared in RPMI 1640 containing 10% FBS and 50 \( \mu \)l of each concentration was plated per well in a 96-well culture plate. Then 50 \( \mu \)l of WR19L, WR19L12a, L5178Y or L5178YA1 cells adjusted to 1\( \times \)10\textsuperscript{5} cells/50 \( \mu \)l was further pipetted thereto, followed by culturing
The SD was analyzed in triplicate by XTT assay, as mentioned above. Absorbance of each well was read at 450 nm. The cell viability of each well was read at 450 nm. Synovial cells prepared as described above were separated into single cells by treatment with a 0.05% trypsin solution at 37°C for 2 min, then dispensed into the wells of a 96-well plate at 2×10^4 cells/well and incubated at 37°C for 6 days. After washing with Hank’s buffer (Gibco/BRL, Grand Island, NY), 200 μl of DMEM containing 10% FBS and various concentrations of HFE7A or CH-11 were added, and the plate was further incubated at 37°C for 20 h. Next, 50 μl of a 1 mg/ml XTT solution (Sigma) and 25 μM phenazine methosulfate (PMS; Sigma) was added. After 4 h of incubation at 37°C, the absorbance of each well was read at 450 nm. The cell viability was analyzed in triplicate by XTT assay, as mentioned above. The SD was <5%. Human primary hepatocytes were obtained from Biowhittaker (Walkersville, MD). Hepatocytes were dispensed into a type I collagen-coated 96-well plate at 4×10^4 cells/well in human epidermal growth factor (hEGF)-omitted hepatocyte culture medium (HCM) (Biowhittaker). After 2 h of incubation at 37°C, the plate was washed with the medium to remove unattached cells. The hepatocytes were incubated with EGF-omitted HCM containing various concentrations of HFE7A or CH-11 for 1 h and washed with the medium 3 times. The cells were cultured for 15 h in the medium containing 5 μg/ml of the goat anti-mouse Ig mAb (Biosource International) and viability of the hepatocytes was determined by XTT assay.

Statistical methods
Statistical analysis was performed by analysis of variance.

Results
Preparation and characterization of anti-human Fas mAb HFE7A
Fas-deficient (Fas−) mice (27) were immunized with a partially purified recombinant human Fas–AIC2A chimera protein (see Methods) and hybridomas were prepared from the splenocytes. Hybridoma-secreting antibodies, which bind to human Fas expressed on mouse T lymphoma WR19L12a cells (6), were selected and subsequently cloned by limiting dilution. Among those, HFE7A was the clone that produced mAb cross-reacting with the Fas molecules of various species ranging from human to mouse (Fig. 1). CH-11 reacted with Fas molecules of human and chimpanzee. It has never been reported that one anti-Fas mAb can react with both the human and murine Fas molecule. The type of heavy chain and light chain of HFE7A were γ1 and κ, respectively.

In vitro and in vivo apoptosis-inducing activity of HFE7A on Fas-expressing lymphoma cells
HFE7A induced death in L5178Y/A1 and WR19L12a cells expressing the murine and human Fas respectively when a secondary antibody was added to the systems (Fig. 2). The reduction in viability was due to apoptosis, as judged by the Annexin V-binding assay and TUNEL assay (data not shown). HFE7A did not demonstrate apoptosis-inducing activity without cross-linking with a secondary antibody in this system (data not shown). Next, to examine the effect of HFE7A in vivo, HFE7A was administered to BALB/c mice. HFE7A induced apoptosis in the thymocytes, as revealed by analysis using the TUNEL method (Table 1 and Fig. 3).

Effect of the administration of HFE7A on BALB/c mice
Intraperitoneal administration of anti-murine Fas mAb Jo2 has been reported to be lethal to mice as this mAb causes severe liver damage (24). To examine the hepatotoxic effects of i.p. administration of HFE7A, we monitored the serum levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) as indices of liver injury. The biochemical study of serum hepatic enzymes clearly showed that HFE7A did not induce any hepatotoxicity within the observation period extending from the day of injection to day 2 after injection (Fig. 4). In neither case of multiple
Table 1. Effect of HFE7A administration on the thymus of BALB/c mice

<table>
<thead>
<tr>
<th>No.</th>
<th>TUNEL-positive cells/mm²</th>
<th>Mean</th>
<th>SD</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>66.9</td>
<td>75.18</td>
</tr>
<tr>
<td>1*</td>
<td></td>
<td>86.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>72.5</td>
<td></td>
</tr>
<tr>
<td>HFE7A 100 µg</td>
<td></td>
<td>3830</td>
<td>1388</td>
</tr>
<tr>
<td>4*</td>
<td></td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Jo2 20 µg</td>
<td></td>
<td>348</td>
<td>258.4</td>
</tr>
<tr>
<td>7*</td>
<td></td>
<td>236</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>191</td>
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</tbody>
</table>

Six-week-old female BALB/c mice (n = 3) were injected with 100 µg of HFE7A or 20 µg of Jo2 i.v. The animals were sacrificed 3 h after the injection and the TUNEL-positive cells in a definite area in the thymus were counted. The representative TUNEL staining photographs of each group (mouse denoted with an asterisk) are shown in Fig. 3.

Fig. 3. Effect of HFE7A administration on the thymus of BALB/c mice. Six-week-old female BALB/c mice were injected with 100µg of HFE7A or 20 µg of Jo2 i.v. The animals were sacrificed 3 h after the injection and the apoptotic cells in the thymus were detected by the TUNEL method. Data are representative of three mice (see Table 1).

Fig. 4. The level of GOT and GPT in the serum after single administration of HFE7A. Six-week-old BALB/c female mice were injected with 200 µg (○) or 500 µg (□) of HFE7A, 100 µg of Jo2 (●) and control PBS (■) i.p. At the indicated times thereafter, serum was collected, and GOT and GPT levels were quantified.

Fig. 5. The level of GOT and GPT in the serum after multiple administration of HFE7A. Six-week old BALB/c female mice were injected with 200 µg of HFE7A i.p. everyday for 7 or 14 days. After the administration period, the mice were sacrificed, and serum GOT (solid bars) and GPT (open bars) levels were measured. The average values are shown with SD (error bars).

Toxicity of HFE7A in marmosets
We also tested the hepatotoxic effects of HFE7A on marmosets because HFE7A binds to marmoset Fas as well as human Fas (Fig. 1). The single 2 mg/body of HFE7A was i.v. injected to five marmosets. Twenty-four hours after treatment, hematological, blood chemistry and pathological examinations were performed, and no evidence indicating hepatic injury was noted (data not shown). The frequency of apoptosis in thymus, spleen and bone marrow also did not increase with the HFE7A treatment (data not shown).

HFE7A did not induce apoptosis in primary human hepatocyte culture in vitro
We examined the killing activity of HFE7A on human primary hepatocytes to elucidate possible hepatotoxicity in clinical application. HFE7A did not show any significant killing activity on human primary cells (Fig. 6), although expression of Fas was detected with HFE7A by flow cytometry (data not shown).

Curative effect of the administration of HFE7A in MRL-lpr/lpr mice
Mice carrying the lpr mutation (Fas antigen) or gld mutation (FasL) develop lymphadenopathy and suffer from auto-
immune diseases such as systemic lupus erythematosus (SLE) (17,18). MRL-\textit{gld/gld} mice develop RA-like arthritis and show SLE-like characteristic symptoms. In order to investigate the possibility that the administration of HFE7A mitigates lymphadenopathy in these mice, MRL-\textit{gld/gld} mice (female, 9 weeks old) were i.p. injected with 0.5 mg of HFE7A. The ratio of abnormal T cells (Thy1.2\textsuperscript{+}B220\textsuperscript{−}) in the thymus decreased with the administration of HFE7A (Table 2), suggesting that HFE7A killed these abnormal T cells in vivo. In addition, the lymph node swelling in MRL-\textit{gld/gld} mice diminished gradually with treatment by HFE7A compared to the control mice (Table 3). The improvement of lymphadenopathy by i.p. administration of HFE7A was more remarkable in young mice (9 weeks old) than in old mice (13 weeks old) (data not shown). The weights of the thymus, spleen and lymph node also diminished with treatment by HFE7A as compared to the control mice (Figs 7 and 8). These results suggest that the ameliorative effect of HFE7A on MRL-\textit{gld/gld} mice might come from induction of apoptosis in the abnormal autoreactive T cells. The serum level of GOT or GPT was not increased by the injection of HFE7A, which indicated that HFE7A caused no liver damage in MRL-\textit{gld/gld} mice (data not shown).

\textbf{In vitro induction of Fas-dependent apoptosis by HFE7A against freshly isolated synovial cells from RA}

We examined the killing activity of HFE7A on RA patient-derived synovial cells to study the possibility of clinical application for RA. As shown in Fig. 9, synovial cells from RA were also dose-dependently killed by HFE7A, to a similar degree as in CH-11, without concurrent addition of a second mAb. These results indicate that HFE7A may be applicable as a therapeutic medicine in RA.

\textbf{Prevention of Jo2-induced hepatitis by HFE7A}

Interestingly, administration of HFE7A in mice inhibited lethal hepatic injury induced by Jo2. BALB/c mice were i.p. injected with various amounts of HFE7A in combination with 0.1 mg of Jo2. As shown in Table 4, whereas all the mice injected with Jo2 alone died within 5 h, administration of HFE7A rescued the mice from Jo2-induced death even when the amount of injected HFE7A was only a half of Jo2. In further tests, Jo2 was administered prior to the injection of HFE7A. When HFE7A was i.p. injected to mice 5, 20 or 60 min after Jo2 injection, none of the mice died (Table 4). However, the injection of HFE7A 120 min after the injection of Jo2 did not rescue the mice (Table 4). Pretreatment of L5178YA1 cells expressing murine Fas with HFE7A did not prevent either Jo2-induced apoptosis or binding of Jo2 (data not shown). These data suggest that the epitope of HFE7A on murine Fas is different from that of Jo2 and HFE7A might prevent Jo2-induced hepatitis in MRL-\textit{gld/gld} mice might come from induction of apoptosis in the hepatic injury induced by Jo2. BALB/c mice were i.p. injected with 0.5 mg of HFE7A or 200 µg of RK-8 i.p. The animals were sacrificed 55 days after injection and the percentage of Thy1.2\textsuperscript{+}B220\textsuperscript{−}, Thy1.2\textsuperscript{−}B220\textsuperscript{−}, Thy1.2\textsuperscript{−}B220\textsuperscript{+} and Thy1.2\textsuperscript{−}B220\textsuperscript{+} thymocytes was quantified by flow cytometric analysis.

\textbf{Table 2. Selective decrease (%) of abnormal T cells in the thymus of MRL-\textit{gld/gld} mice administered with HFE7A}

<table>
<thead>
<tr>
<th></th>
<th>Thy1.2\textsuperscript{−}B220\textsuperscript{+}</th>
<th>Thy1.2\textsuperscript{−}B220\textsuperscript{−}</th>
<th>Thy1.2\textsuperscript{−}B220\textsuperscript{+}</th>
<th>Thy1.2\textsuperscript{−}B220\textsuperscript{+}</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>79.80</td>
<td>15.30</td>
<td>4.50</td>
<td>0.50</td>
</tr>
<tr>
<td>HFE7A 500 µg</td>
<td>13.00</td>
<td>86.10</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>RK-8 200 µg</td>
<td>6.50</td>
<td>92.90</td>
<td>0.30</td>
<td>0.25</td>
</tr>
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</table>

Nine-week-old female MRL-\textit{gld/gld} mice were injected with 500 µg of HFE7A or 200 µg of RK-8 i.p. The animals were sacrificed 55 days after injection and the percentage of Thy1.2\textsuperscript{−}B220\textsuperscript{+}, Thy1.2\textsuperscript{−}B220\textsuperscript{−}, Thy1.2\textsuperscript{−}B220\textsuperscript{+} and Thy1.2\textsuperscript{−}B220\textsuperscript{+} thymocytes was quantified by flow cytometric analysis.

\textbf{Table 3. Curative effect of HFE7A on lymphadenopathy of MRL-\textit{gld/gld} mice}

<table>
<thead>
<tr>
<th></th>
<th>Day 24 (mean ± SD)</th>
<th>Day 55 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
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<tr>
<td>HFE7A, 500 µg (single)</td>
<td>11.5 ± 3.7</td>
<td>9.6 ± 1.3</td>
</tr>
<tr>
<td>HFE7A, 500 µg (control)</td>
<td>1.1 ± 1.0 (P &lt; 0.001)</td>
<td>3.3 ± 3.4 (P &lt; 0.05)</td>
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</table>

Eight-week-old female MRL-\textit{gld/gld} mice were maintained until onset of lymphadenopathy. The mice were injected with 500 µg of HFE7A or PBS on the onset day (n = 5). The extent of swelling of the neck, axillary and inguinal lymph nodes was rated from 0 to 4 for 55 days. We scored the lymph node swelling as follows, no swelling, 0; the diameter of each lymph node < 0.5 cm, 1; 0.5–1.0 cm, 2; 1.0–1.5 cm, 3; > 1.5 cm, 4. Results are expressed as the total score of each lymph node swelling on day 24 and 55.

\textbf{Fig. 7. Average weight of organs removed from MRL-\textit{gld/gld} mice treated with HFE7A.}

Eight-week-old female MRL-\textit{gld/gld} mice were maintained until lymphadenopathy occurred. After that, MRL-\textit{gld/gld} mice were injected with 0.5 mg of HFE7A i.p. once a week for 50 days, and the thymus, spleen and lymph node were removed. The values shown are the average weight of each organ [control, open bars (n = 5); HFE7A treated, solid bars (n = 6)]. The average values are shown with SD (error bars).
An agonistic anti-human Fas mAb without hepatotoxicity

Fig. 8. Comparison of organs removed from MRL-gld/gld mice. After the occurrence of lymphadenopathy, MRL-gld/gld mice were injected with 0.5 mg of HFE7A i.p. once a week for 50 days, and the thymus, spleen and lymph node were removed. Pictures of mice representing each group are shown.

Discussion

It is suggested that the failure of the Fas-FasL system is related to many diseases including RA, SLE, Addison’s disease, Hashimoto’s thyroiditis, scleroderma, Goodpasture’s syndrome, autoimmune hemolytic anemia, multiple sclerosis, graft versus host disease, arteriosclerosis, cardiomyopathy, acute glomerulonephritis, hypoplastic anemia, fulminant hepatitis and AIDS (28–34). Anti-Fas antibodies or FasL are considered to have value as a potential therapeutic protocol for the above diseases because they induce or block apoptosis. However, application of agonistic mAb to human Fas for therapeutic use has two difficulties. (i) It was reported that the administration of agonistic anti-Fas mAb Jo2 and recombinant FasL to mice induced liver failure with symptoms similar to fulminant human hepatitis, quickly followed by death (23). RK-8 induced apoptosis in neutrophils of young mice in vivo (25). Hence, agonistic anti-Fas mAb and FasL may induce severe adverse effect. (ii) It is very difficult to study the hepatotoxicity or therapeutic effects of the anti-human Fas mAb in small animals, because anti-human Fas mAb prepared by conventional methods do not cross-react with Fas in mice.

We immunized human Fas in Fas−/− mice and obtained a mAb HFE7A that cross-reacts with Fas derived from various

Table 4. Protective effects of HFE7A against an acute and lethal hepatic injury induced by Jo2

<table>
<thead>
<tr>
<th>Survival ratioa</th>
<th>Simultaneous injectionb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>50 µg Jo2</td>
</tr>
<tr>
<td></td>
<td>100 µg Jo2 with</td>
</tr>
<tr>
<td></td>
<td>500 µg HFE7A</td>
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<td></td>
<td>100 µg HFE7A</td>
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<td></td>
<td>50 µg HFE7A</td>
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<tr>
<td></td>
<td>25 µg HFE7A</td>
</tr>
<tr>
<td>Jo2, pre-injectionb</td>
<td></td>
</tr>
<tr>
<td>HFE7A (100 µg) injection after Jo2 (50 µg) injection</td>
<td>3/3</td>
</tr>
<tr>
<td>5 min</td>
<td>3/3</td>
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<tr>
<td>20 min</td>
<td>3/3</td>
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<tr>
<td>60 min</td>
<td>3/3</td>
</tr>
<tr>
<td>120 min</td>
<td>0/3</td>
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</table>

*aSix-week-old BALB/c female mice were i.p. injected with 100 µg of Jo2 in combination with 500, 100, 50 or 25 µg of HFE7A.*

*bSix-week-old BALB/c female mice were injected i.p. with 50 µg of Jo2. At 5, 20, 60 or 120 min after the Jo2 injection, 100 µg of HFE7A was i.p. injected to the Jo2-treated mice. As a comparison, 100 or 50 µg of Jo2 alone was i.p. injected to the mice.

For 24 h after Jo2 injection.
species ranging from human to mouse. As far as we know, HFE7A is the first anti-human Fas antibody with effects that can be examined for various diseases in vivo using the conventional mouse model. HFE7A induced Fas-dependent apoptosis in human and mouse-derived T lymphoma in vitro when it is cross-linked by secondary antibody. Although administration of HFE7A did induce apoptosis in thymocytes in mice, it did not cause any hepatotoxicity in mice and marmosets. Although the number of premature neutrophils in bone marrow was reduced by the injection of RK-8 into young BALB/c mice, HFE7A did not affect the number of neutrophils (unpublished result). We examined the effects of HFE7A in MRL- gld/gld mice, one of the mouse models for autoimmune diseases. The lymphadenopathy symptoms in MRL-gld/gld mice were mitigated after administration of HFE7A. HFE7A also showed some ameliorative effect in collagen-induced arthritis in mice (unpublished result). In addition, HFE7A induced apoptosis in freshly isolated synovial cells from patients of RA in vitro. These results suggest that HFE7A have a potential therapeutic usefulness in autoimmune diseases including RA. Furthermore, not only did HFE7A show no toxicity in the liver but it also inhibited hepatic injury induced by Jo2. We have cloned cDNAs encoding HFE7A and have humanized it for possible clinical application. These data will be reported elsewhere.

The finding that HFE7A not only has no hepatic toxicity but also inhibits hepatitis induced by Jo2 in vivo, although it induces apoptosis in thymus, appears somewhat puzzling. This may be related to the difference in environment of the target cells in the thymus and liver. HFE7A did not show apoptotic activity in human T lymphoma in vitro unless cross-linked. HFE7A could be cross-linked in the thymus or lymph node in vivo through Fc receptors expressed in macrophages or dendritic cells. The density of the cells bearing Fc receptors in the liver may be much smaller than that in lymphatic tissues. HFE7A may not be cross-linked in liver and not only may have little hepatotoxic effect, but also may have an antagonistic effect on Fas-mediated apoptosis by abrogating Jo2 binding or conformational changes of the Fas molecule induced by Jo2 including oligomerization of Fas molecule. Although this may explain the tissue-specific apoptosis induction by HFE7A, it does not completely explain the inhibitory effect of HFE7A on Jo2-induced hepatitis because HFE7A did not compete out the binding of Jo2 to murine Fas and Jo2-induced apoptosis in murine Fas-bearing lymphoma in vitro. These data suggest HFE7A may inhibit Jo2-induced hepatitis through a mechanism other than blocking Jo2 binding to Fas.

The structure of the Fas molecule on hepatocytes may be different from that expressed on lymphocytes due to differences in glycosylation, alternative splicing or differences in Fas-associating proteins. It is also reported that the difference in the binding affinity to Fas may lead to a difference in the agonistic or antagonistic characteristics of apoptosis (35,36). The epitopes of RK-8 and HFE7A, which are different from that of Jo2, may be modified in hepatocytes, and this might be implicated in the difference in hepatotoxicity of these anti-Fas mAb. Furthermore, HFE7A may have an antagonistic effect on Jo-2-induced apoptosis in liver due to hepatocyte-specific modification of Fas. Alternatively, signaling through Fas in hepatocytes may not be identical to that in lymphocytes, and this may be involved in the tissue specific biological activity of HFE7A and RK-8 (37–39). In many experimental hepatitis models, activated NK cells or lymphocytes in the liver are implicated in hepatic injury (40,41). HFE7A may induce apoptosis in hepatic NK cells and liver lymphocytes, and this may lead to prevention of hepatocytes from being killed by hepatic NK cells and lymphocytes.

Recently, Takehara and his colleagues reported the hepatoprotective role of TNF against Jo2-mediated hepatocyte apoptosis (42). HFE7A may also inhibit the Jo2-induced apoptosis of hepatocytes by inducing an unidentified survival signal to the hepatocyte, not just by blocking the binding of Jo2 competitively.

In conclusion, we obtained a novel murine-derived anti-human Fas mAb, HFE7A, which cross-reacts with the Fas molecule of various species ranging from human to mouse. This anti-Fas mAb has the opposing effects of inducing apoptosis in lymphocytes and inhibiting Jo2-induced fulminant hepatitis. Administration of HFE7A did not cause any liver damage in mice and marmosets, and it did not induce apoptosis in human primary hepatocytes culture. Furthermore, HFE7A showed curative effects on lymphadenopathy in MRL-gld/gld mice. In addition, HFE7A induced cell death in synovial cells of patients of RA in vivo.

Thus, exploitation of humanized HFE7A may provide a useful therapeutic strategy for the treatment of incurable autoimmune diseases such as RA.

Acknowledgements

We thank Mr Isao Nakayama and Mr Keiichi Fusegawa for their technical support.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>hEGF</td>
<td>human epidermal growth factor</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>GOT</td>
<td>glutamic oxaloacetic transaminase</td>
</tr>
<tr>
<td>GPT</td>
<td>glutamic pyruvic transaminase</td>
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<tr>
<td>HCM</td>
<td>hepatocyte culture medium</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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References

An agonistic anti-human Fas mAb without hepatotoxicity


