Protective effects of HFE7A, mouse anti-human/mouse Fas monoclonal antibody against acute and lethal hepatic injury induced by Jo2

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Abstract HFE7A is a mouse anti-human/mouse Fas monoclonal antibody which, protects mice from fulminant hepatitis induced by Jo2. Herein, we report on the mechanism of the protective effect of HFE7A against Jo2-induced acute and lethal hepatic injury. HFE7A reduced the serum aminotransferase level which was elevated after Jo2 injection. HFE7A also inhibited caspase activation and mitochondrial depolarization in hepatocytes derived from apoptosis induced by Jo2 injection. The protective effect of HFE7A against Jo2-induced apoptosis in mouse hepatocytes was reproducible in vitro. The cell death and caspase activation in isolated mouse hepatocytes were induced by incubating these cells with Jo2 in vitro, and HFE7A inhibited the cell death and caspase activation in mouse hepatocytes in a dose-dependent manner. The affinity of HFE7A to mouse Fas was lower than that of Jo2. The binding of Jo2 to neither recombinant mouse Fas nor mouse hepatocytes was inhibited by an excessive amount of HFE7A. Interestingly, HFE7A

bound to hepatocytes isolated from Fas knockout mice. From these results, it is suggested that HFE7A may exert a protective effect against Jo2-induced hepatitis not by competitively inhibiting the binding of Jo2 to Fas on hepatocytes, and that a distinct molecule other than Fas may possibly be involved in the protective effect of HFE7A against Jo2-induced hepatic injury.

Keywords Fas · HFE7A · Hepatic injury · Jo2 · Apoptosis

Abbreviations

TNF	Tumor necrosis factor
NGF	Nerve growth factor
FasL	Fas ligand
mAb	Monoclonal antibody
HTLV-I	Human T cell leukemia virus type I
RA	Rheumatoid arthritis
SCID	Severe combined immunodeficient
i.v.	Intravenously
i.p.	Intraperitoneally
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactose dehydrogenase
NO	Nitric oxide
DISC	Death inducing signaling complex
MORT	Mediator of receptor-induced toxicity
FADD	Fas-associated protein with death domain
DR	Death receptor
COX	Cyclooxygenase
FcγRII	Fcγ receptor II

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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 (Raff 1992; Ellis et al. 1992; Wyllie et al. 1980). The Fas antigen/Apo-1/CD95 (Fas) molecule is a widely expressed cell surface receptor belonging to the tumor necrosis factor (TNF) receptor/nerve growth factor (NGF) receptor super-family. It can transduce an apoptotic death signal in cells when stimulated by agonistic anti-Fas antibodies or by Fas ligand (FasL) (Yonehara et al. 1989; Trauth et al. 1989; Itoh et al. 1991; Suda et al. 1993; Watanabe-Fukunaga et al. 1992). TNF receptor- and Fas-regulated apoptosis plays an important role in the pathogenesis of immunomediated liver diseases including viral and autoimmune hepatitis, alcoholic liver disease, acute liver failure, and primary biliary cirrhosis (Pinkoski et al. 2000; Canbay et al. 2004). Hepatocytes normally express high levels of Fas, which is believed to be involved in liver cell homeostasis, because Fas-deficient mice develop substantial liver hyperplasia (Adachi et al. 1995).

There have been some reported attempts of the application of anti-Fas antibodies to therapeutic agents against diseases resulting from defects in Fas-mediated apoptosis (Nishimura-Morita et al. 1997; Fujisawa et al. 1996; Nakajima et al. 1995; Firestein et al. 1995; Sakai et al. 1998). The administration of anti-mouse Fas monoclonal antibody (mAb) RK-8 to FasL-deficient MRL*gld/gld* mice reduced the autoimmune symptoms, including those of lymphadenopathy, nephrotic, arthritis and vasculitis (Nishimura-Morita et al. 1997). Intra-articular administration of RK-8 to human T cell leukemia virus type I (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 (Fujisawa et al. 1996). Fas expression was also 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 severe combined

immunodeficient (SCID) mice (Nakajima et al. 1995; Firestein et al. 1995; Sakai et al. 1998). These facts suggest that anti-Fas antibody would be a candidate as a therapeutic agent for autoimmune diseases. On the other hand, administration of Jo2, another hamster anti-mouse Fas antibody, is lethal when administered to mice either intravenously (i.v.) or intraperitoneally (i.p.) (Ogasawara et al. 1993). Administration of Jo2 caused fulminant hepatitis or hemorrhage and death in mice, and Jo2-induced lethality is associated with intensive hepatocyte death and marked elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT; Ogasawara et al. 1993). Injection of a caspase inhibitor protects mice against Jo2-induced apoptosis of hepatocytes and lethality (Rodriguez et al. 1996). These observations suggest that hepatocytes are the primary target and that Jo2-induced death of hepatocytes is the cause of lethality.

We have obtained the unique mouse anti-Fas mAb HFE7A, which reacts with both human and mouse Fas (Ichikawa et al. 2000; Yoshida-Kato et al. 2000). Although administration of HFE7A induced apoptosis in thymocytes, it did not show any signs of hepatotoxicity in mice (Ichikawa et al. 2000). HFE7A showed a therapeutic effect against lymphadenopathy that had developed in MRL*gld/gld* mice and also induced apoptosis in synovial cells from RA patients in vitro (Ichikawa et al. 2000). Interestingly, HFE7A rescued mice from Jo2-induced lethal fulminant hepatitis (Ichikawa et al. 2000). In this study, we investigated the mechanism of the protective effect of HFE7A against Jo2-induced hepatitis and we discovered the possibility that HFE7A may exert a protective effect against Jo2-induced hepatitis by inhibiting apoptosis in hepatocytes without competitively inhibiting the binding of Jo2 to Fas on hepatocytes.

Materials and methods

Animals and antibodies

HFE7A, mouse anti-human/mouse Fas mAb was obtained as mentioned previously (Ichikawa et al. 2000; Yoshida-kato et al. 2000). BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). Fas knockout mice were kindly provided by Dr. Yonehara at Kyoto University. All the mice were

maintained and used in accordance with the guidelines for the care and use of experimental animals of Daiichi Sankyo Co., Ltd. All experiments were approved by the Animal Experimentation Ethics Committee of Daiichi Sankyo Co., Ltd.

Induction of Jo2-hepatitis

BALB/c mice were i.v. injected with 6.7 µg of Jo2, hamster anti-mouse Fas mAb (BD Bioscience, Franklin Lakes, NJ, USA). At 0, 10, 30 or 60 min after the Jo2 injection, 67 µg of HFE7A was additionally i.v. injected. The survival ratio of each group was observed until 24 h after the Jo2 injection.

AST and ALT level in Jo2-treated mice

Twelve-week-old BALB/c female mice were i.v. injected with 6.7 µg of Jo2. At 0, 10 or 60 min after the Jo2 injection, 67 µg of HFE7A was additionally i.v. injected. Serum was collected from each mouse at 6 and 24 h after the Jo2 injection and the AST and ALT levels in the serum were measured as markers of hepatitis.

Isolation of primary hepatocytes

Primary mouse hepatocytes were isolated by a retrograde perfusion method. Briefly, the livers of anesthetized mice were perfused with freshly prepared oxygenated Liver Perfusion Medium (Invitrogen, Carlsbad, CA, USA) with 0.5 mM EGTA via the portal vein for 10 min and then with Hank's balanced salt solution containing 0.05% collagenase I (Boehringer Mannheim, Mannheim, Germany) for 10 min. The perfused livers were removed from the mice and dissociated in ice-cold Hepatocyte Wash Medium (Invitrogen) by agitation. The hepatocytes were washed with ice-cold Hepatocyte Wash Medium 3 times by centrifugation and filtered. The cell viability was consistently >83%, as determined by the trypan blue exclusion method.

Caspase activation in hepatocytes

Six-week-old BALB/c female mice were i.v. injected with 3.3 µg of Jo2 with or without 16.5 µg of HFE7A. At 1, 2, 3 and 4 h after the Jo2 injection, the mice were sacrificed and the primary hepatocytes were isolated by the perfusion method described

above. The activation of caspases-3, -8, and -9 was measured using a Caspase Fluorometric Protease assay kit (MBL, Woburn, MA, USA) according to the manufacturer's instructions. Briefly, 3×10^5 of hepatocytes were resuspended in 50 µL of Cell Lysis buffer and incubated for 10 min on ice. Subsequently, 50 µL of 2× Reaction buffer containing 10 mM DTT and 5 µL of substrate and samples were incubated at 37 °C for 2 h. The fluorescence of each sample was measured at 400 nm excitation and 505 nm emission.

Mitochondria depolarization in hepatocytes

Five-week-old BALB/c female mice were i.v. injected with 10 µg of Jo2 with or without 200 µg of HFE7A. At 30 min after the Jo2 injection, the mice were sacrificed and primary hepatocytes were isolated and incubated with 1 µg/mL of Rhodamine 123 (Invitrogen) at 1×10^6 cells/well in a 24-well plate at 37 °C for 30 min. The cells were washed with PBS twice by centrifugation and resuspended in PBS containing 1 µg/mL of propidium iodide. The samples were analyzed on a FACSCalibur flow cytometer (BD Bioscience) for the Rhodamine uptake. Dead cells stained with propidium iodide were excluded from the analyses.

Apoptosis induction in isolated mouse hepatocytes

Hepatocytes were isolated from 5-week-old BALB/c mice by the perfusion method described above and suspended in Krebs-Hanseleit buffer (118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.9 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.4 mM NaHCO_3) containing 1% BSA. The cells were plated into 24-well plates for suspension culture at 3×10^5 cells/well and incubated with 0.05 µg/mL of Jo2 in the presence of 0.05, 0.5 or 5 µg/mL of HFE7A under conditions of 5% CO_2 and 37 °C. After the appropriate culture period, the hepatocytes and supernatant were collected for the measurement of the caspase activation and lactose dehydrogenase (LDH) release, respectively. The LDH in the culture supernatant was measured using an LDH C II-test kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. Briefly, 0.3 mL of culture supernatant was incubated with 0.5 mL of substrate solution at 37 °C for 10 min. Color development was stopped by adding 5 mL of 0.1 N

HCl and the absorbance at 560 nm was measured using a microplate reader.

Surface plasmon resonance (BIAcore) analysis

The binding affinities of Jo2 and HFE7A for mouse Fas were determined using a BIAcore 3000 apparatus (BIAcore, Uppsala, Sweden). Mouse Fas fused with human IgG Fc portion was obtained as Sf 21 cell-expressed recombinant protein from R&D Systems (Minneapolis, MN, USA). Determinations were conducted at 25 °C using HBS-EP buffer as running buffer. Jo2 or HFE7A were immobilized onto a CM5 chip following the manufacturer's instructions to capture mouse Fas. The mouse Fas was serially diluted from 50 to 1.75 µM in HBS-EP buffer before injection over the surface with the captured Jo2 or HFE7A at 20 µL/min for 2 min. The Jo2 or HFE7A-captured surface was regenerated with 20 µL of 30 mM NaOH or 10 mM glycine, pH 1.5 at 50 µL/min between cycles, respectively. The binding affinities (*KD*) of Jo2 and HFE7A for mouse Fas were determined by nonlinear regression analysis using BIAevaluation 3.1 software (BIAcore).

Competitive ELISA

Flat bottom 96-well microplates (Nunc-Immuno Maxisorp, Nalge Nunc, Roskilde, Denmark) were coated with 1 µg/mL of mouse Fas (R&D Systems) at 4 °C overnight. The plates were washed with PBS-Tween (0.05% Tween 20 in PBS) and incubated with Blocking One (Nacalai tesque, Kyoto, Japan) at 37 °C for 1 h. Then, 0.1 µg/mL of biotin-labeled Jo2 (BD Pharmingen, San Diego, CA, USA) was added to the wells in the presence of Jo2 or HFE7A which were serially diluted from 100 to 0.1 µg/mL. After the plates were incubated at 4 °C overnight and washed, 2000-fold diluted Streptavidin-horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) was added and the plates were incubated at 37 °C for 1 h. A color reaction was developed by exposure to the HRP substrate (Sumitomo Bakelite, Tokyo, Japan) and the absorbance was read at 490 nm with a microplate reader (Berthold, Stuttgart, Germany).

Flow cytometry

Hepatocytes were isolated from 6-week-old female BALB/c mice by the perfusion method described

above. The hepatocytes (5×10^5 cells/sample) were washed with staining buffer (PBS containing 5% FBS and 0.04% NaN₃) and incubated with 20 µg/mL of PE-conjugated Jo2 (BD Pharmingen) with or without 100 µg/mL of unlabelled Jo2 or HFE7A for 20 min on ice. After washing with the staining buffer, the samples were analyzed on a FACSCalibur flow cytometer (BD Bioscience). The data were analyzed using CellQuest Pro 5.2 (BD Bioscience). Dead cells stained with propidium iodide were excluded from the analysis.

Hepatocytes of 8-week-old female Fas knockout mice were similarly prepared. The hepatocytes were incubated with 1 µg/mL of HFE7A for 20 min on ice followed by incubation with FITC-conjugated goat anti-mouse IgG (Organon Teknika, Durham, NC, USA) for 20 min on ice. After washing with the staining buffer, the samples were analyzed on a Cyto ACE-150 flow cytometer (Jasco Corporation, Tokyo, Japan). The data were analyzed using Cyto ACE system program version 3.06 (Jasco Corporation). Dead cells stained with propidium iodide were excluded from the analysis.

Statistical methods

In some experiments, statistical analysis was performed by a *t*-test. *p* values <0.05 indicated statistical significance.

Results

Prevention of Jo2-induced acute and lethal hepatitis by HFE7A

We reported the protective effect of HFE7A against lethal hepatic injury by Jo2 in a previous report where HFE7A prevented Jo2-induced death even when HFE7A was i.p. injected 60 min after the Jo2 i.p. injection (Ichikawa et al. 2000). In this report, we performed a similar examination by an i.v. injection of Jo2 and HFE7A. Whereas all the mice injected with 6.7 µg of Jo2 died within 4 h, administration of HFE7A completely rescued the mice from Jo2-induced death until 24 h after the Jo2 injection, even when it was administered 60 min after the Jo2 injection (Table 1).

Injecting mice with Jo2 caused a time-dependent increase in the AST/ALT plasma level (Ogasawara et al. 1993; Ichikawa et al. 2000). The combinational

Table 1 Protective effects of HFE7A against acute and lethal hepatic injury induced by Jo2

Survival ratio	4 h	24 h
Jo2	0/3	0/3
HFE7A injection after Jo2 injection		
0 min	3/3	3/3
10 min	3/3	3/3
60 min	3/3	3/3

Ten-week-old BALB/c female mice were i.v. injected with 6.7 μ g of Jo2. At 0, 10 or 60 min after the Jo2 injection, 67 μ g of HFE7A was additionally i.v. injected. The survival ratio of each group was observed until 24 h after the Jo2 injection

injection of Jo2 with HFE7A inhibited the increase of the serum AST/ALT level in an HFE7A dose-dependent manner. In particular, 24 h after the Jo2 injection, the AST/ALT level of mice injected with HFE7A even 10 min after the Jo2 injection went down to the basal level (Fig. 1). As mice injected with 6.7 μ g of Jo2 died within 4 h, serum for AST/ALT measurement could not be collected.

Inhibitory effects of HFE7A against Jo2-induced apoptosis in hepatocytes in vivo

To determine whether HFE7A has an inhibitory effect against Jo2-induced apoptosis of hepatocytes, the activation of caspases-3, -8 and -9 of hepatocytes of mice challenged with a sublethal dose of Jo2 was measured. In all the caspases, injection of Jo2 elevated the activation level in a time-dependent

manner and the peak of activation of caspases-8 and -9 was observed at 3 h after the Jo2 injection (Fig. 2a). When HFE7A was simultaneously injected with Jo2, the caspase activation was almost completely suppressed to the basal level (Fig. 2a). HFE7A alone did not induce caspase activation (data not shown).

Hepatocytes are categorized as type II cells in which mitochondrial depolarization and the following cytochrome c release from depolarized mitochondria is observed in the process of apoptosis (Park et al. 2005; Liu et al. 1996; Luo et al. 1998). To investigate mitochondrial depolarization, hepatocytes were isolated from Jo2-injected mice and the mitochondrial membrane potential was measured. When the mitochondrial membrane potential decreased, the uptake of Rhodamine 123 into cells declined. As shown in Fig. 2b, mitochondrial depolarization was observed 60 min after the Jo2 injection, which was inhibited by simultaneous administration of HFE7A.

Inhibitory effects of HFE7A against Jo2-induced apoptosis in hepatocytes in vitro

Next, we investigated whether HFE7A demonstrates protective effects against Jo2-induced apoptosis in isolated hepatocytes in vitro. Hepatocytes which were isolated from BALB/c mice were incubated with Jo2 for 1.5, 3 and 6 h and the LDH release in the culture medium was measured as a marker of cell death. Incubating hepatocytes with Jo2 alone resulted in time-dependent LDH release, about 50% of the hepatocytes had died by 6 h after the Jo2 injection. On the other

Fig. 1 AST and ALT level in the serum after the Jo2 injection with an additional HFE7A injection. Twelve-week-old BALB/c female mice were i.v. injected with 6.7 μ g of Jo2. At 0, 10 and 60 min after the Jo2 injection, 67 μ g of HFE7A was

additionally i.v. injected. At 6 and 24 h after the Jo2 injection, serum was collected from each mouse and the AST and ALT levels were quantified ($n = 2$)

Fig. 2 Effects of HFE7A against Jo2-induced apoptosis in hepatocytes. **a** Caspase activation. Six-week-old BALB/c female mice were i.v. injected with 3.3 μ g of Jo2 with or without 16.5 μ g of HFE7A. At the indicated times thereafter, the animals were sacrificed and the primary hepatocytes were isolated. The activation of caspases-3, -8, and -9 in cell lysate was measured. The experiments were performed in triplicate and the average values are shown by SD (error bars) * $p < 0.05$, ** $p < 0.01$. **b** Mitochondria membrane potential. Five-week-old BALB/c female mice were i.v. injected with

10 μ g of Jo2 with or without 200 μ g of HFE7A. At 30 min after the Jo2 injection, the animals were sacrificed and primary hepatocytes were isolated. The hepatocytes were incubated with Rhodamine 123 for 30 min, and the fluorescence intensity was measured with a flow cytometer. Dead cells stained with propidium iodide were excluded from the analysis. Numbers in the figure indicate the percentage of cells in which the mitochondrial membrane potential decreased. The experiments were performed twice and representative data are shown

hand, LDH release from the hepatocytes treated with Jo2 in the presence of HFE7A was inhibited in an HFE7A dose-dependent manner (Fig. 3a).

Activation of caspases-3, -8 and -9 was also observed in Jo2-treated hepatocytes in vitro. The activation of caspases in the hepatocytes started increasing around 2 h-culture with Jo2 and reached its peak at around 4 h (Fig. 3b). HFE7A inhibited the

activation of caspases in a dose-dependent manner (Fig. 3b).

Affinity of Jo2 and HFE7A to mouse Fas

In order to compare the affinity of Jo2 and HFE7A to mouse Fas, the surface plasmon resonance (BIA core) analysis was performed using recombinant

Fig. 3 Effects of HFE7A against Jo2-induced cytotoxicity in hepatocytes in vitro. Hepatocytes were isolated from 5-week-old female BALB/c mice. The hepatocytes were incubated with 0.05 µg/mL of Jo2 in the presence of 0.05, 0.5, 5 µg/mL of HFE7A at 37 °C. At the indicated times thereafter, **a** LDH

release in culture supernatant and **b** caspase activation were measured. The measurement was performed in triplicate and the average values are shown by SD (error bars) * $p < 0.05$, ** $p < 0.01$

extracellular domain of mouse Fas fused with human IgG Fc. As shown in Table 2, KD of Jo2 and HFE7A were 0.28 and 1.6 nM, respectively, which indicated that affinity of HFE7A to mouse Fas was about 5 times lower than that of Jo2.

HFE7A did not block the binding of Jo2

Next, we investigated whether HFE7A blocks the binding of Jo2 to mouse Fas. First, a competitive

ELISA was performed using recombinant mouse Fas protein which was used in the BIAcore analysis. While the binding activity of biotin-conjugated Jo2 to the extracellular domain of mouse Fas fused with human IgG Fc that was coated to an ELISA plate was blocked by unlabeled Jo2 in a dose-dependent manner, HFE7A did not block the binding of Jo2 to mouse Fas even when a concentration of HFE7A as much as 1,000 times higher existed in the reaction wells (Fig. 4).

Table 2 Affinity of Jo2 and HFE7A to recombinant mouse Fas

	<i>KD</i> (nM)
Jo2	0.28
HFE7A	1.6

Jo2 or HFE7A were immobilized onto a CM5 chip following the manufacturer's instructions to capture mouse Fas. Recombinant mouse Fas was serially diluted from 50 to 1.75 μ M in HBS-EP buffer before injection over the surface with the captured Jo2 or HFE7A at 20 μ L/min for 2 min. The binding affinities (*KD*) of Jo2 and HFE7A for mouse Fas were determined by nonlinear regression analysis using BIAevaluation 3.1 software

Fig. 4 Binding of Jo2 to recombinant mouse Fas was not blocked by HFE7A. Flat bottom 96-well microplates were coated with 1 μ g/mL of mouse Fas at 4 °C overnight. After incubating the plates with Blocking One at 37 °C for 1 h, 0.1 μ g/mL of biotin-labeled Jo2 was added to the wells in the presence of Jo2 or HFE7A which were serially diluted from 100 to 0.1 μ g/mL. After the plates were incubated at 4 °C overnight, 2000-fold diluted Streptavidin-labeled HRP was added and the plates were incubated at 37 °C for 1 h. A color reaction was developed by exposure to the HRP substrate and the absorbance was read at 490 nm with a microplate reader. The assay was performed in triplicate and the average values are shown by SD (error bars) * $p < 0.01$

We had already confirmed by flow cytometric analysis that HFE7A bound to mouse hepatocytes (data not shown). We investigated whether HFE7A inhibited the binding of Jo2 to mouse hepatocytes. Hepatocytes isolated from BALB/c mice were incubated with 20 μ g/mL of PE-conjugated Jo2 in the presence of 100 μ g/mL of unlabeled Jo2 or HFE7A. As shown in Fig. 5, unlabeled Jo2 blocked the

binding of PE-conjugated Jo2 to BALB/c mouse hepatocytes completely. On the other hand, the binding of PE-conjugated Jo2 to BALB/c mouse hepatocytes was not effected by the excess amount of HFE7A (Fig. 5).

HFE7A bound to hepatocytes of Fas knockout mice

From the results shown in Figs. 4 and 5, which indicate that HFE7A did not competitively inhibit the binding of Jo2 to mouse Fas on hepatocytes, it seems possible that HFE7A may have a protective effect on Fas-mediated apoptosis by abrogating conformational changes of the Fas molecule induced by Jo2, including oligomerization of Fas molecule. However, HFE7A did not block Jo2-induced apoptosis in L5178YA1, which is a mouse Fas-bearing lymphoma in vitro (data not shown). It was expected that certain mechanisms other than competitive inhibition over Fas were involved in the protective effect of HFE7A against Jo2-induced hepatic injury. In order to verify the possibility that HFE7A binds not only Fas but also to a distinct molecule on mouse hepatocytes, the binding activity of HFE7A to hepatocytes isolated from Fas knockout mice was determined. As shown in Fig. 6, HFE7A bound to hepatocytes, not to thymocytes, of Fas knockout mice. It was suggested that HFE7A bound to a distinct molecule which was expressed on hepatocytes but not on thymocytes.

Discussion

In the previous study, we reported that HFE7A, mouse anti-human/mouse Fas mAb, had a protective effect against Jo2-induced acute and lethal hepatic injury (Ichikawa et al. 2000). In the present study, we attempted to clarify the mechanism of the protective effect of HFE7A against Jo2-induced hepatitis. It was suggested that HFE7A may exert the protective effect against Jo2-induced hepatitis not by competitively inhibiting the binding of Jo2 to Fas on hepatocytes, and that a distinct molecule other than Fas may possibly be involved in the protective effect.

There are many reports revealing that Fas-associated liver damage and mortality elicited by Jo2 is mediated by a number of separate mechanisms. One of these mechanisms is the direct Fas-mediated

Fig. 5 Binding of Jo2 to hepatocytes was not blocked by HFE7A. Hepatocytes were isolated from 6-week-old female BALB/c mice and incubated with 20 µg/mL of PE-conjugated isotype control antibody (dotted lines) or PE-conjugated Jo2

(solid lines) with or without competitive antibodies (100 µg/mL of unlabeled Jo2 or HFE7A). The fluorescence intensity was measured with a flow cytometer. The experiments were performed twice and representative data are shown

Fig. 6 Binding activity of HFE7A to hepatocytes of Fas knockout mice. Hepatocytes (left histograms) and thymocytes (right histograms) were isolated from 8-week-old female Fas knockout mice, and incubated with (lower histograms) or without (upper histograms) 1 µg/mL of HFE7A for 20 min on ice followed by incubation with FITC-conjugated goat anti-mouse IgG for 20 min on ice. After washing with staining buffer, the fluorescence intensity was measured with a flow cytometer

apoptosis of hepatic parenchymal cells. As evidence, competitive inhibition by soluble Fas against Jo2 binding to cell surface Fas (Matsuki et al. 2002), inhibition of caspase activation by caspase-8 siRNA (Zender et al. 2003) or nitric oxide (NO) derivative of ursodeoxycholic acid (Fiorucci et al. 2001), and inhibition of death inducing signaling complex (DISC) formation by dominant negative mediator of receptor-induced toxicity (MORT)1/Fas-associated protein with death domain (FADD; Schuchmann

et al. 2003) showed resistance to Jo2-induced lethal fulminant hepatitis.

In the present report, it was suggested that HFE7A may possibly have acted directly on hepatocytes in the mechanism of its protective effect against Jo2-induced apoptosis. However, HFE7A did not competitively inhibit the binding of Jo2 to hepatocytes (Fig. 5), suggesting that the protective effect of HFE7A was not the result of simple competitive inhibition against Jo2 binding to Fas on hepatocytes. As evidence, it has been suggested that the epitope of HFE7A on mouse Fas is different from that of Jo2 (Ichikawa et al. 2000). Although HFE7A may possibly abrogate Jo2-induced conformational changes of Fas, such as oligomerization, which is necessary to transduce an apoptotic death signal, it could not explain the inhibitory effect of HFE7A on Jo2-induced hepatitis because HFE7A did not inhibit Jo2-induced apoptosis in mouse Fas-bearing lymphoma in vitro (data not shown). FACS analysis using hepatocytes isolated from Fas knockout mice indicated that HFE7A bound to hepatocytes of Fas knockout mice but not to thymocytes (Fig. 6). These results suggested the possibility that HFE7A displays an inhibitory effect on Jo2-induced hepatic injury via binding a certain molecule other than Fas that is expressed on hepatocytes, such as transducing an anti-apoptotic signal into hepatocytes. Since HFE7A did not show cross reactivity to other TNF receptor family proteins, such as TNFR1, TNFR2, death receptor (DR) 4, DR5, lymphotoxin β receptor and

decoy receptor 3 (data not shown), these receptors may not be the targets of HFE7A in the mechanism of its protective effect on Jo2-induced hepatitis.

Another mechanism of Fas-associated liver damage and mortality elicited by Jo2 is TNF- α -mediated liver injury. Fas stimulation recruits neutrophils and activates Kupffer cells that secrete TNF- α , which aggravates Fas-mediated liver injury (Cazanave et al. 2008; Costelli et al. 2003; Mizuhara et al. 1994; Matsuki et al. 2002). On the other hand, Jodo et al. reported that Jo2 delivers a Fas-mediated apoptosis signal to sinusoidal lining cells prior to hepatic parenchymal cells and this apoptosis signal is dependant on Fc γ RII expression on sinusoidal lining cells. The destruction of sinusoids, which was induced by apoptosis of sinusoidal lining cells and subsequent liver hemorrhage, leads to damage of the hepatic parenchymal cells (Jodo et al. 2003). Since Jo2 induced apoptosis in isolated mouse hepatocytes and HFE7A inhibited an apoptosis in vitro assay system which contained neither infiltrated neutrophils and activated cytotoxic lymphocytes nor Fc γ RII positive sinusoidal lining cells, it is suggested that the hypothetic mechanism of the direct Fas-mediated apoptosis of hepatic parenchymal cells is certainly involved in Jo2-induced fulminant apoptotic liver damage in mice. The possibility still remains that the second and third hypothetic mechanisms via TNF- α and Fc γ RII on sinusoidal lining cells, respectively, might be involved in the protective effect of HFE7A against Jo2-induced liver damage. HFE7A possibly inhibits TNF- α production or its activity through inducing apoptosis in neutrophils or Kupffer cells that are the source of TNF- α , and/or inducing apoptosis of cytotoxic lymphocytes activated by TNF- α . Furthermore, there is a possibility that HFE7A might competitively inhibit the binding of Jo2 to Fc γ RII, resulting in the blockade of Jo2-induced lethality.

Identifying the cell surface molecule(s) that are the target of HFE7A and elucidating the mechanisms of the protective effect of HFE7A against Jo2-induced hepatic injury may provide a useful therapeutic strategy for anti-Fas agonistic reagents.

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References

- Adachi M, Suematsu S, Kondo T, Ogasawara J, Tanaka T, Yoshida N, Nagata S (1995) Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat Genet* 11:294–300
- Canbay A, Friedman S, Gores GJ (2004) Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 39:273–278
- Cazanave S, Vadrot N, Tinel M, Berson A, Letteron P, Larosche I, Descatoire V, Feldmann G, Robin MA, Pessayre D (2008) Ibuprofen administration attenuates serum TNF- α levels, hepatic glutathione depletion, hepatic apoptosis and mouse mortality after Fas stimulation. *Toxicol Appl Pharmacol* 231:336–343
- Costelli P, Aoki P, Zingaro B, Carbo N, Reffo P, Lopez-Soriano FJ, Bonelli G, Argiles JM, Baccino FM (2003) Mice lacking TNF- α receptors 1 and 2 are resistant to death and fulminant liver injury induced by agonistic anti-Fas antibody. *Cell Death Differ* 10:997–1004
- Ellis RE, Yuan J, Horvitz HR (1992) Mechanisms and functions of cell death. *Annu Rev Cell Biol* 7:663–698
- Fiorucci S, Mencarelli A, Palazzetti B, Soldato PD, Morelli A, Ignarro LJ (2001) An NO derivative of ursodeoxycholic acid protects against Fas-mediated liver injury by inhibiting caspase activity. *Proc Natl Acad Sci* 98:2652–2657
- Firestein GS, Yeo M, Zvaifler NJ (1995) Apoptosis in rheumatoid arthritis synovium. *J Clin Invest* 96:1631–1638
- Fujisawa K, Asahara H, Okamoto K, Aono H, Hasunuma T, Kobata T, Iwakura Y, Yonehara S, Sumida T, Nishioka K (1996) Therapeutic effect of the anti-Fas antibody on arthritis in HTLV-1 tax. *J Clin Invest* 98:271–278
- Ichikawa K, Yoshida-Kato H, Ohtsuki M, Ohsumi J, Yamaguchi J, Takahashi S, Tani Y, Watanabe M, Shiraishi A, Nishioka K, Yonehara S, Serizawa N (2000) A novel murine anti-human Fas mAb which mitigates lymphadenopathy without hepatotoxicity. *Int Immunol* 12:555–562
- Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66:233–243
- Jodo S, Kung JT, Xiao S, Chan DV, Kobayashi S, Tateno M, Lafyatis R, Ju ST (2003) Anti-CD95-induced lethality requires radioresistant Fc γ RII⁺ cells. *J Biol Chem* 278:7553–7557
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147–157
- Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94:481–490
- Matsuki Y, Li L, Hsu HC, Yang PA, Zheng R, Edwards CK III, Chaudry IH, Zhang HG, Mountz JD (2002) Soluble Fas gene therapy protects against Fas-mediated apoptosis of

- hepatocytes but not the lethal effects of Fas-induced TNF- α production by Kupffer cells. *Cell Death Differ* 9:626–635
- Mizuhara H, O'Neil E, Seki N, Ogawa T, Kusunoki C, Otsuka K, Satoh S, Niwa M, Senoh H, Fujiwara H (1994) T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J Exp Med* 179:1529–1537
- Nakajima T, Aono H, Hasunuma T, Yamamoto K, Shirai T, Hirohata K, Nishioka K (1995) Apoptosis and functional Fas antigen in rheumatoid arthritis synoviocytes. *Arthritis Rheum* 38:485–491
- Nishimura-Morita Y, Nose M, Inoue T, Yonehara S (1997) Amelioration of systemic autoimmune disease by the stimulation of apoptosis-promoting receptor Fas with anti-Fas mAb. *Int Immunol* 9:1793–1799
- Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S (1993) Lethal effect of the anti-Fas antibody in mice. *Nature* 364:806–809
- Park KT, Mitchell KA, Huang G, Elferink CJ (2005) The aryl hydrocarbon receptor predisposes hepatocytes to Fas-mediated apoptosis. *Mol Pharmacol* 67:612–622
- Pinkoski MJ, Brunner T, Green DR, Lin T (2000) Fas and Fas ligand in gut and liver. *Am J Physiol Gastrointest Liver Physiol* 278:G354–G366
- Raff MC (1992) Social controls on cell survival and cell death. *Nature* 356:397–400
- Rodriguez J, Matsuura K, Ody C, Nagata S, Vassalli P (1996) Systemic injection of a tripeptide inhibits the intracellular activation of CPP32-like proteases in vivo and fully protects mice against Fas-mediated fulminant liver destruction and death. *J Exp Med* 184:2067–2072
- Sakai K, Matsuno H, Morita I, Nezuka T, Tsuji H, Shirai T, Yonehara S, Hasunuma T, Nishioka K (1998) Potential withdrawal of rheumatoid synovium by the induction of apoptosis using a novel in vivo model of rheumatoid arthritis. *Arthritis Rheum* 41:1251–1257
- Schuchmann M, Varfolomeev EE, Hermann F, Rueckert F, Strand D, Koehler H, Strand S, Lohse AW, Wallach D, Galle PR (2003) Dominant negative MORT1/FADD rescues mice from CD95 and TNF-induced liver failure. *Hepatology* 37:129–135
- Suda T, Takahashi T, Golstein P, Nagata S (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75:1169–1178
- Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, Debatin KM, Krammer PH (1989) Monoclonal antibody—mediated tumor regression by induction of apoptosis. *Science* 245:301–305
- Watanabe-Fukunaga R, Brandon CI, Yonehara S, Cropland NG, Jerkins NA, Nagata S (1992) The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J Immunol* 148:1274–1279
- Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251–306
- Yonehara S, Ishii A, Yonehara M (1989) A cell killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J Exp Med* 169:1747–1756
- Yoshida-Kato H, Ichikawa K, Yamaguchi J, Watanabe K, Ohsumi J, Yonehara S, Serizawa N (2000) Cloning and expression of a novel murine anti-human Fas antibody. *Biosci Biotechnol Biochem* 64:1903–1908
- Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F, Kubicka S (2003) Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc Natl Acad Sci* 100:7797–7802