

# mHFE7A, a newly identified monoclonal antibody to Fas, induces apoptosis in human melanoma cells *in vitro* and delays the growth of melanoma xenotransplants

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**Abstract.** The Fas receptor is a potentially valuable therapeutic target in cancer treatment. However, the clinical application of antibodies directed to this target is hindered by their serious side effects *in vivo*, including liver toxicity. One murine monoclonal antibody, mHFE7A, binds both to human Fas and murine Fas, without inducing any obvious side effects. However, the potential therapeutic effects of mHFE7A are unclear in human cancer cells or tumors. Here, we determined whether mHFE7A could induce apoptosis *in vitro*, and assessed its effects on major apoptotic pathways in a human melanoma cell line, MMN9. We also investigated its anti-cancer effects on transplanted melanoma MMN9 tumors in BALB/c nude mice. Treatment of mHFE7A cross-linking with Ig induced cell death similar to CH-11 treatment. Apoptosis induced by mHFE7A was defined by Hoechst 33342 DNA staining and DNA fragmentation assay. Furthermore, mHFE7A-mediated apoptosis was dependent on activation of a caspase signaling cascade involving caspases-8 and -3. Administration of mHFE7A also delayed the growth of melanoma xenografts. Thus, we provide the first evidence that the murine anti-Fas monoclonal antibody, mHFE7A, induces apoptosis of human malignant melanoma cells *in vitro* and is anti-tumorigenic *in vivo*.

## Introduction

The Fas (1) antigen is a type I cell surface receptor that belongs to the tumor necrosis factor (TNF) receptor/nerve growth factor (NGF) receptor superfamily. When the Fas ligand (FasL) or anti-Fas antibody binds to the Fas antigen, the caspase apoptotic pathway is activated, and this cysteine protease plays an essential role in the proteolytic cascade that finally

leads to apoptosis (2-4). On the other hand, mitochondrial apoptotic pathway that depended on caspase-2 is also related to Fas-induced apoptosis (5). Mitochondrial membrane permeabilization reflects alterations in the balance between pro-apoptotic and anti-apoptotic members of the bcl-2 protein family. Upon its release, cytochrome *c* cooperates with Apaf-1 to activate caspase-9, thereby initiating amplification of the effector caspase cascade (6-8).

While monoclonal antibodies against Fas can exert powerful anti-tumor actions against various xenografted human tumors (9), a major limitation in their potential clinical application is the severe, apoptosis-mediated hepatotoxicity they induce *in vivo* (10). A murine monoclonal antibody (mAb) HFE7A (mHFE7A), raised against human Fas, binds to both human and mouse Fas (11,12). Notably, mHFE7A induced apoptosis in thymocytes in mice without any overt signs of hepatotoxicity, which is known to occur with other anti-Fas agents including the hamster anti-murine Fas mAb, JO2 (10). Moreover, administration of mHFE7A to mice prevents hepatotoxicity induced by JO2. These observations suggested that mHFE7A may have possible clinical application as an anti-cancer agent without inducing serious adverse effects. Furthermore, since mHFE7A was found to induce apoptosis in murine T-cell lymphoma cells expressing murine or human Fas (11), it may also have application as a treatment for autoimmune diseases, such as rheumatoid arthritis. However, the potential therapeutic effects of mHFE7A in human malignant cells or tumors are unclear and its anti-proliferative activity also remains to be elucidated.

In this study, we investigated whether mHFE7A could induce apoptosis in a human melanoma cell line, established from oral melanoma, and assessed the involvement of major apoptotic pathways in its cytotoxicity. We demonstrate that mHFE7A-induced apoptosis involves activation of caspase-8 and its downstream effector caspases. Furthermore, administration of mHFE7A delays the growth of xenografted melanoma tumors. These findings suggest that mHFE7A may have potential as a new chemotherapeutic agent.

## Materials and methods

**Cells culture and animals.** We employed a human melanoma cell line, MMN9, which we established from oral mucosa, as

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reported previously (13). Cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin (Gibco) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

Female BALB/c nu/nu mice aged 4-5 weeks were purchased from CLEA Japan (Tokyo, Japan). The mice were housed in specific pathogen-free facilities, and water and food were provided *ad libitum*. All animal experiments were conducted in accordance with Institutional Guidelines for Animal Experiments of Mie University.

**Antibody and agents.** The murine anti-Fas antibody, mHFE7A, was kindly provided by Sankyo, Co., Ltd., (Tokyo, Japan). Goat anti-mouse immunoglobulin (Ig) was purchased from Biosource (Camarillo, CA). As this commercial Ig exhibited cytotoxicity of approximately 10%, we dialyzed it against PBS to eliminate potentially toxic preservation agents. CH-11, the murine anti-Fas antibody used for positive control, was purchased from MBL Co., Ltd., (Nagoya, Japan). Anti-human antibodies to bcl-2, bcl-x<sub>L</sub>, bak (mouse monoclonal, Oncogene, Boston, MA), and bax (mouse monoclonal, Sigma) were used. A mAb to  $\beta$ -actin was used to control for equal protein loading.

Human recombinant IFN $\gamma$  (imunomax- $\gamma$ <sup>®</sup>, Shionogi & Co., Ltd., Osaka, Japan) was dissolved in phosphate-buffered saline (PBS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>, and was added to the culture medium at 100 or 1,000 Japanese Regular units/ml (JRU, one JRU equals approximately 1.5 IU).

**mHFE7A treatment in vitro.** Although mHFE7A is IgG type, differentiated from IgM type CH-11, cross-linking by Ig is needed to induce apoptosis. We compared the following two methods for cross-linking.

**Cross link (CL):** cells were prepared in RPMI culture medium containing 5% FBS at a density of 1x10<sup>5</sup> cells/ml. A 50  $\mu$ l cell suspension (5x10<sup>3</sup> cells) was pre-incubated for 48 h in a 96-well flat-bottomed plate. Subsequently, 50  $\mu$ l of culture medium containing various concentrations of mHFE7A was added (total volume 100  $\mu$ l), and the cells were incubated for 4 h. After discarding the culture medium and washing the plate twice with PBS, 100  $\mu$ l of complete medium with goat anti-mouse Ig mAb (1  $\mu$ g/ml) was added for cross-linking.

**Plate cross link (PCL):** the 96-well flat-bottomed plate was pre-coated with various concentrations of Ig mAb for 24 h, at 4°C. After discarding the Ig mAb and washing the plate twice with PBS, 50  $\mu$ l of culture medium containing various concentrations of mHFE7A was added, and incubated for 4 h. Subsequently, a 50  $\mu$ l cell suspension (5x10<sup>3</sup> cells) was added (total volume 100  $\mu$ l).

**Cytotoxicity assay.** The anti-proliferative effect of mHFE7A was assessed using an MTT assay. Cells were treated with mHFE7A, as described above. After 24 h incubation with mHFE7A, 10  $\mu$ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added, and the cells were further incubated for 3 h. Subsequently, 100  $\mu$ l of 10% SDS solution was added, and the cells were incubated for another 8 h. Absorbance at 540 nm was measured with an EIA reader (Bio-Rad, Hercules, CA). The cell survival ratio was expressed as percentage activity, which was calculated

according to the following formula: % activity = target cell absorbance/control cell absorbance x 100.

**Detection of apoptosis.** Apoptosis induced by mHFE7A was confirmed following two methods: i) DNA staining with Hoechst 33342: after treatment with cross-linked mHFE7A, cells were fixed with 1% glutaraldehyde and assessed by staining with Hoechst 33342 (Sigma). Nuclear morphology was observed with a fluorescence microscope (AX80 T, Olympus, Tokyo, Japan); ii) DNA fragmentation assay: DNA fragmentation was measured by quantitation of cytosolic mono- and oligonucleosome-bound DNA using a Cell Death Detection ELISA kit (Roche Diagnostics GmbH, Penzberg, Germany), according to the manufacturer's instructions. Briefly, the cytosolic fraction (13,000 x g supernatant) isolated from cells following treatment with or without cross-linked mHFE7A for 6, 12, 14 or 18 h were used as an antigen source in a sandwich ELISA which employed a primary anti-histone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. From the absorbance values, the percentage of fragmentation in comparison to controls (untreated MMN9 cells) was calculated according to the following formula:

$$\% \text{ of control} = \frac{(\text{absorbance}_{\text{samples cells}} - \text{absorbance}_{\text{blank}})}{(\text{absorbance}_{\text{control cells}} - \text{absorbance}_{\text{blank}})} \times 100$$

**Measurement of caspase activity.** The specific activities of caspase-8, -9 and -3 were measured by assay using the following colorimetric substrates: N-acetyl-Ile-Glu-Thr-Asp-p-nitroanilide (Ac-IETD-pNA) (Biomol, Plymouth Meeting, PA, USA) for caspase-8, N-acetyl-Ile-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA) (Sigma) for caspase-9, and Ac-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) (Promega, Madison, WI) for caspase-3. Harvested cells treated with cross-linked mHFE7A for 1, 4, 8, 12 and 24 h were washed and resuspended in cell lysis buffer (20 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 5 mM DTT, 0.1% NP-40, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM PMSF). Cells were lysed by freezing and thawing, and cell extracts were collected by centrifugation at 15,000 x g for 20 min. The protein content of the cell extract was determined by the Bradford method using BSA as a standard. A 2-mg/ml cell extract (40  $\mu$ l) was placed in a 96-well plate, following which 32  $\mu$ l of caspase assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS), 10  $\mu$ l of 100 mM DTT, 2  $\mu$ l of DMSO and 2  $\mu$ l of a 10 mM solution of the appropriate caspase substrate were added (total volume 100  $\mu$ l per well). After 4 h incubation at 37°C, absorbance was measured at 405 nm.

**Western blotting.** Whole cell extracts were prepared for Western blotting by suspending the cell pellets in lysis buffer containing 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X, 0.1% SDS, 0.5% sodium deoxycholate, 20  $\mu$ g/ml aprotinin and 20  $\mu$ g/ml leupeptin. After incubation at 4°C for 30 min, the protein content of the supernatant was determined by BCA-200 protein assay (Pierce, Rockford, IL). Protein extracts (8 mg/ml) were boiled in SDS sample buffer

**SPANDIDOS PUBLICATIONS** lucing agents and applied to 10-15% SDS-amide gradient gels. The proteins were electrophoretically transferred to PVDF membranes using Phast System (Amersham Biosciences, Piscataway, NJ). Blotted membranes were blocked with 10% dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature, and then incubated for 1 h in TBS-T solution containing specific antibodies. After incubation, the membranes were extensively washed in TBS-T and incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG. Band detection was carried out using an enhanced chemiluminescence system (ECL; Amersham Biosciences).

**Measuring anti-tumor growth effects of mHFE7A in BALB/c nude mice.** To establish transplantable MMN9 xenograft tumors,  $1 \times 10^7$  MMN9 cells/200  $\mu$ l/mouse were injected subcutaneously into the back of BALB/c nude mice. When calculated tumor volume reached 100-150 mm<sup>3</sup>, the tumor was excised, necrotic tissues were completely removed and the remaining tumor tissue was cut into 2-3 mm sections. These sections were implanted subcutaneously on the back of nude mice. The day on which the tumor volume reached 100-150 mm<sup>3</sup> was defined as day 0. From day 0 to day 4, animals in the mHFE7A group received an intra-tumoral injection of 10  $\mu$ g/50  $\mu$ l/day mHFE7A for q.d., with the control group receiving an injection of 50  $\mu$ l saline alone. Each group comprised six mice. Body weight and tumor volume were measured twice weekly. Tumor volume (V) was calculated as  $V = (a) \times (b)^2/2$  where i) is the width (large diameter) and ii) the length (small diameter) of the tumor in millimeters. The body weight was measured in grams, and the relative body weight (RBW) was calculated as  $RBW = W_x/W_i$ , where  $W_x$  is the mean body weight at any given time and  $W_i$  is the mean initial body weight at the start of treatment.

**Statistical analysis.** All experiments were performed in triplicate and results were statistically analyzed using the Student's t-test. Significance was defined as a calculated p-value of <0.01 or 0.05.

## Results

**Antiproliferative effect of mHFE7A in MMN9 cells.** The MMN9 cells responded to mHFE7A with both cross-linking methods. However, PCL was more sensitive than CL, the 1  $\mu$ g/ml mHFE7A inhibition ratio was 16.1% on PCL and 7.7% on CL. Thus, we used the PCL method for following experiments. MMN9 cells responded to mHFE7A in a dose-dependent manner, with a dose of 1  $\mu$ g/ml resulting in 16.0% growth inhibition and 0.1  $\mu$ g/ml producing 12.5% inhibition in MMN9 growth (Fig. 1). Treatment with mHFE7A alone and Ig alone produced cell death in 0.27 and 1.25% of cells, respectively. The antiproliferative effect of mHFE7A was increased significantly by cross-linking. Based on these results, we used both mHFE7A and Ig at 1  $\mu$ g/ml in subsequent experiments. Compared with our previously report (13) that anti-proliferative effect of 0.1  $\mu$ g/ml CH-11 was 17%, CH-11 with Ig showed 15.7% growth inhibition. Similar MMN9 cell growth inhibition ratio was observed with mHFE7A, CH-11 and the combination of CH-11 with Ig.

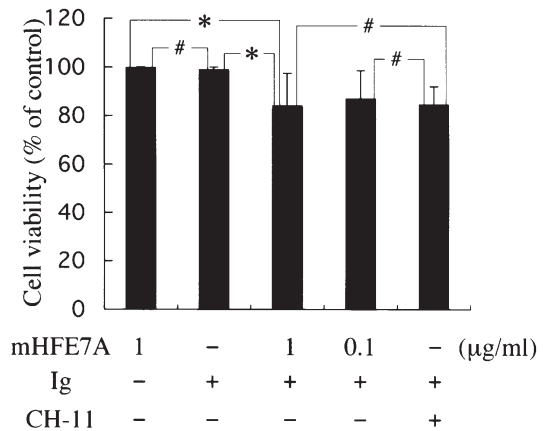


Figure 1. Cell viability of MMN9 cells treated by mHFE7A or CH-11 with or without Ig. The values were expressed as percent of control in which cells were cultured untreated. Values given represent the mean  $\pm$  standard deviation (SD) of three independent experiments. The number of mHFE7A represents the dose of mHFE7A. The dose of Ig and CH-11 is 1.0 and 0.1  $\mu$ g/ml, respectively. #, no significant difference; \*p<0.05, \*\*p<0.01.

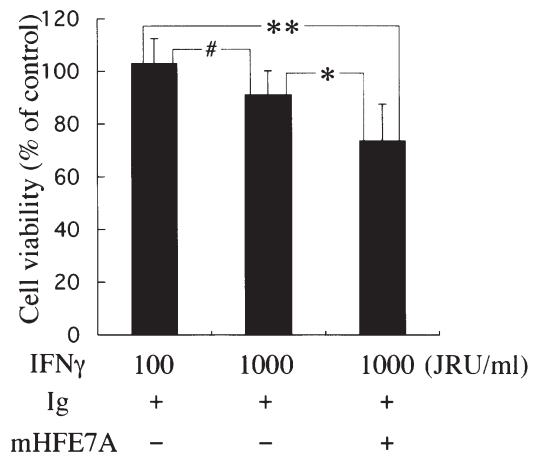


Figure 2. Cell viability of 100 and 1000 JRU/ml IFN $\gamma$ , and combination therapy with IFN $\gamma$  and mHFE7A. The columns represent % activity after an exposure period of 24 h (Ig 1.0  $\mu$ g/ml). Values given represent the mean  $\pm$  SD of three independent experiments. #, no significant difference; \*p<0.05, \*\*p<0.01.

**Combined therapy of MMN9 cells with IFN $\gamma$  and mHFE7A.** Marked synergistic cell growth inhibition effect was observed with treatment of CH-11 and IFN $\gamma$ , we examined whether IFN $\gamma$  enhanced the antiproliferative effect of mHFE7A. While 100 JRU IFN $\gamma$  without Ig induced 35% growth inhibition on MMN9 cells (13), no inhibition was observed on IFN $\gamma$  with Ig. Furthermore, combined therapy with IFN $\gamma$  and CH-11 showed 70% inhibition of growth (13), but combined therapy with IFN $\gamma$  and mHFE7A only 26.4% (Fig. 2). These results indicate that IFN $\gamma$  was inactivated in Ig pre-coated culture plates.

**Detection of apoptosis induced by mHFE7A.** Morphological examination was used to detect apoptotic MMN9 cells, in which fragmented nuclei were observed by Hoechst 33342 staining (Fig. 3).

For more sensitive assessment of apoptosis, DNA fragmentation was measured at 6, 12, 18 and 24 h after treatment with

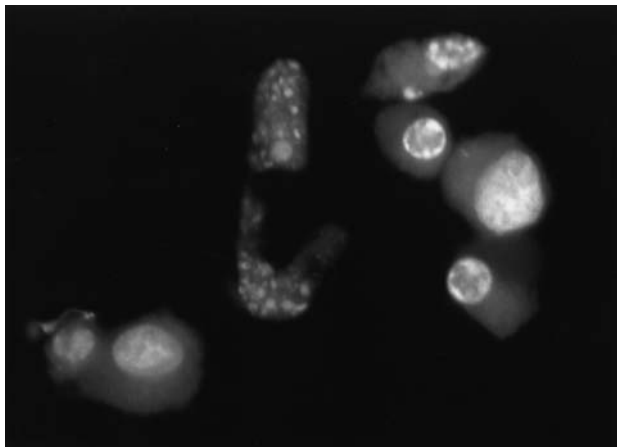


Figure 3. Morphological analysis of MMN9 cells treated with 1.0  $\mu\text{g/ml}$  mHFE7A cross-linked by 1.0  $\mu\text{g/ml}$  Ig for 12 h. Cells were stained with Hoechst 33342 and those with fragmented nuclei were deemed to be apoptotic cells. Original magnification x200.

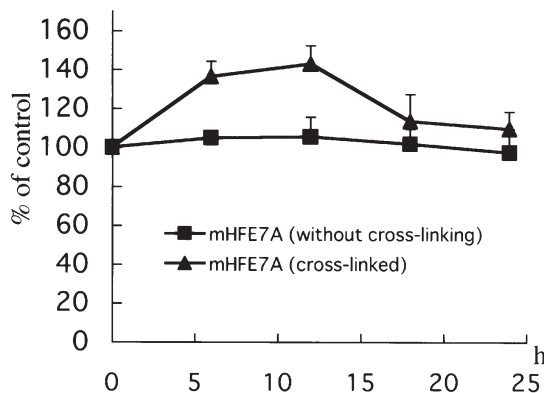


Figure 4. Time course of DNA fragmentation. DNA fragmentation was determined by quantifying the amount of mono- and oligo-nucleosome-bound DNA by ELISA at 6, 12, 18 and 24 h after mHFE7A treatment. SD was <10%.

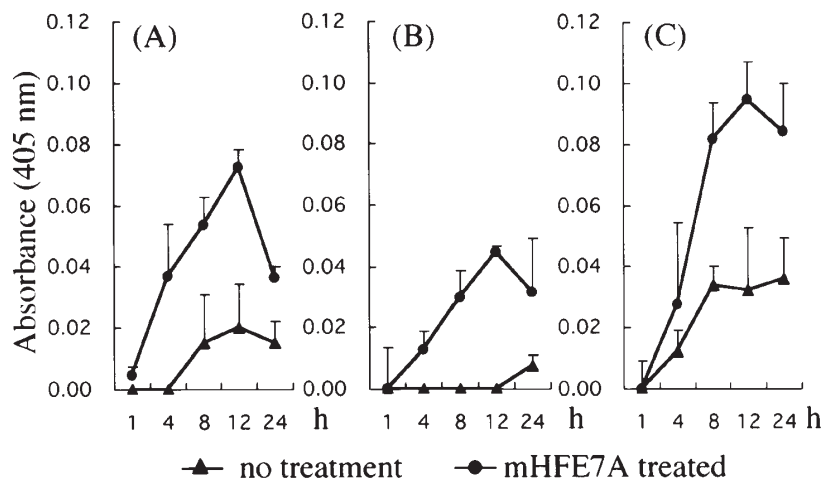


Figure 5. Measurement of caspase-8, -9 and -3 activity in MMN9 cells following treatment with cross-linked mHFE7A. (The relative absorbance values) = (absorbance of mHFE7A treated sample) - (absorbance of control sample). (A), caspase-8; (B), caspase-9; (C), caspase-3.

mHFE7A by quantitation of cytosolic mono- and oligo-nucleosome bound DNA via sandwich ELISA. DNA fragmentation emerged at 6 h after cross-linking, with peak fragmentation of 143% occurring after 12 h (Fig. 4). After 18 h, fragmentation had reduced to 113%, and it was further reduced to 109% at 24 h. No significant cleavage of DNA into oligonucleosomal fragments was detectable in the absence of Ig cross-linking.

*Time-course of caspase activation.* We examined the specific activities of caspase-8, -9 and -3 following induction of apoptosis at time-points 1, 4, 8, 12 and 24 h after treatment with mHFE7A (Fig. 5).

Caspase-8, which is known to be activated in the initial stages of Fas-mediated apoptosis, was elevated after 1 h of treatment, and its activity peaked at 12 h. Caspase-3, which is normally a final activation step in the caspase cascade, was markedly elevated after 8 h of treatment and peaked at 12 h. Caspase-9, which is implicated in an alternative apoptotic pathway was activated up to 12 h after exposure to mHFE7A, although the degree of its activation was significantly smaller than that observed for caspase-8 and caspase-3, and this activity was attenuated by the 24-h time-point. In the absence of cross-linking, mHFE7A still increased caspase-8, -3 and -9 activities, albeit to a lesser extent than was observed with cross-linking.

*Expression of bcl-2 family proteins after treatment with mHFE7A.* To determine the effects of mHFE7A on apoptosis-related oncoprotein levels, we investigated the expression of bcl-2, bcl-x<sub>L</sub>, bax and bak by Western blotting in MMN9 cells following their exposure to Ig cross-linked mHFE7A for 24 h. Although bcl-2 expression was slightly down-regulated, no significant alteration of bcl-x<sub>L</sub>, bak or bax was observed (Fig. 6).

*mHFE7A inhibits melanoma growth in athymic nude mice.* mHFE7A needs cross-linker to transduce the apoptotic signal *in vitro*. However, mHFE7A induced apoptosis in

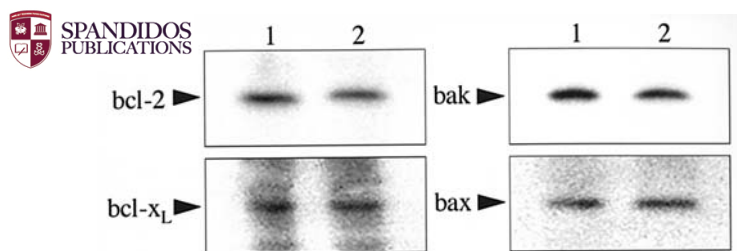


Figure 6. Expression of bcl-2 family members (bcl-2, bcl-x<sub>L</sub>, bak and bax) in MMN9 cells determined by Western blotting. Cells were exposed to mHFE7A (1.0  $\mu$ g/ml) and cross-linked with 1.0  $\mu$ g/ml Ig. Lane 1, untreated cells; lane 2, mHFE7A-treated cells.

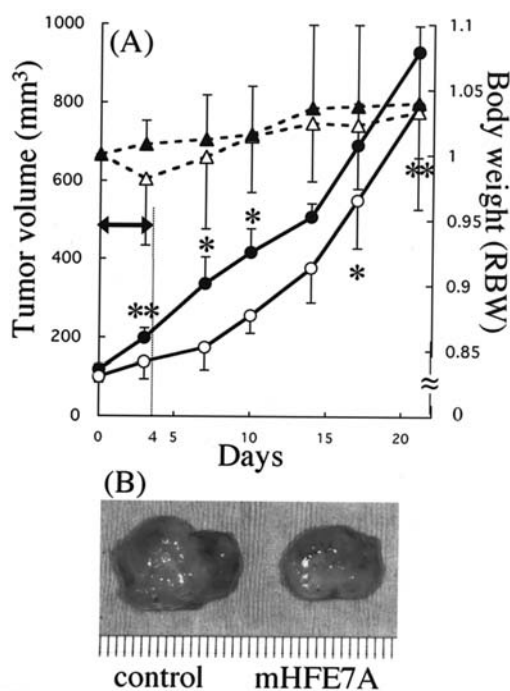


Figure 7. mHFE7A inhibits melanoma growth *in vivo*. (A), The graph shows the progression of mean tumor size (mm<sup>3</sup>, solid line) and the increase of body weight (relative mean ratio, dotted line) from days 0 to 21. Control group, closed circles and triangles; mHFE7A group, open circles and triangles. Values represent the mean of each group, which comprised six mice,  $\pm$ SD. \* $p$ <0.05; \*\* $p$ <0.01. (B), Photographs of enucleated MMN9 tumors were taken on day 21. Treatment with mHFE7A delayed tumor growth, and larger tumors had developed when mice were left untreated. All mice within one group showed similar responses although one representative example of each group is shown here.

BALB/c mouse without any cross linker. Then we examined whether mHFE7A induced anti-proliferative effect against xenografted MMN9 tumors in BALB/c-nu/nu mouse. The MMN9 tumor volume reached 100-150 mm<sup>3</sup> approximately two weeks after innoculation. Upon reaching this volume threshold, anti-melanoma mHFE7A treatment was initiated. In a group of six animals, mHFE7A significantly delayed tumor growth for a period of seven days (Fig. 7) and after that time-point, the growth curves paralleled each other. On day 21, the mHFE7A-treated group exhibited a significantly smaller tumor volume (20.5%) than the control group. Although the body weight was decreased during mHFE7A treatment, no significant differences were found between mHFE7A-treated and control group. Furthermore, no

abnormal serological findings were recorded, hepatotoxicity was observed on day 21 (data not shown).

## Discussion

The Fas receptor is a potentially valuable therapeutic target for autoimmune diseases and cancer. However, the clinical application of anti-Fas antibodies is hindered by the serious side effects they induce *in vivo*, including liver toxicity. In this study, we used a murine anti-human Fas mAb, mHFE7A, that binds not only to human Fas but also to murine Fas, without inducing any overt side effects (10,11). Although other anti-Fas-antibodies have been screened in various cancer cell lines, the potential chemotherapeutic activity of mHFE7A has not been tested in human malignant cells or tumors. In the present study, we demonstrate the anti-tumor activity of mHFE7A *in vitro* and *in vivo*, using the human melanoma cell line, MMN9, as a model.

It is reported that mHFE7A which is IgG mAb required cross-linking by anti-mouse goat Ig to induce apoptosis on target cells *in vitro*. On the contrary, in the absence of a cross-linker mHFE7A did not induce cell death (11). Anti-mouse Ig might bind to Fc portion of mHFE7A and act as a cross-linker of mHFE7A bound Fas on target cells. It is assumed that binding of the cross-linked form of mHFE7A, which is an anti-Fas mAb of the IgG1 subclass, induces Fas receptor oligomerization followed by transduction of a cytolytic signal into the cell and apoptosis *in vitro* (11). On the other hand, mHFE7A can induce apoptosis without cross-linker *in vivo*, because Fc $\gamma$  receptor (Fc $\gamma$ R) is assumed to act as a cross-linker. It is hypothesized that inflammatory macrophages or neutrophils probably work as Fc $\gamma$ R positive cells to decrease the number of inflammatory cells in human rheumatoid arthritis/SCID mouse chimera (14).

The antiproliferative effect of mHFE7A with Ig cross-linker was almost equal to the effect of CH-11. The cell death caused by mHFE7A treatment was apoptosis indicating nuclear staining and DNA fragmentation assay. The level of DNA fragmentation increased gradually up to 12 h of exposure and declined after 24 h, with no DNA fragmentation being detected without cross-linking. These results verified that a cross-linker Ig is required for efficient induction of apoptosis by mHFE7A *in vitro*.

Since caspase activation occurs as a result of initiation of the Fas receptor-mediated cell death process, the activities of caspases up-regulated in response to mHFE7A binding to Fas were investigated. In addition, the major signaling pathways implicated in mHFE7A-mediated apoptosis were clarified. Binding of cross-linked mHFE7A to Fas induced an initial activation of caspase-8 at 1 h, followed by caspase-3 activation after 4 h of treatment. These results suggested that mHFE7A-mediated apoptosis is dependent on a caspase cascade involving caspase-8 and caspase-3. On the contrary, caspase-9 activity was low, suggesting that a little of mitochondrial membrane disruption pathway is activated on mHFE7A-mediated apoptosis. Furthermore, caspase-8, -3 and -9 activities were all significantly lower in the absence of cross-linking.

Bcl-2 family members are known to modulate apoptosis in response to activation of cell surface death receptors. High expression of bcl-2 or bcl-x<sub>L</sub> protects cells from Fas-induced

apoptotic cell death (15-17), while the bak and bax proteins play pro-apoptotic roles (18-20) in Fas-mediated apoptosis. In human melanoma cells, the bax/bcl-2 ratio determined the susceptibility to CD95/Fas-mediated apoptosis (20). Thus, we investigated alterations in expression of several bcl-2 family proteins (bcl-2, bcl-x<sub>L</sub>, bak and bax) after exposure to cross-linked mHFE7A by Western blotting. Notably, mHFE7A treatment reduced bcl-2 expression, but resulted in no significant change in the expression of other bcl-2 family proteins. The result of bax/bcl-2 ratio and caspase-9 examination suggests that limited mitochondrial apoptotic pathway is activated on mHFE7A treatment.

We previously reported that in MMN9 cells grown *in vitro*, a pronounced synergistic antiproliferative effect is observed following combined exposure to IFN $\gamma$  and CH-11 compared with their individual effects (13). On the other hand, IFN $\gamma$  is known to sensitize melanoma (21), multiple myeloma (22), cholangiocarcinoma (23) and other tumor cells (24,25) to Fas-induced apoptosis *in vitro*. These reports, including ours, defined the marked synergistic mechanism that IFN $\gamma$  enhanced the expression of cell surface Fas antigen. Thus, we investigated the combined effect of IFN $\gamma$  and mHFE7A after confirming the expression of Fas on the cell surface *in vitro*. However, IFN $\gamma$  treatment with Ig drastically reduced the growth inhibition effect in MMN9 cells. While 100 JRU IFN $\gamma$  without Ig induced 35% growth inhibition of MMN9 cells (13), no inhibition was observed with IFN $\gamma$  and Ig. Further investigation will be needed concerning whether IFN $\gamma$  is inactivated or neutralized by Ig.

Various studies reporting anti-cancer effects of anti-Fas antibodies *in vitro* have suggested their potential anti-tumorigenic activity *in vivo*. However, only a few *in vivo* experimental chemotherapy studies have been reported. Decaudin *et al* (9), reported that when CH-11 is injected into nude mice bearing human osteosarcoma, neuroblastoma, prostatic cancer and glioblastoma cells, significant inhibition of the growth of the former three cell types are recognized, but no efficacy is observed on glioblastoma, with a relationship between *in vitro* and *in vivo* sensitivity to CH-11. Our recent results using CH-11 *in vivo* showed no efficacy on MMN9 tumor (26). The dose of CH-11 in both studies was in total 10  $\mu$ g per mouse, in one, two, or four intratumor, subcutaneous, or intravenous injections. Hence, we investigated the anti-tumor effects of mHFE7A on transplantable MMN9 melanoma tumors in nude mice. Administration of mHFE7A, 10  $\mu$ g per mouse for 5 days, delayed the growth of these xenotransplant tumors but, importantly, no overt signs of adverse effects were observed in the normal organ tissue. This suggests that mHFE7A may induce an anti-tumor action *in vivo* without producing serious side effects. While here we employed a total dose of 50  $\mu$ g mHFE7A, another report in which injection of 500  $\mu$ g mHFE7A i.p. to BALB/c mice or 2.0 mg i.v. to marmosets was used also revealed no evidence of hepatic injury (11). This suggests that the dose of mHFE7A could be increased safely. Increasing either the dose or duration of HFE7A administration may result in stronger or more prolonged inhibition of tumor growth.

Humanization of murine antibodies has been adopted as one approach for overcoming their clinical limitations (27),

and humanization of HFE7A has already been performed (28). Thus, further investigations of HFE7A as a potential anti-cancer therapy are certainly warranted.

In conclusion, we provide the first unequivocal evidence that the murine anti-Fas mAb, mHFE7A, induces apoptosis in human malignant melanoma cells *in vitro*. Similar to apoptosis mediated by other anti-FasL, mHFE7A-induced apoptosis was dependent on the major caspase pathway and involves sequential activation of caspases-8 and -3. Intratumoral injection of mHFE7A delayed the growth of melanoma xenotransplants *in vivo*, without exhibiting any overt signs of hepatotoxicity. Although the anti-tumor effects of mHFE7A were not particularly strong, either *in vitro* or *in vivo*, increasing the dose of mHFE7A or using it in combination with other agents may produce more powerful anti-tumorigenic effects. Overall, HFE7A may provide a promising strategy for the treatment of melanoma.

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