Article

Combination of Human Fas (CD95/Apo-1) Ligand with Adriamycin Significantly Enhances the Efficacy of Antitumor Response

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The prognosis of hepatocellular carcinoma (HCC) is poor, even with the combined treatment of curative resection and adjuvant chemoradiotherapy. To solve this problem, many biologic therapies have been investigated. Fas ligand (FasL, CD95L) is mainly expressed in activated T lymphocytes and natural killer (NK) cells, and plays a central role in both cell-mediated immunity and immune downregulation. Several studies have shown that FasL is expressed in HCC. In the present report, we prepared recombinant human pET-22b(+)/FasL protein and investigated the effect of FasL on HCC cells *in vitro* and on tumor growth in a murine HCC tumor model. The well-known cytotoxic chemotherapeutic reagent adriamycin (ADM) served as a control. We found that FasL effectively suppressed the viability of H22 tumor cells and significantly induced the apoptosis of H22 cells. The apoptotic levels of cells treated with FasL-ADM were significantly higher than those treated with FasL or ADM alone, and the FasL-ADM combination resulted in a more than additive effect on tumor growth delay in this model. The results suggested that combined treatment of FasL and other chemotherapeutic agents may be a new approach to improve the efficacy of chemotherapy for HCC. *Cellular & Molecular Immunology*. 2009;6(3):167-174.

Key Words: hepatocellular carcinoma, combination therapy, FasL, ADM, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is an aggressive and rapidly fatal malignancy representing the fifth most common cancer worldwide, and has been ranked the second most common cancer killer in China since the 1990s (1, 2). Despite many advances have been made in the clinical study of HCC and the achievement of long-term survival of patients in some clinical centers, only a definitive subset of cases is cured by surgery, and the overall dismal outcome of patients with HCC has not changed (3). Hence, exploration of more effective and safer therapeutic modalities is needed.

FasL (CD95 ligand) is a member of the tumor necrosis factor (TNF) superfamily of cytokines. Similar to other members of the TNF superfamily, FasL is synthesized as a 40-kDa type 2 transmembrane protein and acts in a juxtacrine manner (4, 5). This protein is expressed by activated T lymphocytes (6) and natural killer (NK) cells (7) and by a small number of non-immune cells, mainly from immune-privileged tissues such as testis (8), cornea (9), trophoblasts (10), and cancer cells (11, 12). By engaging its receptor (Fas), membrane-bound FasL induces apoptosis in the target cells. In this manner, FasL plays a central role in both cell-mediated immunity and immune downregulation (13, 14).

FasL can be cleaved by several matrix metalloproteinases, resulting in a soluble form of FasL (sFasL) (15-17). In this process, sFasL is not cytotoxic, and it consists of the largest part of the extracellular domain; it can even competitively bind to its receptor with membrane-bound FasL. After cross-linking, the pro-apoptotic property of sFasL is restored. Several studies have shown that FasL expression is implicated in HCC and is enhanced in regions with infiltrating inflammatory cells on the margins of the cancerous tissue (18-20). *In vitro* studies have revealed that the capacity of naturally processed sFasL to induce apoptosis is less potent than that of cell surface FasL, possibly due to decreased capability to cross-link Fas (21, 22). On the other hand, *in vivo* studies have shown that injection of sFasL can induce tumor cell apoptosis and hepatocyte apoptosis, which

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cause liver failure (23, 24). Furthermore, sFasL has been implicated in patients with lymphocytic leukemia and NK cell lymphomas (25). Therefore, FasL is important in the regulation of apoptosis in hepatocytes and plays a role in the pathogenesis of liver diseases, including liver injury, hepatitis, cirrhosis, and HCC.

In the present study, we produced biologically active, human sFasL and investigated the effect of FasL on HCC cells and tumor growth in a murine HCC tumor model. We also demonstrated the antitumor effect of the combination of FasL with adriamycin (ADM) (FasL-ADM) in the murine HCC tumor model.

Materials and Methods

Mice

All the experiments were performed by using 6-8 weeks female BALB/c mice purchased from Model Animal Research Center of Medical College Xiamen University. All the animals were housed under specific pathogen free conditions with constant access to water and chow. Mice were allowed acclimatize for at least one week before experiments. Briefly, all experimental procedures were carried out following approval of the Institutional Animal Care Committee.

Cell culture

The mouse hepatocellular carcinoma cell line, H22, was maintained in our laboratory. Cells were cultured in flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C in an humidified atmosphere of 5% CO_2. To facilitate attachment and growth the culture medium was changed 4 h later; then the culture medium change was performed every 2-4 days. After immediately isolation, the viable hepatocytes were at least 90% to 95%.

Reagents

DMEM, FBS and penicillin-streptomycin were purchased from Hyclone Corporation, Utah, USA. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), anti-rabbit FasL mAb, and dimethyl sulphoxide (DMSO) were purchased from Sigma Corporation, USA. TUNEL reagent kit and hematoxylin-and-eosin (H&E) assay kit were purchased from Chemicon International, Inc (Temecula, CA, USA).

Recombinant construction, expression and purification of human FasL

The full-length nucleotide sequences of FasL were obtained by overlapping PCR; the restriction sites, including plasmid *Nco* I and *Xho* I were added. The obtained DNA sequence of FasL and the plasmid *Nco* I and *Xho* I sequences were digested by restriction enzymes *Nco* I and *Xho* I separately, and the digested products were purified and recovered by the DNA gel extraction kit. The obtained full-length FasL gene was then cloned into the plasmid pET-22b(+) by T4 DNA ligase. This recombinant plasmid pET-22b(+)/FasL DNA for rmFasL expression was transformed into *Escherichia coli* strain *Rosseta-gami.* The positive colony was selected and cultured at 37°C in 3 ml Luria-Bertani broth (LB medium) for 8-12 h. For large-scale preparation, a 3-ml aliquot of the overnight culture was added to 300 ml of fresh LB medium, and *E. coli Rosseta-gami* cells were grown in 1-L shaker flasks at 37°C and shaken at 250 rpm. After 7 h, when the cell density reached OD_{590} = 0.6-0.8, the bacteria were harvested and isopropyl-β-D-thiogalactoside (IPTG) was added to 300 ml of LB medium to induce expression. The harvested *E. coli Rosseta-gami* cells from shaker flasks were disintegrated with ultrasonication (300 W, 20 min).

The inclusion bodies of *E. coli Rosseta-gami* were then precipitated by centrifugation (12,000 rpm, 20 min). The target protein was mainly present in inclusion bodies. The inclusion bodies were washed at 4°C in a washing fluid containing 2 mol/L urea. After washing for 7-8 h, the inclusion bodies were dissolved in a solution containing 8 mol/L urea. The solution was centrifuged, and the supernatant was applied to a Ni-NTA affinity column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to purify the protein. After loading and eluting, the targeted fractions were pooled and identified by Tricine SDS-PAGE. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA).

FasL protein characterization assay by ELISA

The protocol of FasL ELISA is as follows: 96-well polystyrene microtiter plates (Immuno II; Dynatech, South Windham, ME, USA) were coated overnight with the purified pET-22b(+)/FasL in 0.05 M $NaHCO_3$ buffer (100 μl/well) at 4°C. After washing with PBS containing 0.1% Tween 20, the plates were blocked with BSA (10 g/L, 200 μl/well) for 1 hour at 37°C. Anti-rabbit FasL monoclonal antibodies (mAbs) were then added at a concentration of 100 μl/well and incubated for 1 h at 37°C. The plates were then washed and reacted with horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ anti-rabbit IgG (1:500 dilution) (100 μg/L, 200 μl/well) for 30 min at 37°C.

After additional washes, a freshly prepared color development mixture (0.5 mg/ml enzyme substrate *o*-phenylenediamine, Sigma Corporation, USA, dissolved in 0.1 M citrate/phosphate buffer, pH 5.5, containing H_2O_2) was added to the plates (100 μl/well). After 15 min of incubation in the dark at 37°C, the reaction was stopped by adding 2 mol/l H_2SO_4 (50 μl/well). The absorbance at 490 nm (A_{490}) was measured with Attomole Luminometer. Negative and blank controls were included in each test. The P/N value was calculated according to the following formula: P/N value = (A value of the examined sample minus A value of the blank control)/(A value of the negative control minus A value of the blank control). The sample was judged as positive only if the P/N value was more than 2.1.

Cytotoxic effects of FasL and ADM on H22 cells

Cell proliferation was measured by a colorimetric assay

utilizing MTT. In brief, the H22 cells were seeded in 96-well plates in triplicate at 5×10^3 cells/well and incubated in culture medium overnight. Then the cells were treated with serial dilutions of FasL (0.25, 0.50, 1.0, 2.0, 3.0 mg/ml), ADM (1.0, 2.5, 5.0, 10.0, 15.0 mg/ml) respectively in a total volume of 0.2 ml each well for 12 h. The H22 cells treated with equal amount of normal DMEM instead of drugs served as the control group. Thereafter, 20 μl of the indicator dye MTT solution (5 mg/ml) was added to each well and cultures were continued for 48 h at 37°C, 5% CO_2. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved with 0.15 ml of dimethyl sulphoxide (DMSO) and then the optical density (OD) value A490 was measured by the multiscanner autoreader (Dynatech MR 5000, Chantilly, VA). The following formula was used: cell proliferation inhibited (%) = [1-(OD of the experimental samples/OD of the control) × 100%].

Detection of apoptosis by flow cytometric analysis

The H22 cells were collected and incubated in 24-well plates in triplicate at 5×10^5 cells/well. As mentioned in MTT analysis, the cells were treated with a serial of dilutions of FasL and ADM respectively. The cells treated with equal amount of normal DMEM instead of drugs served as the control group. Twelve hours later, the treated cells were washed with phosphate buffer saline (PBS) and resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of 1×10^6 cells/ml. After incubation, 195 μl of the solution was transferred to a 5 ml culture tube with 5 μl annexin V-FITC added. The tube was then incubated for 30 min at room temperature in the dark. The cells were washed with binding buffer and resuspended in 190 μl binding buffer, with 10 μl propidium iodide (PI) added. Finally, the tube was gently vortexed and incubated for another 30 min in the dark, and the stained cells were analyzed by FACSCalibur (Becton Dickinson, USA) with CellQuest software within 1 h.

In vivo animal tumor model experiment

Tumors were generated in male BALB/c mice by intramuscular *(i.m.)* injection of H22 cells (1.0×10^5 cells in 100 μl PBS) into the right hind leg of each mouse. Tumor measurements were converted to tumor volume (V) by the formula ($L \times W^2 \times 0.52$), where L and W are the length and width, respectively. Measurements were made with a vernier caliper. All tumor-bearing mice were divided randomly into groups (6 mice/group). The treatments were initiated on day 4 when tumor volume reached about 40-50 mm^3 (designated as day 0) and were performed once every two days for two weeks. The drugs were injected into mice subcutanesly (*s.c.*). One group was selected as the control with sham-exposed, and the others were treated with FasL, ADM, and FasL-ADM. Mice were monitored daily after injection for clinical signs and survival.

H&E staining analysis

To distinguish antitumor effect of FasL *in vivo*, H&E staining was used. Tumor and liver tissues were harvested after mice died (n = 6/group). The H&E stained tissues were examined for necrotic cells, tumor cells, or apoptotic cells. H&E-stained sections were viewed using an Olympus BHT microscope (Melville, NY, USA).

TUNEL assay

To detect apoptosis *in vivo* treatments, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using the Apop Tag *in situ* apoptosis detection kit as described previously (26). This method can detect fragmented DNA ends of apoptotic cells. Necrotic or injured cells are defined by the only uptake of PI (indicating damage to the cell membrane) and the lack of nuclear condensation and fragmentation with the DNA ends of dUTPs. The apoptotic cells are defined by the uptake of PI (red) and FITC (green) (indicating the damage to the cell membrane) and the presence of clear nuclear condensation and/or fragmentation with DNA ends of dUTPs. After TUNEL staining, any of the above two categories could be TUNEL positive (yellow) or TUNEL negative. These are admittedly narrow yet very powerful operational definitions of the complex processes of apoptosis and necrosis. They are well suited for a high throughput quantitation of apoptosis in tissues that is very reproducible and accurate. Briefly, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. The sections were treated with 20 μg/ml of proteinase K in distilled water for 10 min at room temperature. The tumor and liver tissues were fixed in 1% paraformaldehyde for 10 min. To block endogenous peroxidase, the slides were incubated in methanol containing 0.3% hydrogen peroxide for 20 min. The remaining procedures were performed according to the instructions provided by the manufacturer. For quantification of apoptosis, five microscopic fields were randomly selected at high power magnification (200× or 400×) and the average counts of TUNEL-positive cells were calculated.

Statistical analysis

Data were presented as mean ± SD. The significance of the difference between the groups was assessed by Student's two-tailed *t*-test. Probability value of less than 0.05 was considered significant. All means were calculated from at least three independent experiments.

Results

Gene construction, expression and purification of FasL

The full-length FasL gene was obtained from 12 long primer segments by the fourth round of PCR. PCR cycle conditions were as follows: one cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 58°C for 45 s, and 72°C for 1 min. The reactions were terminated for 10 min at 72°C. The PCR products were visualized by UV illumination after electrophoresis on a 1% agarose gel. The lengths of the resulting bands were consistent with the theoretical values

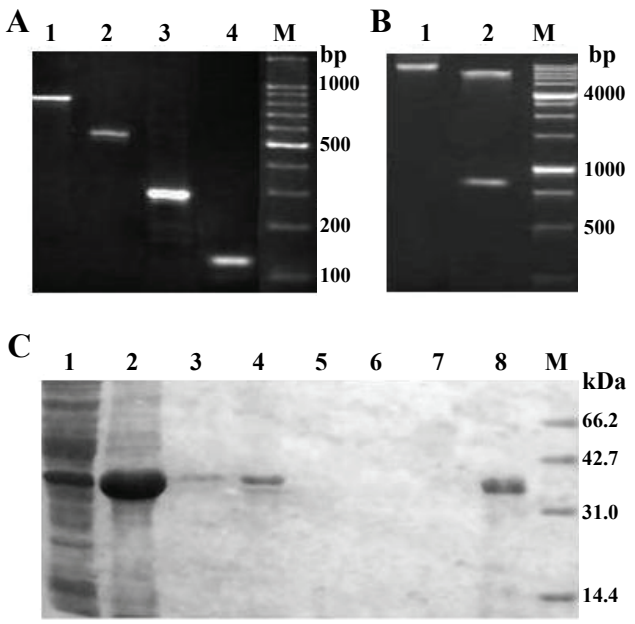

Figure 1. Gene construction, expression and purification of FasL. (A) PCR products were purified by gel electrophoresis. F12, F10, and F7 were the primer segments. Lane M, DNA markers; Lane 1, F12 (861 bp); Lane 2, F10 (585 bp); Lane 3, F7 (304 bp). (B) Confirmation of FasL amplification by restriction digestion. Recombinant pET22b(+)/FasL plasmid was digested by *Nco* I and *Xho* I. Lane M, DNA markers; Lane 1, recombinant pET22b(+)/FasL plasmid; Lane 2, *Nco* I and *Xho* I restriction fragment pET22b(+)/FasL plasmid. (C) SDS-PAGE analysis of FasL expression and purification. Lane 1, supernatant; Lane 2, sediment; Lane 3, column liquid; Lane 4, binding buffer; Lanes 5, 6, and 7, wash buffer; Lane 8, eluting buffer; Lane M, protein markers.

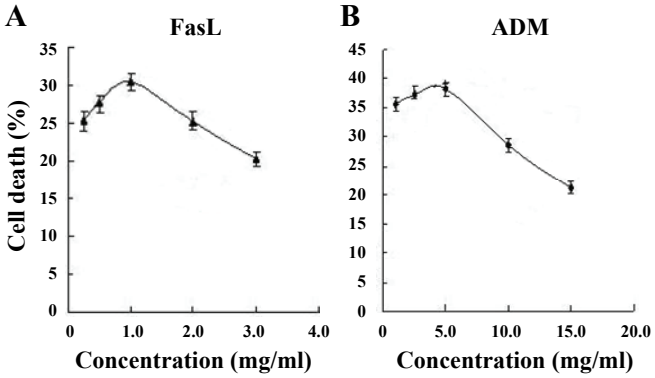

Figure 2. **FasL and ADM induced apoptosis of H22 cells.** Cell proliferation was measured by a colorimetric assay using MTT. (A) FasL in concentrations ranging from 0.25 mg/ml to 3.0 mg/ml was added into cell culture and incubated for 12 h; the optimal sensitivity was reached at 1.0 mg/ml. (B) ADM used here was in the concentrations ranging from 1.0 mg/ml to 15.0 mg/ml, and incubated H22 cells for 12 h.

(Figure 1).

E. coli Rosseta-gami cells expressing rhFasL were subjected to ultrasonication and urea dissolution, and the target protein was concentrated in a small buffer volume, with a significant removal of contaminants. In the ion exchange purification step, rhFasL was eluted as a single homogenous protein at 100 mM NaCl. After the final step, the desired level of product purity (> 98%) was achieved. On tricine SDS-PAGE, the mobility of the purified protein was found to correspond to a molecular mass of 40 kDa (Figure 1C). The purity of the protein was approximately 95% as determined by scan analysis. Then we examined the binding activity of the purified FasL by ELISA, and the P/N values were 3.43, 3.39, and 3.28, all of which were more than 2.1. Thus, FasL was successfully expressed and purified.

FasL and ADM induced cell death

The sensitivities of H22 cells to FasL and ADM induced cell death were statistically different and depended on the concentration. A dose-response experiment was performed with FasL concentrations ranging from 0.25 to 3.0 mg/ml. It was found that the optimal sensitivity was reached at 1.0 mg/ml (Figure 2A). ADM was tested using concentrations of 1.0-15.0 mg/ml; ADM was optimal at 5.0 mg/ml (Figure 2B).

Flow cytometry analysis of cell apoptosis

To investigate whether FasL induces apoptosis in H22 cells, we performed double-staining flow cytometry with annexin V and PI. As shown in Figure 3, after treatment with 0.5, 1.0, or 2.0 mg/ml FasL, the percentage of H22 cells positive for annexin V and PI were 22.4%, 23.6%, and 23.9%; while the percentage of H22 cells positive for annexin V but negative for PI were 0.04%, 0.35%, and 0.43% respectively. After treatment with 2.5, 5.0, or 10.0 mg/ml ADM, the percentage of H22 cells positive for annexin V and PI were 13.1%, 23.2%, 0.16%, respectively; while the percentage of H22 cells positive for annexin V but negative for PI were 0.62%, 0.01%, and 0.00%, respectively. Thus, H22 cells were significantly inhibited by FasL and ADM.

FasL and ADM suppress the growth of H22 in mice

We constructed mice tumor model to determine the effects of FasL, ADM, and FasL-ADM on tumor development and mortality in mice. Three groups of mice were treated with 0.02 mg FasL, 0.5 mg ADM, or a combination of 0.01 mg FasL and 0.25 mg ADM in a total amount of 0.1 ml. A control group was treated with 0.1 ml saline. Two weeks later, mice were euthanized by CO_2 inhalation, and the livers and tumors were removed, weighed, and divided onto 1-2 mm slices. The slices were apportioned for subsequent H&E staining and TUNEL assay. Tumor volume and weight were suppressed by FasL and ADM as shown in Figure 4A. Body weights of the mice were also remarkably suppressed as shown in Figure 4B. The combination of FasL and ADM was the most effective in suppressing the growth of H22 tumors.

The effect of FasL and ADM on tumor tissues and the livers of mice analyzed by H&E

We have found that FasL can prolong mice survive (data not

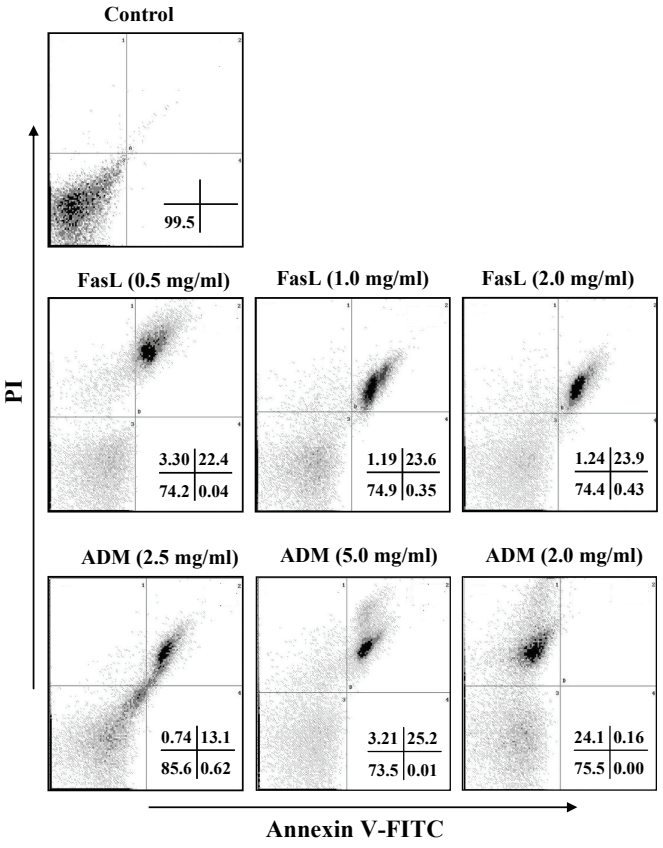

Figure 3. Flow cytometry analysis of cell apoptosis of H22 cells treated with FasL or ADM for 12 h. H22 cells were treated with FasL (0.5, 1.0, 2.0 mg/ml) or ADM (2.5, 5.0, 10.0 mg/ml), then stained with annexin V and PI and analyzed by flow cytometry.

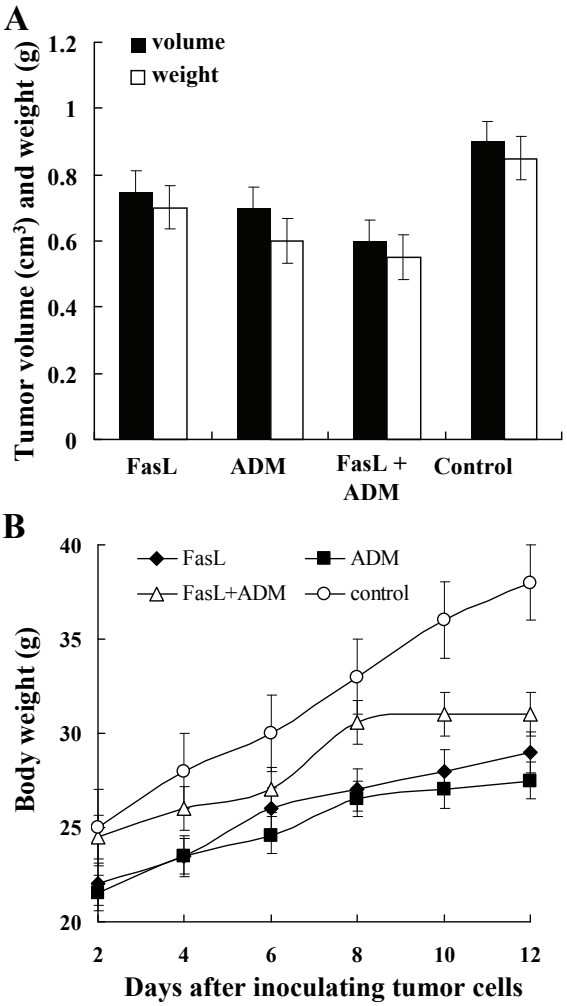

Figure 4: FasL and ADM suppressed the growth of H22 cells. (A) Tumor volumes and weights were obtained from each group. Tumor growth was measured every alternate day. (B) The body weights of tumor-bearing mice. Similar results were obtained in three independent experiments. Data are shown as means ± SD for six mice in each group.

shown) and decrease tumor growth, so, we wanted to research the mechanism related to it. H&E stain showed that the treatment groups had increased necrosis or apoptosis cells compared to the control group. The amount of necrotic cells in the group receiving the combination treatment was increased. The apoptotic and necrotic cell population of the FasL and ADM groups was smaller than that of the FasL-ADM group (Figure 5A). Hepatic tissue section stained with H&E showed that while the FasL group had fewer apoptotic and necrotic hepatic cells, the ADM group had large colored cells. There were fewer apoptotic and necrotic hepatic cells in the combination treatment group than in the single treatment groups (Figure 5B). The combination therapy FasL and ADM had less cytotoxicity on normal hepatic cells than treatment with FasL or ADM alone; this is because the amount of necrosis in the hepatic cells was clearly decreased.

The effect of FasL and ADM on tumor tissues and the livers of mice analyzed by TUNEL staining

In order to detect and quantify apoptosis from all experimental cells in tumor tissue, we detected DNA strand breaks by TUNEL. Apoptosis was observed to be induced in the cell lines in response to FasL and ADM by FCM, In tumor tissue, cells undergoing apoptosis were subsequently examined using a fluorescent microscope after TUNEL staining. ADM and FasL-treated tumor cells showed nuclear chromatin condensation and fragmentation with positive TUNEL signal, Red staining indicates DNA stained by PI. Green indicates dUTP end-labeled by FITC. Cells in which the red and green staining overlap are apoptotic, in tumor tissue treated by FasL indicated apoptotic cells, there were more cells in tissue in which the red and green staining overlap, more TUNEL-positive cells were detected among the cells treated with a combination of FasL and ADM than among tissue treated with the single agent alone. As shown in Figure 5D, TUNEL-positive cells and PI-positive cells from the combination of FasL-ADM were fewer than the other groups in the liver specimens.

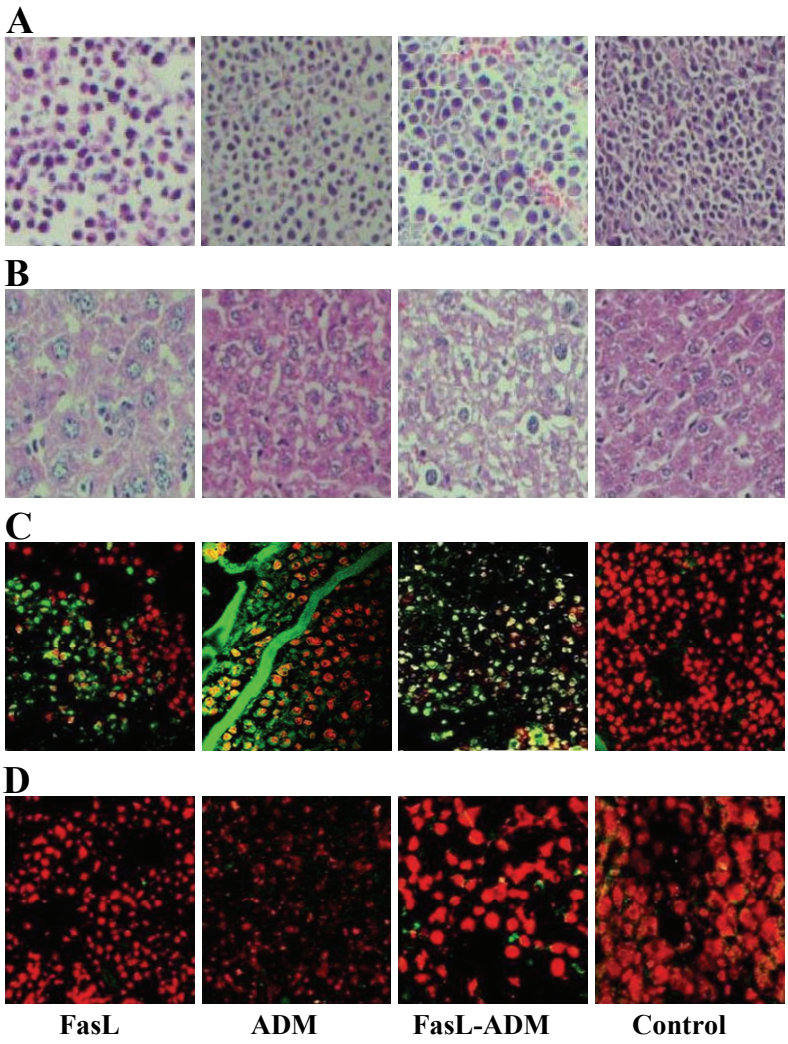

Figure 5. **The effect of FasL and ADM on tumor and the livers of mice examined by H&E and TUNEL staining.** Red staining indicates DNA stained by PI. Green indicates dUTP end-labeled by FITC. Cells in which the red and green staining overlap are apoptotic. (A) Tumor sections. The tumor tissue slices showed that apoptotic and necrotic cells were more in the combination treatment FasL-ADM group than those in the FasL and ADM groups. (B) Liver tissue sections by HE staining from each group. The hepatic tissue slices showed that the FasL-ADM group had fewer apoptotic and necrotic hepatic cells than the FasL and ADM groups. (C) TUNEL staining of resected samples from the tumors of the treated mice. (D) TUNEL staining of resected samples from the mouse liver tissues from each group.

Discussion

Tumor occurrence, development, and clonal expansion have been demonstrated to result from dysregulation of cell death (apoptosis) and intrinsic proliferation (cell division). Failure of apoptosis could allow the survival of transformed cells that are prone to undergo further genetic damages to play an important role in the pathogenesis of tumors (27, 28). Thus, apoptotic processes to remove harmful or unnecessary cells are indispensable for the progression of cell development, differentiation, proliferation, and protection. Apoptotic cell death is initiated *via* a variety of extracellular and intracellular death signals (29, 30). For well-recognized extrinsic apoptotic pathways, FasL induces apoptosis by activating the cell surface death receptor Fas (31). Activated Fas recruits the adaptor protein Fas-associated death domain protein (FADD) that cleaves procaspase-8 and initiates an active form of caspase-8, which activates the downstream effector caspase-3 that leads to apoptosis (31). Several studies have shown that FasL expression is implicated in HCC and that it is enhanced in regions with infiltrating inflammatory cells on the margins of the cancerous tissue (18-20). Moreover, *in vivo* studies have shown that injection of sFasL can induce tumor cell apoptosis and hepatocyte apoptosis, which cause liver failure (23, 24).

In this study, we successfully constructed the FasL gene and expressed and purified FasL, and the P/N values of FasL exceeded 2.1 examined by ELISA. We also demonstrated that the viability of H22 cells could be inhibited by FasL at different concentrations. As observed in the MTT assay, the

optimal dose of FasL and ADM to inhibit the growth of H22 cells was 1.0 and 5.0 mg/ml, respectively. In addition, we determined the apoptotic index induced by FasL and ADM *in vitro* by using flow cytometry. The double-staining flow cytometry experiment showed the same results as those obtained in the MTT assay. At the optimal concentration, the apoptotic percentages of H22 cells were 30.7% in the FasL group and 32.3% in the ADM group. In other words, H22 cells were significantly inhibited by FasL and ADM *in vitro*. To further determine the effects of FasL on tumor development and mortality of mice, the combination of FasL-ADM was also used. *In vivo* studies showed that therapy with FasL or ADM alone or a combination of FasL-ADM could suppress tumor volume and weight (Figure 4A); the body weights of the treated mice were also remarkably reduced (Figure 4B). The combination of FasL and ADM was the most effective in suppressing the growth of H22 tumors compared with FasL or ADM alone. H&E staining of tumor tissue showed that apoptotic and necrotic cells were more in the FasL-ADM group than in the FasL and ADM groups (Figure 5A). Hepatic tissue section stained with HE showed that the FasL-ADM group had fewer apoptotic and necrotic hepatic cells than the FasL and ADM groups (Figure 5B). The TUNEL-stained slices also confirmed the results (Figures 5C and 5D). Combination treatment with FasL-ADM had the best antitumor effect and the least cytotoxicity on H22 cells *in vivo* in this study, though the mechanism by which apoptosis is induced or enhanced by FasL in combination with ADM is not clear.

In summary, this study suggests that FasL significantly enhanced the antitumor effect on HCC cells *in vitro* and *in vivo*. Combination treatment of FasL-ADM had better antitumor effect than FasL alone. Combination treatment involving FasL and the chemotherapeutic agent ADM may be a potent adjuvant therapeutic approach for the treatment of HCC.

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