Partial Contribution of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)/TRAIL Receptor Pathway to Antitumor Effects of Interferon-α/5-Fluorouracil against Hepatocellular Carcinoma

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ABSTRACT

Purpose: **Our purpose was to explore the contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of IFNα and 5-fluorouracil (5-FU) combination therapy for hepatocellular carcinoma (HCC).**

Experimental Design: **Susceptibility of HCC cell lines to TRAIL and/or 5-FU was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The effects of 5-FU, IFNα, or both on the expression of TRAIL receptors (R1, R2, R3, and R4) on HCC cells or TRAIL in peripheral blood mononuclear cells (PBMC) were examined by flow cytometry. IFNα-induced cytotoxic effects of PBMC on HCC cell lines were examined by ^{51}Cr release assay. TRAIL expression in peripheral blood mononuclear cells and liver tissue from patients was examined by real-time reverse transcription-PCR or immunohistochemistry.**

Results: **HLE and HepG2 were sensitive to TRAIL, but HuH7, PLC/PRF/5, and HLF were resistant. 5-FU had synergistic effect on TRAIL in HLF and additive effect in four other HCC cell lines. TRAIL receptors on HCC cells were up-regulated by 5-FU, and IFNα induced TRAIL on CD4$^+$ T cells, CD14$^+$ monocytes, and CD56$^+$ NK cells. Treatment of effector cells by IFNα and target HCC cells by 5-FU enhanced the cytotoxicity of CD14$^+$ monocytes and CD56$^+$ NK cells against HCC cells via a TRAIL-mediated pathway. TRAIL mRNA overexpression was noted in PBMC of HCC patients who clinically responded to IFNα/5-FU combination therapy, and TRAIL$^+$ mononuclear cells were found in cancer tissue of a responder.**

Conclusion: **Our results suggest that modulation of TRAIL/TRAIL receptor-mediated cytotoxic pathway might partially contribute to the anti-HCC effect of IFNα and 5-FU combination therapy.**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide (1). The prognosis of HCC is still poor despite newly developed therapeutic modalities such as radiofrequency ablation and microwave coagulation therapy (2, 3). HCCs with macroscopic tumor thrombi in the major branches of the portal vein (Vp3–4) are extremely aggressive (4). Most HCC patients with Vp3–4 tumors develop recurrences, and half of them die within 1 year after surgery even if curative resection is done (5). The prognosis of unresectable cases with Vp3–4 is much worse, and most patients die within several months (6). Therefore, the development of new antitumor therapy for HCC patients is urgent and mandatory.

In general, HCCs are resistant to anticancer drugs (7). However, recent studies including those from our group showed an excellent clinical response to the combination therapy of IFNα and 5-fluorouracil (5-FU) in HCC patients complicated with Vp3–4 (8). Although the exact mechanism of action of this combination therapy has not yet been established, it has been reported that IFNα enhances the expression of thymidine phosphorylase in colon carcinoma cells, which converts 5-FU to an active metabolite and enhances the DNA damage by 5-FU (9, 10). We also showed previously that IFNα and 5-FU synergistically reduced tumor cell proliferation through cell cycle arrest (11). Although IFNα also exerts immunomodulatory effects by stimulating T cells, NK cells, and monocytes (12, 13), the involvement of its immunomodulatory properties in the IFNα/5-FU combination therapy remains to be determined.

Recently, IFNα was reported to up-regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in T cells, NK cells, and monocytes (14–16). TRAIL is a newly identified member of the tumor necrosis factor superfamily, which induces apoptosis of transformed cells but not normal cells (17, 18). TRAIL binds to four distinct membrane-bound TRAIL receptors (TRAIL-R1, R2, R3, and R4) and a soluble receptor, osteoprotegerin. TRAIL-R1 and R2 act as death-inducing recep-

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tors, whereas TRAIL-R3, -R4, and osteoprotegerin may act as decoy receptors (19–24). An important role for TRAIL in tumor immunotherapy has been suggested in murine models (25). Therefore, the TRAIL/TRAIL receptor system may be involved in the antitumor effect of the IFNα and 5-FU combination therapy.

In the present study, we investigated the effects of IFNα combined with 5-FU on the expression and function of TRAIL and TRAIL receptors in human peripheral blood mononuclear cells (PBMC) and HCC cell lines. We also investigated clinical relevance of the *in vitro* findings by using clinical samples from HCC patients who received the combination therapy. The results suggest the partial contribution of TRAIL/TRAIL receptor-mediated cytotoxic pathway to the tumoricidal effects of IFNα and 5-FU combination therapy on HCC.

MATERIALS AND METHODS

Cells. Human HCC cell lines (HuH7, PLC/PRF/5, HLE, HLF, and HepG2) were obtained from Japan Cancer Research Resources Bank (Osaka, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin at 37°C in a humidified incubator under 5% CO_2 in air. PBMC were obtained from healthy subjects and prepared by Ficoll-Hypaque centrifugation. $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, $CD56^+$ NK cells, $CD19^+$ B cells, $CD4^-CD8^-$ cells, $CD4^-CD8^-CD14^-$ cells, $CD4^-CD8^-CD56^-$ cells, and $CD4^-CD8^-CD14^-CD56^-$ cells were isolated from PBMC by using anti-CD4, anti-CD8, anti-CD14, anti-CD56, and anti-CD19 immunomagnetic beads and Magnetic Cell Sorting (Miltenvi Biotec, Bergisch Gladbach, Germany). The purity of each subset was >90% as determined by flow cytometry. These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin.

Reagents. Purified human IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan), and 5-FU was a kind gift from Kyowa Hakko Co. (Tokyo, Japan). Antihuman TRAIL monoclonal antibody (mAb; RIK-2) was prepared as described previously (26). mAbs against TRAIL receptors were obtained from eBioscience (San Diego, CA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay. The sensitivity of each HCC cell line to recombinant TRAIL and the effect of 5-FU on TRAIL-induced death were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. HCC cells (3×10^3 in 100 μL medium) were seeded in 96-well microplates. After 24 hours, 100-μL medium containing soluble human TRAIL (Alexis Corp., Lausen, Switzerland) and/or 5-FU (Kyowa Corp., Tokyo) were added, and the plates were incubated for 48 hours. Cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma Chemical, St. Louis, MO) according to the instructions provided by the manufacturer, and each well was measured spectrophotometrically on a dual beam microtiter plate reader at 550 nm with a 650-nm reference.

Flow Cytometry. Cells (1×10^6) were incubated with 1 μg of biotinylated mAb against TRAIL and TRAIL receptors or control IgG for 30 minutes at 4°C, followed by phycoerythrin-labeled avidin (BD PharMingen, San Diego, CA). After washing with PBS, the cells were analyzed on a fluorescence-activated cell sorter (FACScan, BD PharMingen), and data were processed with Cell Quest software (BD PharMingen). For detection of TRAIL on $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells, PBMCs were double-stained with biotinylated antihuman TRAIL mAb followed by phycoerythrin-labeled avidin and FITC-labeled antihuman CD4, CD8, CD14, or CD56 mAb (BD PharMingen). The mean fluorescence intensity was measured in the experiment of alteration of TRAIL receptors.

^{51}Cr Release Assay. Target HCC cells (1×10^6) were labeled with 40 μCi $Na^{51}CrO_4$ for 45 minutes at 37°C. ^{51}Cr-labeled target cells (1×10^4) and effector cells ($CD4^+$ T cells, $CD8^+$ T cells, $CD4^-CD8^-$ cells, $CD4^-CD8^-CD14^-$ cells, $CD4^-CD8^-CD56^-$ cells, or $CD4^-CD8^-CD14^-CD56^-$ cells) were mixed in U-bottomed wells of a 96-well microplate at the indicated E:T ratios. After 8 hours of incubation, cell-free supernatants were collected and counted on a gamma counter. The following formula was used to calculated the percentage of specific cytotoxicity: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Total or spontaneous release was determined in the presence of 1% NP40 or medium alone, respectively. In some experiments, anti-TRAIL mAb was added at a final concentration of 10 μg/mL.

Quantitative RT-PCR for TRAIL mRNA. Between April 1998 and December 2001, 23 patients with unresectable HCC associated with multiple intrahepatic tumors and Vp3–4 received IFNα/5-FU combination therapy at the Department of Surgery, Osaka University Hospital. The treatment regimen consisted of subcutaneous injection of IFNα (5×10^6 units) on days 1, 3, and 5 of every week for 4 weeks and continuous infusion of 5-FU (450–500 mg/day) for 2 weeks through a catheter introduced into the proper hepatic artery (8). Peripheral blood samples were obtained from 12 patients who received this combination therapy. The study protocol was approved by the Human Ethics Review Committee of Osaka University School of Medicine, and a signed consent form was obtained from each patient before participation in the study. PBMC were prepared by Ficoll-Hypaque centrifugation. We extracted total cellular RNA from PBMC using TRIzol reagent (Molecular Research Center, Cincinnati, OH), using the instructions provided by the manufacturer. Isolated RNA was quantitated and assessed for purity by UV spectrophotometry. cDNA was synthesized with avian myeloblastosis virus reverse transcriptase according to the protocol provided by the supplier (Promega, Madison, WI). One μg of RNA was incubated at 70°C for 5 minutes and then placed on ice before the addition of reverse transcription (RT) reaction reagents with oligodeoxythymidylic acid primer. The RT reaction was done at 42°C for 90 minutes, followed by heating at 95°C for 5 minutes. The LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification and quantification. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TRAIL PCR products, LightCycler DNA Master SYBR Green I (Boehringer Mannheim) was used. We did real-time PCR reactions in a sample mixture containing 0.2 μmol/L of each primer, 1× LC-DNA Master SYBR Green I, 4 mmol/L $MgCl_2$, and 2 μL of cDNA as a template using the following primers: human GAPDH

(forward, 5′-CAACTACATGGTTTACATGTTC-3′; reverse, 5′-GCCAGTGGACTCCACGAC-3′); and human TRAIL (forward, 5′-CAACTCCGTCAGCTCGTTAGAAAG-3′; reverse: 5′-TTAGACCAACAACTATTTCTAGCACT-3′), yielding products of 182 and 443 bp, respectively. GAPDH PCR cycle condition was set up as follows: one cycle of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 10 seconds, and 72°C for 20 seconds. TRAIL PCR cycle condition was set up as follows: one cycle of 95°C for 10 minutes followed by 35 cycles of 95°C for 15 seconds, 55°C for 10 seconds, and 72°C for 25 seconds. Fluorescence was acquired at the end of every 72°C extension phase. We did quantitative analysis of data using the LightCycler analysis software (Roche Diagnostics). The standard curves for GAPDH and TRAIL were constructed by using 10-fold serial dilutions of cDNA prepared from IFNα-stimulated PBMC from a healthy subject. Expression of TRAIL mRNA was reported relative to GAPDH. In the experiment investigating TRAIL mRNA expression in $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells, we did quantitative RT-PCR for TRAIL using the same method.

Immunohistologic Staining for TRAIL-R1 and R2. Paraffin-embedded sections (5-μm thick) of liver cancer tissues from three patients with HCC who responded to IFNα/5-FU combination therapy (responders) and one patient who did not respond to the same therapy (nonresponder) were deparaffinized in xylene and rehydrated before analysis. Endogenous peroxidase was quenched with 0.5% hydrogen peroxide. After washing with PBS, slides were blocked with 1% normal goat serum or normal rabbit serum and then incubated overnight at 4°C at 1:40 dilution in PBS of rabbit anti-DR4 (TRAIL-R1) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-DR5 (TRAIL-R2) polyclonal antibody (Santa Cruz Biotechnology), or normal rabbit IgG (as a control). En-Vision ABC detection kit (Dako, Tokyo, Japan) containing horseradish peroxidase was used to detect antibody binding. Diaminobenzidine was used as the substrate. Slides were counterstained with hematoxylin.

Immunohistologic Staining for TRAIL. Paraffin-embedded sections (5-μm thick) of liver cancer tissues from three responders and one nonresponder and normal liver tissues from a patient with focal nodular hyperplasia were deparaffinized in xylene and rehydrated before analysis. After washing with PBS, slides were blocked with 2-nitro-5-thiobenzoate blocking buffer (NEN Life Science Products, Boston, MA) and then incubated overnight at 4°C with goat antihuman TRAIL antibody K-18 (Santa Cruz Biotechnology) diluted in 2-nitro-5-thiobenzoate blocking buffer at a concentration of 5 μg/mL. After washing with PBS, slides were incubated with biotinylated donkey antigoat IgG antibody (Chemicon, Temecula, CA) for 30 minutes. Alexa 568-labeled streptavidin (Molecular Probes, Inc., Eugene, OR) was used to detect antibody binding. Slides were then covered with VECTASHIELD (Vector Laboratories, Burlingame, CA) and examined under a confocal laser scanning microscope (model LSM 510, Carl Zeiss, Oberkochen, Germany). A single beam from a helium/neon laser (543 nm) was used for excitation. Emission (603 nm) was detected through a long pass filter (>560 nm) and displayed in red. For control staining, the immunizing peptide K-18p (Santa Cruz Biotechnology) was included at 4 μg/mL in the primary antibody incubation. Placental tissue was used as a positive control (27). The number of TRAIL-positive cells was counted in more than six 360 magnification fields of confocal laser scanning microscope.

Statistical Analysis. Data are represented as mean ± SD or SEM. Differences between groups were examined for significant differences by unpaired *t* test. In Fig. 1*A*, differences in the mean percentages of cell viability in the presence of TRAIL alone or TRAIL plus 5-FU were compared by the Dunnett post hoc procedure. The level of statistical significance was set at $P < 0.05$.

Evaluation of TRAIL/5-FU Cooperative Effects. Calculation of the synergistic cytotoxicity of soluble human TRAIL and 5-FU was determined by isobolographic analysis of Berenbaum (28).

RESULTS

Sensitivity of HCC Cell Lines to TRAIL. We did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using soluble human TRAIL to examine the sensitivity of HCC cell lines to TRAIL-mediated cytotoxicity. HuH7, PLC/PRF/5, HLE, HLF, and HepG2 cells were incubated with or without various concentrations of soluble TRAIL and/or 5-FU for 48 hours. Sensitivity to TRAIL or 5-FU was different among five HCC cell lines (Fig. 1*A*). HLE and HepG2 cells were sensitive to soluble human TRAIL, but HuH7, PLC/PRF/5, and HLF cells were rather resistant. On the other hand, 5-FU alone exhibited dose-dependent cytotoxicity against HuH7, PLC/PRF/5, HLF, and HepG2 cells, whereas HLE cells were relatively resistant. The percentage of cell viabilities was significantly decreased by the combined treatment of cells with TRAIL and 5-FU at various doses of these agents as compared with TRAIL alone. Isobologram analysis showed that the combination of TRAIL and 5-FU exhibited a synergistic effect in HLF cells and an additive effect in HuH7, PLC/PRF/5, HLE, and HepG2 cells (Fig. 1*B*).

Expression of TRAIL Receptors on HCC Cell Lines. Using flow cytometry, we investigated whether the difference in HCC cell sensitivity to TRAIL correlated with the expression levels of TRAIL-R1, R2, R3, and R4 on the surface of HCC cell lines (Fig. 2*A*). Low expression levels of TRAIL-R1 were noted on the surface of PLC/PRF/5, HLE, and HLF cells. In addition, TRAIL-R2 was expressed on all HCC cell lines, the expression level was higher on TRAIL-sensitive HCC cells (HLE and HepG2 cells) than on TRAIL-resistant HCC cells (HuH7, PLC/PRF/5, and HLF cells). TRAIL-R3 and R4 were not detectable on the surface of any HCC cell line, regardless of their TRAIL sensitivity status. These results indicate that the basal expression of TRAIL-R2 correlates with the sensitivity to TRAIL.

Effect of 5-FU and/or IFNα on TRAIL Receptor Expression on HCC Cell Lines. To examine the effect of 5-FU and/or IFNα on TRAIL receptor expression on HCC cell lines, the cells were preincubated with or without 5-FU (0.5 μg/mL) and/or IFNα (500 units/mL) for 48 hours followed by determination of expression of TRAIL receptors by flow cytometry. As shown in Fig. 2*B*, preincubation with 5-FU for 48 hours increased the expression of TRAIL-R1, R2, R3, and R4 on HepG2 cells. In contrast, IFNα did not alter the expression of any

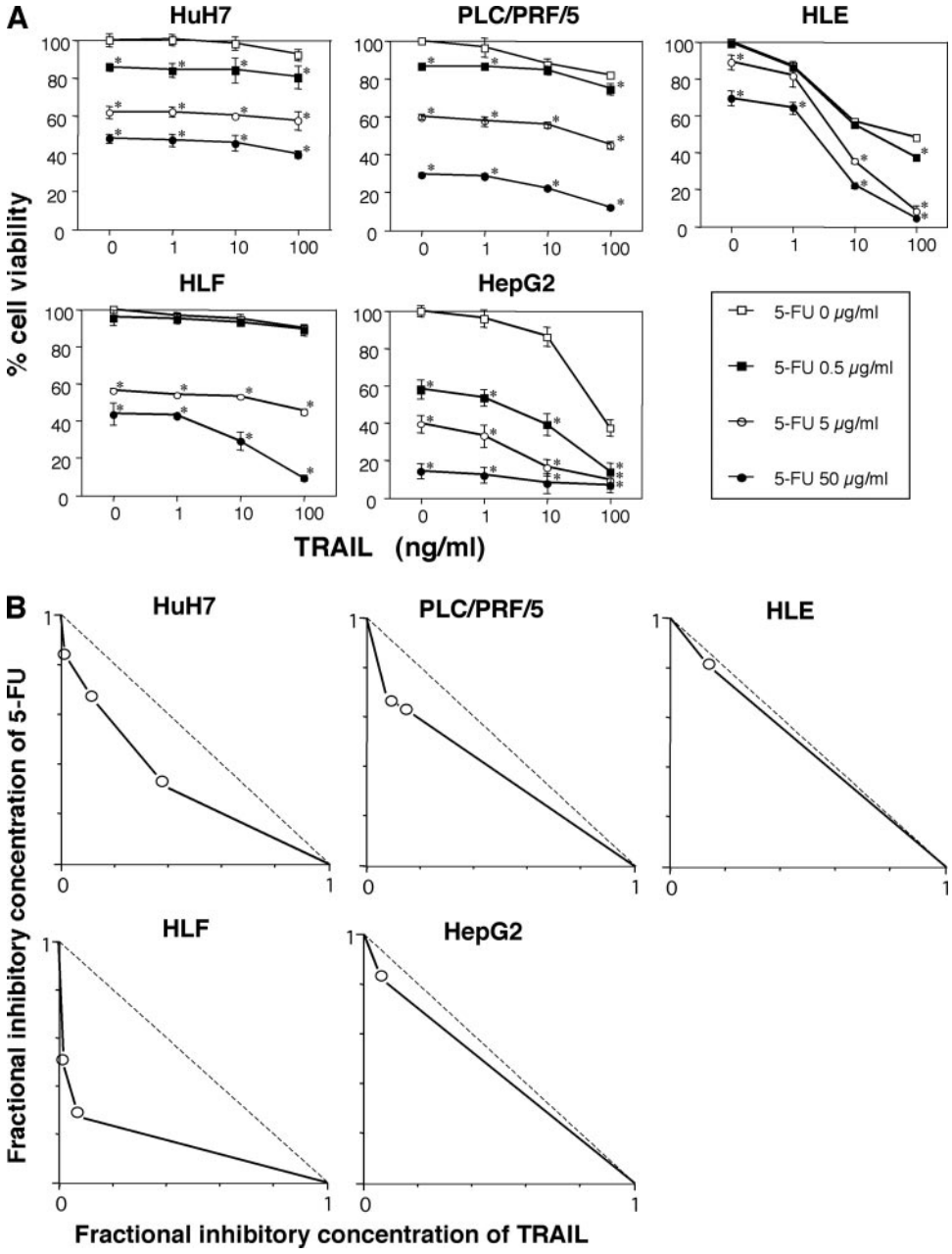

Fig. 1 Effects of 5-FU on TRAIL-induced cytotoxicity in HCC cell lines. *A*, HuH7, PLC/PRF/5, HLE, HLF, and HepG2 cells were treated simultaneously with the indicated concentrations of soluble human TRAIL and 5-FU for 48 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done to evaluate cell viability. Data are represented as mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Differences in the percentage of cell viability between TRAIL alone and TRAIL plus 5-FU were determined by the Dunnett post hoc procedure. *, $P < 0.05$. *B*, synergy was estimated by isobolographic analysis.

TRAIL receptor. The effect of combination of 5-FU and IFNα was nearly equal to that of 5-FU alone. As summarized in Fig. 2*C*, the effects of 5-FU and/or IFNα on the expression of TRAIL receptors on other HCC cell lines were similar to the HepG2 case, except for HLE cells, which showed no changes in response to either 5-FU or IFNα. These results indicate that 5-FU, but not IFNα, can significantly increase the expression of TRAIL-R1 and R2 on some HCC cell lines.

Effect of 5-FU and/or IFNα on TRAIL Expression on PBMC. We next examined the effects of 5-FU and/or IFNα on TRAIL expression on PBMC subpopulations (Fig. 3*A*). TRAIL expression was not detected on freshly prepared PBMC by flow cytometry. Incubation with IFNα (500 units/mL) for 24 hours markedly induced TRAIL expression on $CD14^+$ monocytes and $CD56^+$ NK cells. $CD4^+$ T cells, but not $CD8^+$ T cells, also expressed TRAIL at a low level after IFNα stimulation. On the other hand, 5-FU (0.5 μg/mL) did not induce TRAIL expression alone or altered the effect of IFNα on TRAIL expression. IFNα treatment resulted in overexpression of TRAIL mRNA in $CD14^+$ monocytes and $CD56^+$ NK cells. In contrast, no such increase was noted in $CD19^+$ B cells (Fig. 3*B*).

Involvement of TRAIL in IFNα-Stimulated PBMC Cytotoxicity against 5-FU-Treated HCC Cells. We did the ^{51}Cr release assay to investigate the involvement of TRAIL in

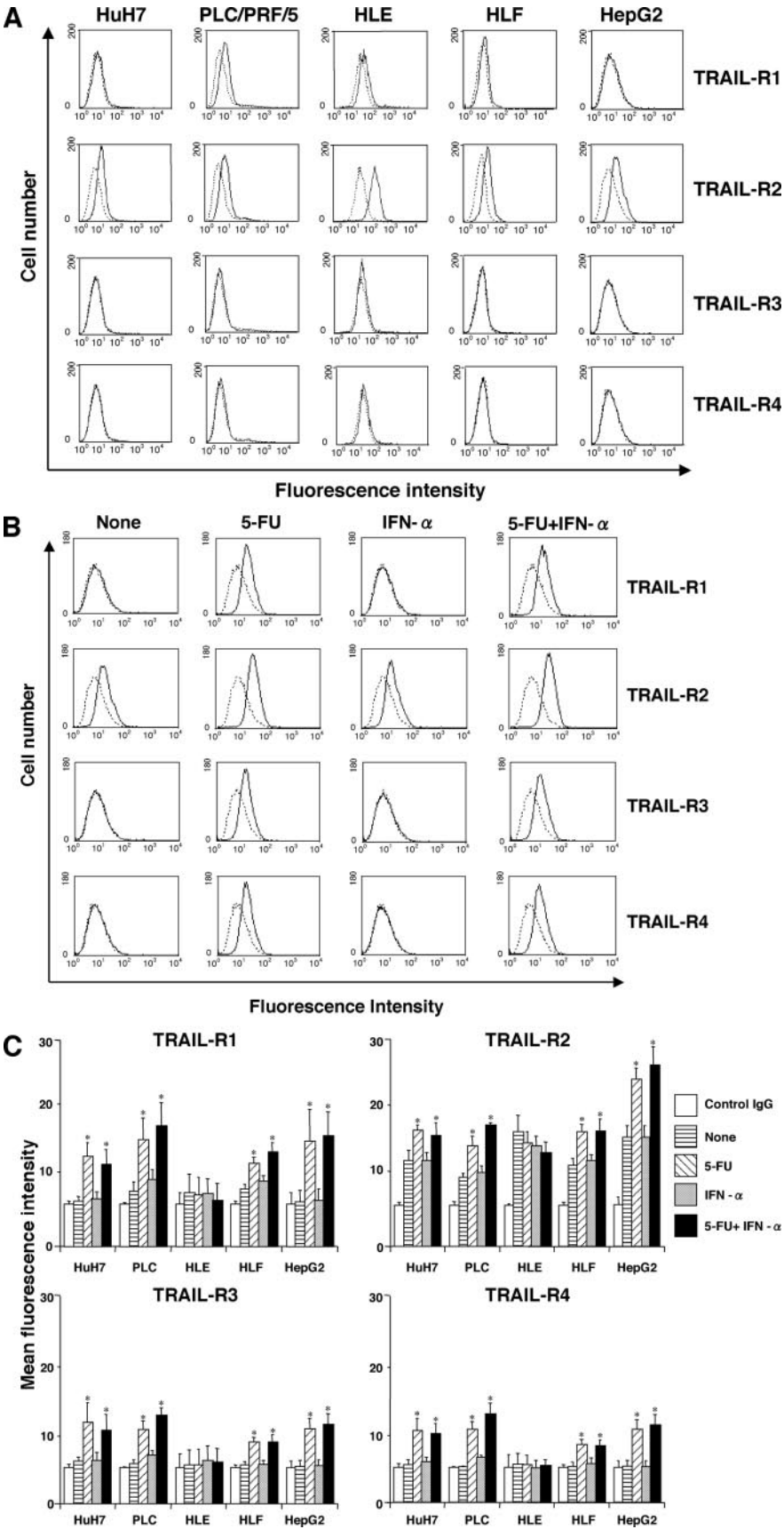

Fig. 2 *A*, Expression of TRAIL receptors on the surface of HCC cell lines. *Dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated mAb against the indicated TRAIL receptors. *B* and *C*, effects of 5-FU and/or IFNα on TRAIL receptor expression on HCC cell lines. HepG2 cells (*B*) and the indicated HCC cells (*C*) were treated with 5-FU (0.5 μg/mL) and/or IFNα (500 units/mL) for 48 hours. *B*, *dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated mAb against the indicated TRAIL receptors. Similar results were obtained in three independent experiments. *C*, *open bars*, the mean fluorescence intensity of staining with biotinylated control IgG; *other bars*, the mean fluorescence intensity of staining with biotinylated mAbs against the indicated TRAIL receptors. Data are mean ± SD of three independent experiments. *, $P < 0.05$ compared with untreated cells.

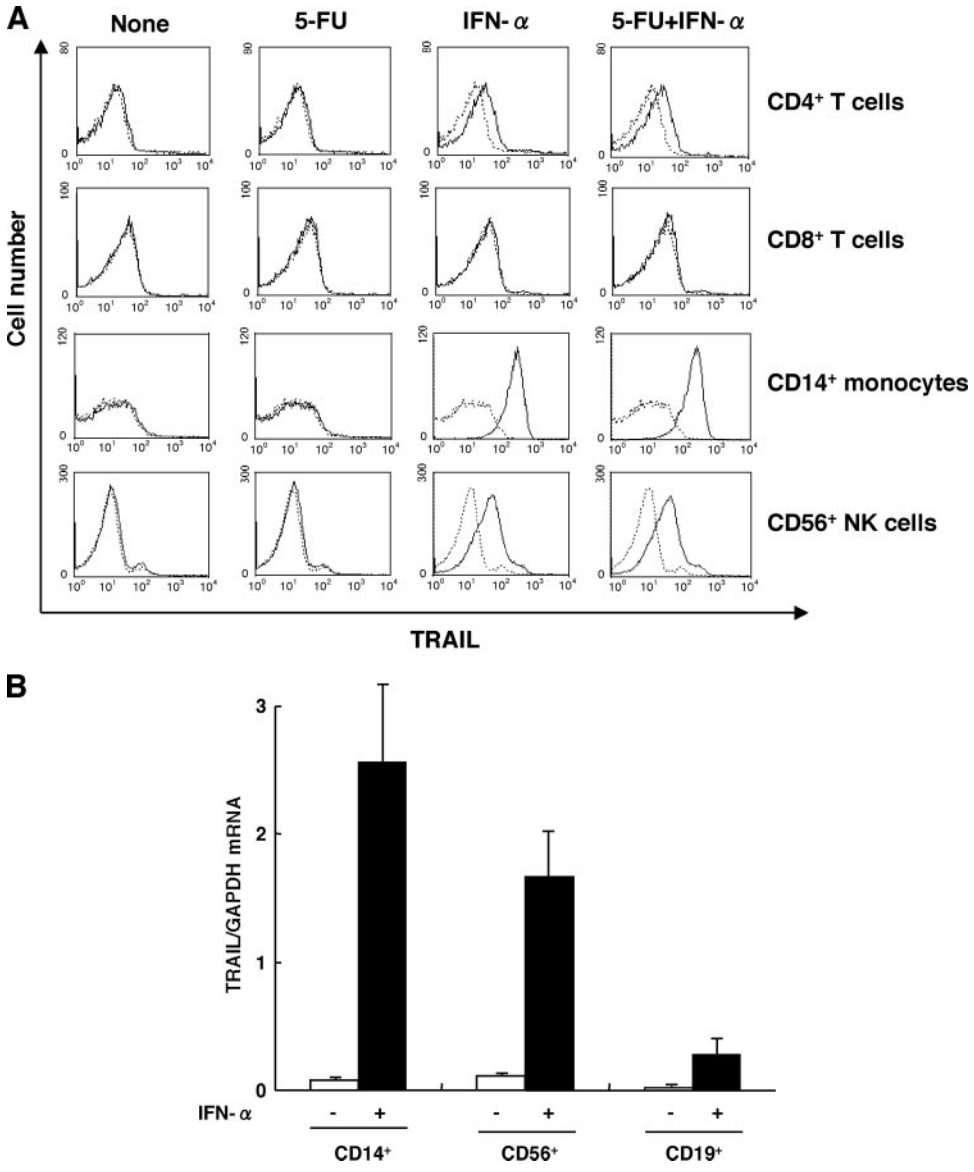

Fig. 3 *A*, regulation of TRAIL expression on $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells in PBMC by 5-FU and/or IFNα. PBMC from healthy subjects were cultured in the presence or absence of 5-FU (0.5 μg/mL) and/or IFNα (500 units/mL) for 24 hours and then stained with biotinylated anti-TRAIL mAb followed by phycoerythrin-labeled avidin and FITC-labeled antihuman CD4, CD8, CD14, or CD56 mAb. *Dotted lines*, staining with biotinylated control IgG; *solid lines*, staining with biotinylated anti-TRAIL mAb. Similar results were obtained in three independent experiments. *B*, TRAIL mRNA expression in $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells. Cells were isolated from PBMC by using anti-CD14, anti-CD56, and anti-CD19 immunomagnetic beads and Magnetic Cell Sorting and cultured in the presence (*closed bars*) or absence (*open bars*) of IFNα (500 units/mL) for 24 hours. LightCycler PCR and detection system were used to do quantitative RT-PCR for TRAIL, and expression of TRAIL mRNA is indicated relative to GAPDH. Data are mean ± SD of three independent experiments.

IFNα-stimulated PBMC cytotoxicity against untreated or 5-FU–treated HCC cell lines for which 5-FU and soluble TRAIL exhibited synergistic (HLF) or additive (HepG2) effect. We first used $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells isolated from PBMC as the effector cells after incubation with or without IFNα for 24 hours. As shown in Fig. 4*A*, $CD4^-CD8^-$ cells, but not $CD4^+$ or $CD8^+$ T cells, were the main effector cells that exhibited substantial cytotoxicity against HLF and HepG2. When $CD4^-CD8^-$ cells were stimulated by IFNα, the cytotoxicity was significantly increased from 3.6 to 18% against HLF and from 43 to 63% against HepG2 at an E:T ratio of 20. Moreover, when the target cells were pretreated with 5-FU, IFNα enhanced the cytotoxicity of $CD4^-CD8^-$ cells from 19 to 54% against HLF and from 48 to 66% against HepG2. These results indicate a synergistic effect against HLF and an additive effect against HepG2 of the combination of IFNα and 5-FU on $CD4^-CD8^-$ cell-mediated cytotoxicity.

To investigate which subset of $CD4^-CD8^-$ cells was responsible for the IFNα-induced cytotoxicity, we prepared effector cells that were depleted of $CD14^+$ monocytes and/or $CD56^+$ NK cells from $CD4^-CD8^-$ cells. As shown in Fig. 4*B*, $CD4^-CD8^-CD14^-$ cells (mainly composed of NK cells and B cells) and $CD4^-CD8^-CD56^-$ cells (mainly composed of monocytes and B cells) exhibited higher cytotoxicity against HLF cells than $CD4^-CD8^-CD14^-CD56^-$ cells (mainly composed of B cells). IFNα significantly enhanced the cytotoxicity of $CD4^-CD8^-CD14^-$ cells, and pretreatment of the target cells with 5-FU significantly enhanced the cytotoxicity by both $CD4^-CD8^-CD14^-$ cells and $CD4^-CD8^-CD56^-$ cells. These results suggested that both $CD56^+$ NK cells and $CD14^+$ monocytes were the major effector cells that largely contributed to the IFNα-induced cytotoxicity of PBMC against 5-FU–treated HCC cells.

Finally, we examined the contribution of TRAIL by using

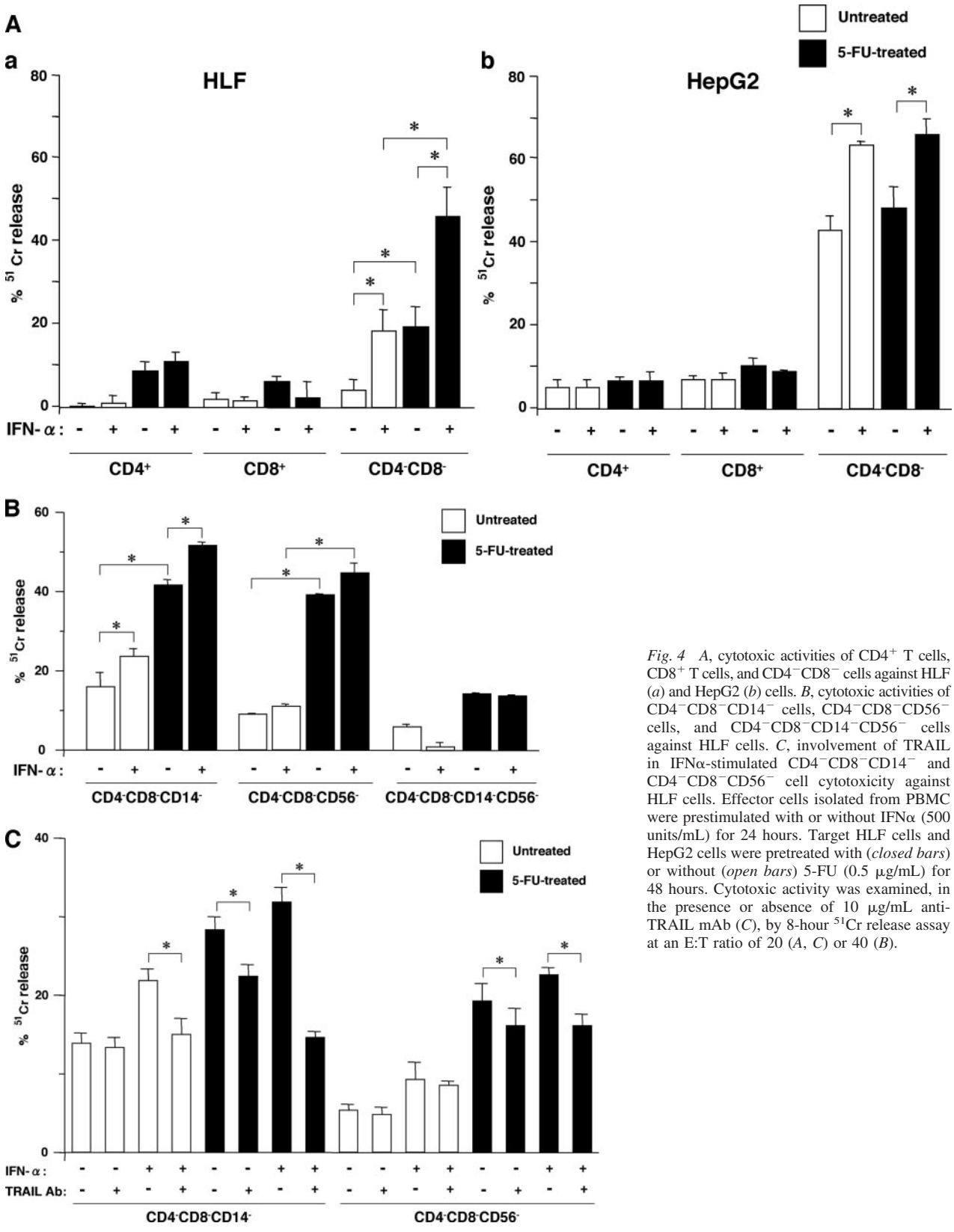

Fig. 4 *A*, cytotoxic activities of $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^-CD8^-$ cells against HLF (*a*) and HepG2 (*b*) cells. *B*, cytotoxic activities of $CD4^-CD8^-CD14^-$ cells, $CD4^-CD8^-CD56^-$ cells, and $CD4^-CD8^-CD14^-CD56^-$ cells against HLF cells. *C*, involvement of TRAIL in IFNα-stimulated $CD4^-CD8^-CD14^-$ and $CD4^-CD8^-CD56^-$ cell cytotoxicity against HLF cells. Effector cells isolated from PBMC were prestimulated with or without IFNα (500 units/mL) for 24 hours. Target HLF cells and HepG2 cells were pretreated with (*closed bars*) or without (*open bars*) 5-FU (0.5 μg/mL) for 48 hours. Cytotoxic activity was examined, in the presence or absence of 10 μg/mL anti-TRAIL mAb (*C*), by 8-hour ^{51}Cr release assay at an E:T ratio of 20 (*A*, *C*) or 40 (*B*).

neutralizing anti-TRAIL mAb (RIK-2). As shown in Fig. 4*C*, IFNα-induced cytotoxicity of $CD4^-CD8^-CD14^-$ cells was almost completely abrogated by anti-TRAIL mAb, irrespective of pretreatment of HLF target cells with 5-FU. In addition, the enhanced cytotoxicity of $CD4^-CD8^-CD56^-$ cells against 5-FU-treated HLF cells was also significantly inhibited by anti-TRAIL mAb. These results suggest that IFNα-induced cytotoxicity of $CD56^+$ NK cells is predominantly mediated by TRAIL and that 5-FU–enhanced cytotoxicity of $CD14^+$ monocytes is at least partly mediated by TRAIL.

Increased Expression of TRAIL mRNA in PBMC of HCC Patients Who Responded to IFNα/5-FU Therapy. The above *in vitro* results suggest the possible contribution of TRAIL to IFNα/5-FU combination therapy. In the next step, we addressed the clinical relevance of the *in vitro* studies by using clinical samples. PBMC from 12 patients with unresectable HCC associated with multiple intrahepatic tumors and Vp3–4, who were treated with IFNα/5-FU combination therapy, were collected 48 hours after the last subcutaneous injection of IFNα. The characteristics of these 12 patients are shown in Table 1. Before starting the therapy, blood samples were also collected as control from all patients. Six patients (responders) showed clinical benefits (complete response [CR] in one of six and partial response [PR] in five of six) whereas the other six (nonresponders) did not (progressive disease [PD] in six of six) according to the Eastern Cooperative Oncology Group criteria. The age of the patients was from 32 to 80. The mean age of the responders was higher (56.8) than that of nonresponders (46.6). All 12 patients had chronic hepatitis that progressed to liver cirrhosis except cases 12. Five patients had hepatitis B virus infection, and seven patients had hepatitis C. Nine of the 12 patients were classified as Child-Pugh class B, whereas two patients were class C, and one patient was class A. In all patients, HCCs were diagnosed as unresectable because of multiple intrahepatic metastasis and the presence of tumor thrombi in the major branch (Vp3) or trunk (Vp4) of the portal vein. Moreover, two patients had tumors that invaded the bile duct. Extrahepatic metastases were not found, and the stage of the disease was IVA ($T_4N_0M_0$) in all 12 patients. Five of the six responders had received >4 treatment cycles, which was associated with prolongation of prognosis (from 6 to 23 months), whereas all six nonresponders could not be treated for >2 cycles and died within 6 months. There were no apparent technical side effects resulting from catheter insertion and subcutaneous implantation of the reservoir. Grade 3 leukopenia was observed in three patients (cases 3, 4, and 11) and was well managed by the administration of granulocyte colony-stimulating factor. Grade 3 thrombocytopenia was observed in one patient (case 10) and temporarily necessitated discontinuation of therapy. Three patients reported diarrhea, and it was managed by binding medicine, but was not the reason for interruption of therapy. Case 11 developed gastric ulcer during therapy in addition to diarrhea and leukopenia, and the patient decided to stop the therapy. In contrast, 6 of the 12 patients showed no remarkable adverse effects. Fever was commonly observed but easily controlled by nonsteroidal anti-inflammatory drugs.

The expression of TRAIL mRNA in peripheral blood of these patients was measured by real-time RT-PCR. Although the expression levels of TRAIL mRNA in PBMC varied from one individual to another, the average TRAIL mRNA level increased by 1.5- to 2.5-fold after initiation of therapy in the patients who clinically showed a complete response or partial response(Fig. 5*A*). In contrast, no such increase was observed in the patients who clinically showed PD. We also measured the fold index of TRAIL/β-actin, and the result was consistent (data not shown). These results suggest that induction of TRAIL in PBMC correlates with the clinical response to IFNα/5-FU combination therapy.

Expression of TRAIL Receptor and TRAIL in HCC Tissues. To confirm the involvement of TRAIL/TRAIL receptor system in the liver of IFNα/5-FU–treated patients, we further investigated the expression of TRAIL-R1, R2, and TRAIL in HCC tissues of the three patients who exhibited

Table 1 Characteristics of the patients with unresectable hepatocellular carcinoma associated with multiple intrahepatic tumors and Vp3–4, who were treated with IFNα/5-FU combination therapy

Case	Age	Gender	Hepatitis	Child-Pugh classification	Tumor pathology	Stage of disease	Treatment cycles	Response	Side effects *	Outcome (mos)
1	55	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	6	PR	Diarrhea	23, dead
2	70	M	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PR	-	17, dead
3	65	F	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	5	PR	Leukopenia	13, dead, lung metastasis
4	54	M	HBV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	6	PR	Leukopenia	13, dead
5	65	F	HCV	C	Vp3, multiple	$T_4N_0M_0$ Stage IVA	4	PR	-	9, dead
6	32	M	HBV	C	Vp3, multiple, B (+)	$T_4N_0M_0$ Stage IVA	4	CR	-	6, dead
7	51	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	2	PD	-	5, dead
8	57	M	HCV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PD	-	4, dead
9	43	M	HBV	B	Vp4, multiple	$T_4N_0M_0$ Stage IVA	3	PD	-	4, dead
10	49	F	HBV	B	Vp3, multiple	$T_4N_0M_0$ Stage IVA	2	PD	Thrombocytopenia	4, dead
11	80	F	HCV	B	Vp3, multiple, B (+)	$T_4N_0M_0$ Stage IVA	1	PD	Diarrhea, gastric ulcer leukopenia	3, dead
12	70	M	HCV	A	Vp3, multiple	$T_4N_0M_0$ Stage IVA	1	PD	Diarrhea	2, dead

Abbreviations: Vp4, tumor thrombosis in the trunk of the portal vein; Vp3, tumor thrombosis in the major branches of the portal vein; B (+), bile duct invasion; CR, complete response; PR, partial response; PD, progressive disease.

* Thrombocytopenia and leukopenia represent platelet and leukocytecounts $<0.4 \times 10^5/\mu L$ and $2{,}000/\mu L$, respectively.

partial response to IFNα/5-FU combination therapy (responders) and one patient who exhibited PD (nonresponder). Both TRAIL-R1 and R2 were expressed in HCC cells of all IFNα/5-FU–treated patients regardless of their clinical benefits. Expression of TRAIL-R1 and R2 was located mainly in the cytoplasm and plasma membrane. Representative specimens are shown in Fig. 5*B-a* and 5*B-b*. Immunohistologic studies also showed TRAIL expression in tumor-infiltrating mononuclear

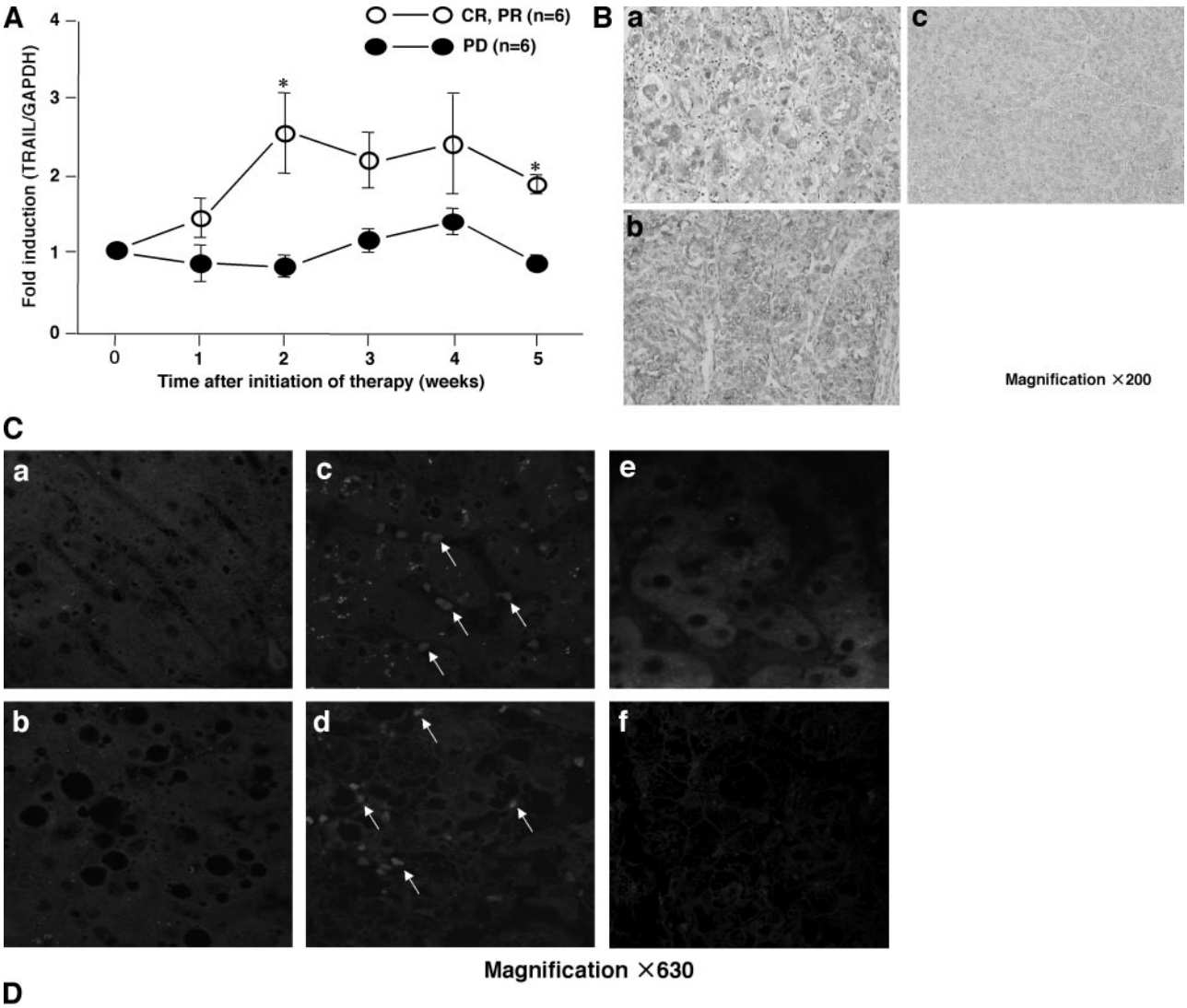

Fig. 5 *A*, TRAIL mRNA expression in PBMC from patients with HCC who received the IFNα/5-FU combination therapy. *Open circles*, patients who showed a clinical response [complete response (CR) or partial response (PR)]; *closed circles*, patients who showed no clinical response [progressive disease (PD)]. Data are mean ± SEM of six patients in each group. *, $P < 0.05$ compared with the PD groups. *B*, immunohistochemical staining of liver specimens with (*a*) antihuman TRAIL-R1 antibody, (*b*) antihuman TRAIL-R2 antibody, and (*c*) normal rabbit IgG (original magnification ×200). TRAIL-R1 and TRAIL-R2 were expressed in all HCC tissues of all four patients. *C*, expression of TRAIL in tumor-infiltrating mononuclear cells. For immunofluorescent staining, the tissue was stained with antihuman TRAIL antibody and Alexa 568-labeled streptavidin. TRAIL-positive cells appear *red* in color (*arrows*; original magnification ×630). Nontumor area of the liver tissue from the nonresponder (*a*), tumor site of the liver tissues from the nonresponder (*b*), nontumor site of liver tissue from a representative responder (*c*), tumor site of liver tissue from a representative responder (*d*), normal liver tissue (*e*), negative control staining with the immunizing peptide in the TRAIL-antibody (*f*), *D*, TRAIL-positive cell counts in liver tissues. NT, nontumor site; T, tumor site. Data are mean ± SD of more than six 360 magnification fields. *, $P < 0.05$ compared with the normal liver.

cells of three responders. Representative specimens are shown in Fig. 5*C-c* and 5*C-d*. In contrast, TRAIL-expressing cells were not detected in the nonresponder or in the normal liver (Fig. 5*C-a*, *b*, *e*). Quantitative analysis revealed significantly higher numbers of TRAIL-positive mononuclear cells in the tumor site and nontumor site of liver tissues of responders compared with those of nonresponder and normal liver tissue (Fig. 5*D*). These results suggest the possible contribution of TRAIL at the tumor site.

DISCUSSION

HCC often invades major adjacent vessels such as the portal vein, causes intra-hepatic metastasis, and finally becomes uncontrollable despite repetitive and multimodal therapy (1). We have previously reported the efficacy of IFNα and 5-FU combination therapy against advanced HCC based on its high responsive rate and low incidence of side effects (8). Although some reports have characterized the synergistic action of IFNα and 5-FU against colon carcinoma cells (9, 10), the reasons for IFNα enhancement of the antitumor effect of 5-FU against HCC has not yet been elucidated. In the present study, we showed in our *in vitro* experiments that TRAIL receptors on HCC cells were up-regulated by 5-FU and that IFNα induced TRAIL on $CD4^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells. Furthermore, the cytotoxic action of $CD14^+$ monocytes and $CD56^+$ NK cells against HCC cells was enhanced through the TRAIL-mediated pathway after treatment of effector cells by IFNα and target HCC cells by 5-FU. These *in vitro* results suggest the possible contribution of TRAIL/TRAIL receptor-mediated antitumor effector mechanism by host innate immune cells to the antitumor effect of IFNα/5-FU combination therapy. Consistent with this notion, a positive correlation between the clinical response and expression of TRAIL in PBMC was observed in patients with HCC who received the combination therapy. Moreover, TRAIL-R1 and R2 were expressed in HCC cells of all IFNα/5-FU–treated patients, and TRAIL-expressing tumor infiltrating cells were found in those patients who responded to the combination therapy.

It is conceivable that the TRAIL-mediated antitumor activity requires the expression of death-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, on tumor cells (29, 30). However, it is still not clear whether the basal expression level of TRAIL-R1 or TRAIL-R2 correlates with sensitivity to TRAIL. Previous studies suggested a positive correlation between TRAIL sensitivity and TRAIL-R1 or TRAIL-R2 expression in melanomas, renal cell carcinomas, and Jurkat clones (29–31), whereas other studies suggested no correlation in breast carcinomas and multiple myelomas (32, 33). In HCC cell lines, it has been reported that their TRAIL sensitivity did not correlate with the basal expression level of TRAIL receptors (34, 35). In the present study, three of five HCC cell lines (HuH7, PLC/PRF/5, and HLF) were rather resistant and two of five HCC cell lines (HLE and HepG2) were highly sensitive to soluble TRAIL. Compared with TRAIL-resistant HCC cell lines, the TRAIL-sensitive HCC cell lines expressed higher basal levels of TRAIL-R2. In contrast, the basal expression of TRAIL-R1 did not correlate with TRAIL sensitivity. All five HCC cell lines showed negative basal expression of TRAIL-R3 and R4, despite different TRAIL sensitivity. These results indicate that the basal expression level of TRAIL-R2 correlates positively with TRAIL sensitivity of HCC cells.

Treatment of HCC cells with TRAIL and 5-FU showed a variable sensitivity to TRAIL among the five HCC cell lines. A synergistic effect was observed against one cell line (HLF) and an additive effect was observed against the other four cell lines. It has been reported that chemotherapeutic agents, such as doxorubicin, camptothecin, and cisplatin, also synergized with TRAIL to induce apoptosis in HCC cell lines (34, 35). These results are consistent with the report that coadministration of recombinant TRAIL and 5-FU exhibited a synergistic antitumor effect in a mouse model (36). Concerning the modulation of TRAIL receptor expression by 5-FU, all TRAIL receptors, including TRAIL-R1 and TRAIL-R2, were significantly up-regulated in four of five HCC cell lines. A similar up-regulation of TRAIL-R2 was observed previously with DNA-damaging chemotherapeutic drugs such as doxorubicin, cisplatin, and etoposide (33, 37, 38). Previous studies suggested that the synergistic effect of TRAIL and chemotherapeutic agents might be induced through the induction of TRAIL-R2 expression (29, 33, 38). Our result also showed that 5-FU synergistically enhanced TRAIL-mediated cytotoxicity through up-regulation of TRAIL-R1 and R2 in HLF cells. Although up-regulation of TRAIL-R1 and R2 by 5-FU was observed, 5-FU additively enhanced TRAIL-mediated cytotoxicity in HuH7, PLC/PRF/5, and HepG2 cells. In addition, neither up-regulation of TRAIL-receptors nor synergy of 5-FU and TRAIL were observed in HLE cells. These results imply that alteration of TRAIL-receptor expression might depend on differences between HCC cell lines. Previous studies suggested that the alternation of other intracellular factors determines the TRAIL sensitivity (31, 39). It has been shown that TRAIL-induced apoptosis could be inhibited by FLIP and Bcl-xL (40, 41). Recent reports showed the importance of Bax and Bak, which regulate the release of cytochrome *c* and Smac/DIABLO from the mitochondria during TRAIL-induced apoptosis (42, 43). Additional studies are needed to address the possible effect of 5-FU on these intracellular molecules in HCC cells.

We showed that IFNα markedly induced TRAIL expression on $CD14^+$ monocytes and $CD56^+$ NK cells in PBMC. These results are consistent with previous reports (16, 44). 5-FU did not affect the induction of TRAIL by IFNα. We showed that $CD14^+$ monocytes and $CD56^+$ NK cells were the major effector cells that kill target HCC cells *in vitro*, and their cytotoxicity was enhanced by IFNα. This enhancement was predominantly mediated by TRAIL, because neutralizing anti-TRAIL mAb mostly abrogated it. Moreover, when target HCC cells were pretreated with 5-FU, the cytotoxicity by IFNα-stimulated $CD14^+$ monocytes and $CD56^+$ NK cells was markedly enhanced, which was also at least partly mediated by TRAIL. These results suggested that the induction of TRAIL by IFNα in innate immune effector cells, such as NK cells and monocytes, and the modulation of TRAIL sensitivity of HCC target cells may be involved in the beneficial effect of IFNα/5-FU combination therapy. Because FasL and perforin, in addition to TRAIL, are key cytotoxic effector molecules used by IFNα-

activated NK cells (45, 46), additional studies are needed to clarify the contribution of these molecules.

Among the HCC patients who received IFNα/5-FU combination therapy, the expression of TRAIL mRNA in PBMC was significantly higher in clinical responders than in nonresponders. This suggests that up-regulation of TRAIL mRNA in PBMC may be a predictor of the clinical response to the combination therapy. Consistently, Lancaster *et al.* (47) reported recently that overexpression of TRAIL mRNA was associated with favorable survival after chemotherapy in ovarian cancers. In addition, we showed the infiltration of TRAIL-expressing mononuclear cells into the TRAIL-R1– and TRAIL-R2–expressing HCC tissue in responders to the combination therapy. These results support the possible contribution of TRAIL to the clinical response.

Despite the development of various treatment modalities, the prognosis of patients with advanced HCC associated with Vp3 remains extremely poor (6). One of the major obstacles is the low sensitivity of this type of HCC to chemotherapeutic drugs (7). Therefore, the development of combination therapy with various agents exerting differential antitumor effects is urgent and mandatory. In this respect, IFNα-induced TRAIL-mediated cytotoxicity by immune effector cells may be promising, because TRAIL exerts antitumor activity without significant toxicity against normal tissues (37, 48, 49). Consistent with this notion, no measurable hepatotoxicity has been observed in patients receiving IFNα/5-FU combination therapy.

In conclusion, the results of our *in vitro* and *in vivo* studies suggest that modulation of the TRAIL/TRAIL receptor-mediated cytotoxic pathway might partially contribute to the antitumor effects of IFNα and 5-FU combination therapy against HCC. Strategies aimed at further increasing TRAIL expression in immune effector cells and further sensitizing tumor cells to TRAIL are expected to augment the therapeutic effect of such treatment.

REFERENCES

1. Farmer DG, Rosove MH, Shaked A, Busuttil RW. Current treatment modalities for hepatocellular carcinoma. Ann Surg 1994;219:236–47.

2. Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002;122:1609–19.

3. Barnett CC Jr, Curley S. A. Ablative techniques for hepatocellular carcinoma. Semin Oncol 2001;28:487–96.

4. Fujii T, Takayasu K, Muramatsu Y, et al. Hepatocellular carcinoma with portal tumor thrombus: analysis of factors determining prognosis. Jpn J Clin Oncol 1993;23:105–9.

5. Ikai I, Yamaoka Y, Yamamoto Y, et al. Surgical intervention for patients with stage IV-A hepatocellular carcinoma without lymph node metastasis: proposal as a standard therapy. Ann Surg 1998;227:433–9.

6. Fan J, Wu ZQ, Tang ZY, et al. Multimodality treatment in hepatocellular carcinoma patients with tumor thrombi in portal vein. World J Gastroenterol 2001;7:28–32.

7. Shen DW, Lu YG, Chin KV, Pastan I, Gottesman MM. Human hepatocellular carcinoma cell lines exhibit multidrug resistance unrelated to MRD1 gene expression. J Cell Sci 1991;98:317–22.

8. Sakon M, Nagano H, Dono K, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer (Phila) 2002;94:435–42.

9. Schwartz EL, Hoffman M, O'Connor CJ, Wadler S. Stimulation of 5-fluorouracil metabolic activation by interferon-alpha in human colon carcinoma cells. Biochem Biophys Res Commun 1992;182:1232–9.

10. van der Wilt CL, Smid K, Aherne GW, Noordhuis P, Peters GJ. Biochemical mechanisms of interferon modulation of 5-fluorouracil activity in colon cancer cells. Eur J Cancer 1997;33:471–8.

11. Eguchi H, Nagano H, Yamamoto H, et al. Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res 2000;6:2881–90.

12. Brassard DL, Grace MJ, Bordens RW. Interferon-alpha as an immunotherapeutic protein. J Leukoc Biol 2002;71:565–81.

13. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998;67:227–64.

14. Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K, Yagita H. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J Exp Med 1999;189:1451–60.

15. Sato K, Hida S, Takayanagi H, et al. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. Eur J Immunol 2001;31:3138–46.

16. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J Exp Med 1999;189:1343–54.

17. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995;3:673–82.

18. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996;271:12687–90.

19. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science (Wash DC) 1997;276:111–3.

20. Walczak H, Degli-Esposti MA, Johnson RS, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997;16:5386–97.

21. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science (Wash DC) 1997;277:815–8.

22. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science (Wash DC) 1997;277:818–21.

23. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997;7:813–820.

24. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 2003;63:912–6.

25. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. Nature's TRAIL-on a path to cancer immunotherapy. Immunity 2003;18:1–6.

26. Kayagaki N, Yamaguchi N, Nakayama M, et al. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. J Immunol 1999;162:2639–47.

27. Phillips TA, Ni J, Pan G, et al. TRAIL (Apo-2L) and TRAIL receptors in human placentas: implications for immune privilege. J Immunol 1999;162:6053–9.

28. Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269–335.

29. Griffith TS, Fialkov JM, Scott DL, et al. Induction and regulation of tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand-mediated apoptosis in renal cell carcinoma. Cancer Res 2002;62:3093–9.

30. Nguyen T, Zhang XD, Hersey P. Relative resistance of fresh isolates of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Clin Cancer Res 2001;7:966s–973s.

31. Jang YJ, Park KS, Chung HY, Kim HI. Analysis of the phenotypes of Jurkat clones with different TRAIL-sensitivities. Cancer Lett 2003; 194:107–17.

32. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59:734–41.

33. Mitsiades CS, Treon SP, Mitsiades N, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 2001;98:795–804.

34. Yamanaka T, Shiraki K, Sugimoto K, et al. Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 2000;32:482–90.

35. Shin EC, Seong YR, Kim CH, et al. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 2002;34:114–22.

36. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Investig 1999;104:155–62.

37. Wu GS, Burns TF, McDonald ER 3rd, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141–3.

38. Nagane M, Pan G, Weddle JJ, Dixit VM, Cavenee WK, Huang HJ. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res 2000;60:847–53.

39. Lacour S, Hammann A, Wotawa A, Corcos L, Solary E, Dimanche-Boitrel MT. Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res 2001;61:1645–51.

40. Okano H, Shiraki K, Inoue H, et al. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Investig 2003;83:1033–43.

41. Kim IK, Jung YK, Noh DY, et al. Functional screening of genes suppressing TRAIL-induced apoptosis: distinct inhibitory activities of Bcl-XL and Bcl-2. Br J Cancer 2003;88:910–7.

42. Kandasamy K, Srinivasula SM, Alnemri ES, et al. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. Cancer Res 2003;63:1712–21.

43. Kim JY, Kim YH, Chang I, et al. Resistance of mitochondrial DNA-deficient cells to TRAIL: role of Bax in TRAIL-induced apoptosis. Oncogene 2002;21:3139–48.

44. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94–100.

45. Kirou KA, Vakkalanka RK, Butler MJ, Crow MK. Induction of Fas ligand-mediated apoptosis by interferon-alpha. Clin Immunol 2000;95: 218–26.

46. Kaser A, Enrich B, Ludwiczek O, Vogel W, Tilg H. Interferon-alpha (IFN-alpha) enhances cytotoxicity in healthy volunteers and chronic hepatitis C infection mainly by the perforin pathway. Clin Exp Immunol 1999;118:71–7.

47. Lancaster JM, Sayer R, Blanchette C, et al. High expression of tumor necrosis factor-related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. Clin Cancer Res 2003;9:762–6.

48. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 1999;5:157–63.

49. Lawrence D, Shahrokh Z, Marsters S, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med 2001;7:383–5.