

Cyclooxygenase-2 inhibitor and interferon- β synergistically induce apoptosis in human hepatoma cells *in vitro* and *in vivo*

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Abstract. Recent clinical trials have shown that interferon (IFN) is effective for chemoprevention against hepatocellular carcinoma (HCC). However, it remains controversial as to whether IFN exerts direct cytotoxicity against HCC. Cyclooxygenase (COX)-2 also plays a role in hepatocarcinogenesis and may mediate resistance to apoptosis in HCC. Therefore, we aimed to elucidate the combined effect of COX-2 inhibitor, NS-398, and IFN on *in vitro* growth suppression of HCC using 3 hepatoma cell lines (HepG2, PLC/PRF/5, and Huh7) and *in vivo* nude mouse xenotransplantation model using Huh7 cells. Only minimal growth inhibition was observed after treatment with IFN- β alone in the 3 hepatoma cell lines. In contrast, treatment with NS-398 and IFN- β synergistically inhibited cell proliferation in dose- and time-dependent manner. Apoptosis was identified by 4',6-diamidino-2-phenylindole dihydrochloride and fluorescent staining. IFN- β up-regulated the expression of TRAIL, while NS-398 increased the expression of TRAIL receptors (especially of death receptor 5). Subsequently, activation of caspase-8 and caspase-3 was observed following the treatment with NS-398 and IFN- β . Blockade of TRAIL with a specific antibody attenuated this apoptosis. Furthermore, we found that IFN- β up-regulated COX-2 expression in Huh7 cells, and NS-398 might suppress the up-regulated COX-2 activity downstream of IFN signaling. *In vivo* experiment showed the combined regimen with NS-398 and IFN- β reduced the growth of xenotransplanted HCCs in nude mice. In conclusion, NS-398 is sufficient to overcome IFN resistance in hepatoma cells through the TRAIL/TRAIL receptor pathway, therefore, the

combination would appear to be a new therapeutic regimen for HCC.

Introduction

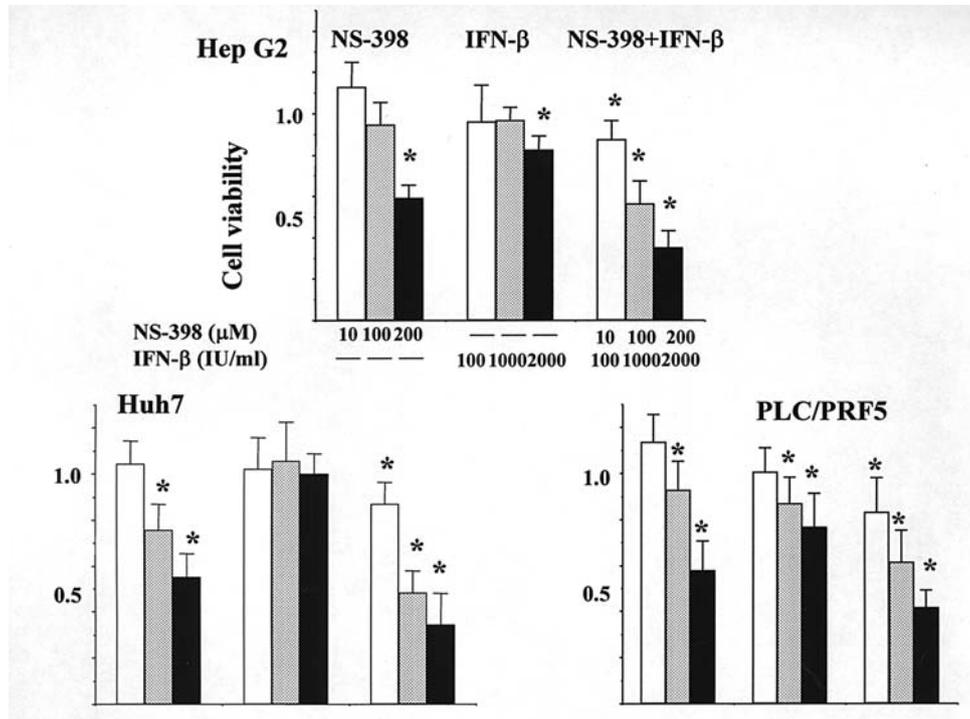
Interferon (IFN) plays an essential role in both antiviral and antitumor host defenses. Recent clinical trials have shown that IFN is effective in preventing the development of hepatocellular carcinoma (HCC) (1,2), but its precise mechanism has not yet been clarified. Recently it was found that IFN induces apoptosis by up-regulating tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in immune cells (3), melanoma cells (4), and even in solid tumor cells (5,6). These results suggest that autocrine TRAIL production is involved in the induction of apoptosis. TRAIL binds to two apoptosis-inducing receptors, TRAIL R1 (DR4) and TRAIL R2 (DR5); and two additional cell-bound receptors incapable of transmitting an apoptotic signal, TRAIL R3 (DcR1) and TRAIL R4 (DcR2). Both DR4 and DR5 have an intracellular death domain to transmit the apoptotic signal. Stimulation of DR4 and DR5 results in recruitment and activation of caspase-8 via Fas-associated death domain (FADD) (7). Activated caspase-8 then directly activates caspase-3, -6, and -7 (8) or activates the intrinsic mitochondria-mediated pathway through Bid cleavage (9). In HCC, it is unclear as to whether IFN is directly cytotoxic, and indeed, most hepatoma cell lines are resistant to IFN-mediated apoptosis *in vitro* (10). Consequently, combination agents are necessary to overcome IFN resistance.

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cell proliferation and induce apoptosis in a variety of cancer cell types (11-13) by inhibiting cyclooxygenase (COX). Two isoforms of COX, COX-1 and -2, have been identified. COX-1 is constitutively expressed in most tissues and is associated with PG production required for normal physiological function. In contrast, COX-2 is induced at the site of inflammation by mitogens, cytokines, and other factors. Overexpression of COX-2 has been reported in many human cancers (14-16), and a role in an early stage of hepatocarcinogenesis has also been described (17,18). Recent studies demonstrated that a selective COX-2 inhibitor reduced the growth of a variety of cancers, including HCC, *in vivo* (19,20)

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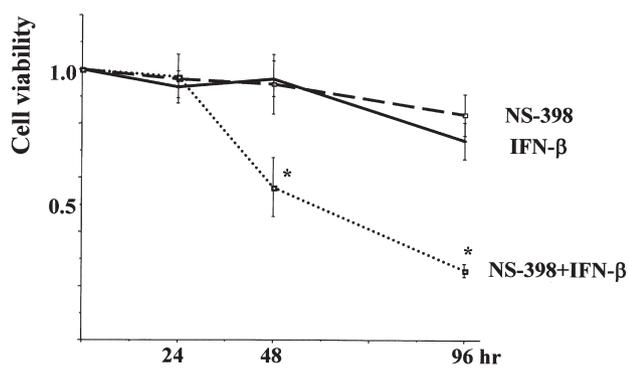
Key words: TRAIL, death receptor, Prostaglandin E2, nude mice, Huh7

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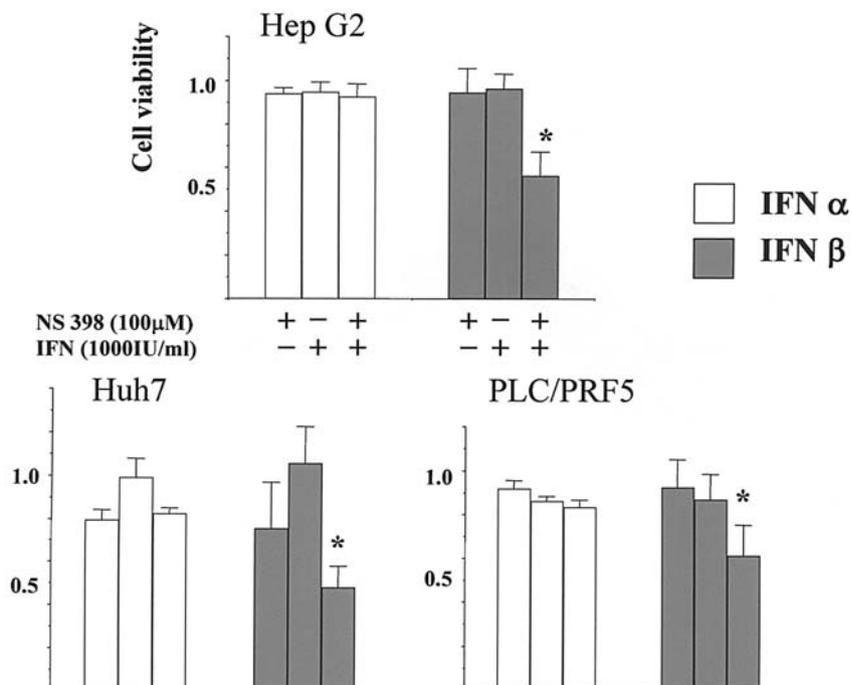
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Hep G2



and *in vitro* (21). The mechanism accounting for its anti-proliferative effect in cancer cells includes: inhibition of COX-2 and subsequent PGE2 activity (a COX-2-dependent pathway) (22), inactivation of the protein kinase, AKT (11), inhibition of Ca^{2+} influx into the endoplasmic reticulum (23), down-regulation of Bcl-2 (24), and induction of cell cycle arrest (25). Recently Liu *et al* (26) reported that celecoxib, a selective COX-2 inhibitor, induced DR5 up-regulation and subsequent apoptosis in human non-small lung cell carcinoma.

C



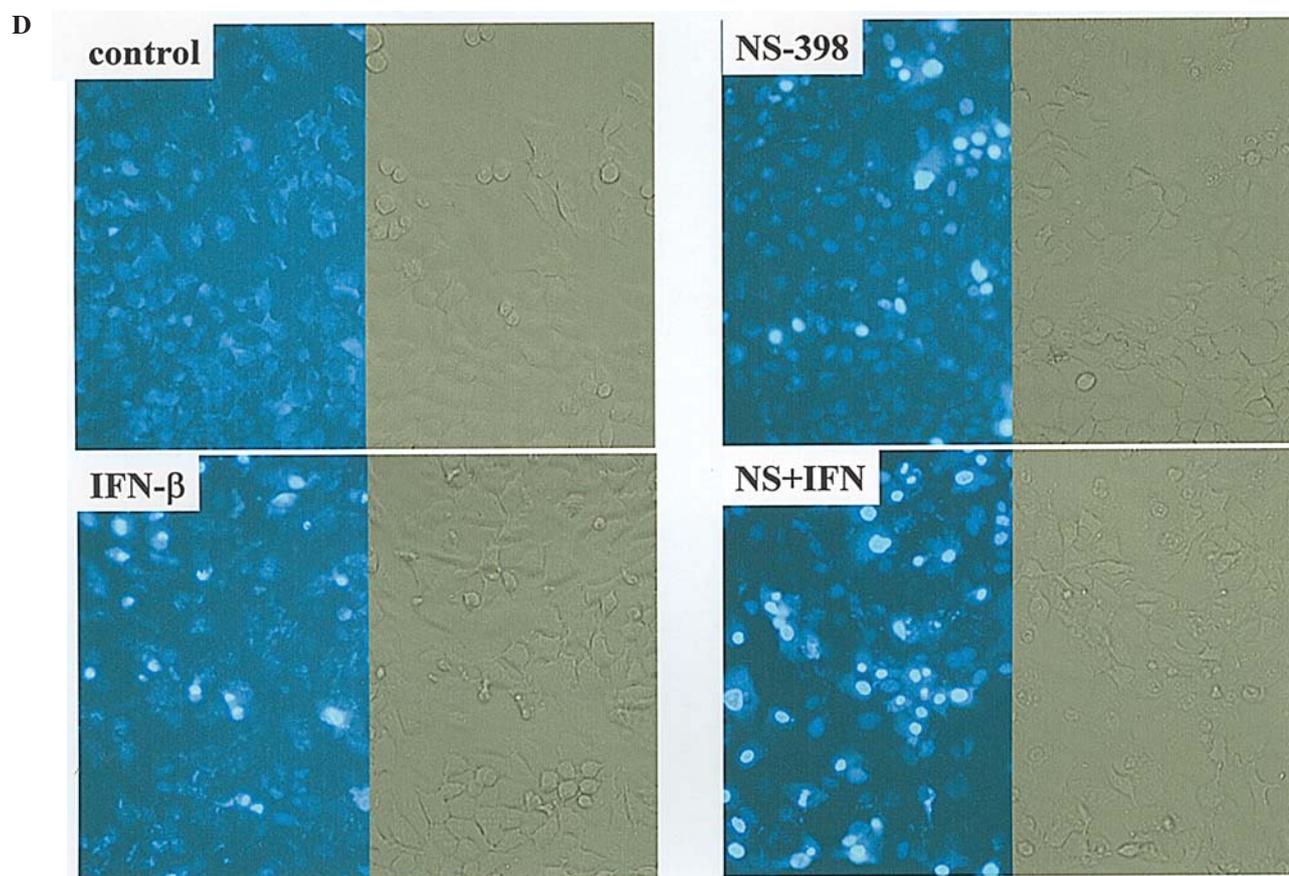


Figure 1. Effect of IFNs and NS-398 on cell growth and apoptosis in hepatoma cell lines. A, HepG2, Huh7, and PLC/PRF cells were treated with IFN- β (100, 1000, 2000 IU/ml) and/or NS-398 (10, 100, 200 μ M) for 48 h. Cell viability was determined by MTS assay. Data represent mean \pm SD of 12 samples. * p <0.05 compared with control cells in each cell line. B, HepG2 cells were treated with IFN- β (1000 IU/ml) and/or NS-398 (100 μ M). Cell viability at 24, 48, and 96 h following the administration was determined by MTS assay to assess time-dependence. Data represent mean \pm SD of 6 samples. * p <0.001 compared with cells treated with NS-398 or IFN- β alone at the indicated time-points. C, The growth-inhibitory effect of IFN- α (1000 IU/ml) in combination with NS-398 (100 μ M) at 48 h post-treatment was compared with that of IFN- β (1000 IU/ml) in the 3 hepatoma cell lines. Data represent mean \pm SD of 6 samples. * p <0.01 compared with cells treated with NS-398 or IFN- β alone in each cell line. D, DAPI staining was performed to detect apoptosis in PLC/PRF/5 cells 48 h following each treatment. The result shown is one representative example from three experiments. A similar effect was observed in Huh7 and HepG2 cells.

These observations suggest that the combination of IFN with COX-2 inhibitors might amplify apoptosis of cancer cells by up-regulating the expression of both TRAIL and DR5. To test this hypothesis, we examined the apoptosis-inducing effect of combination treatment with IFN and a selective COX-2 inhibitor, NS-398, on human HCC cell lines both *in vitro* and *in vivo*.

Materials and methods

Reagents. NS-398 was purchased from Cayman Chemical (Ann Arbor, MI, USA). NS-398 was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C . Human recombinant IFN- α was purchased from Shering-Plough K.K. (Osaka, Japan) and IFN- β was purchased from Mochida Pharmaceutical Co. (Tokyo, Japan). Human recombinant TRAIL and neutralizing monoclonal anti-TRAIL was purchased from Alexis Biochemicals (San Diego, CA, USA), and PGE2 was purchased from Cayman Chemical.

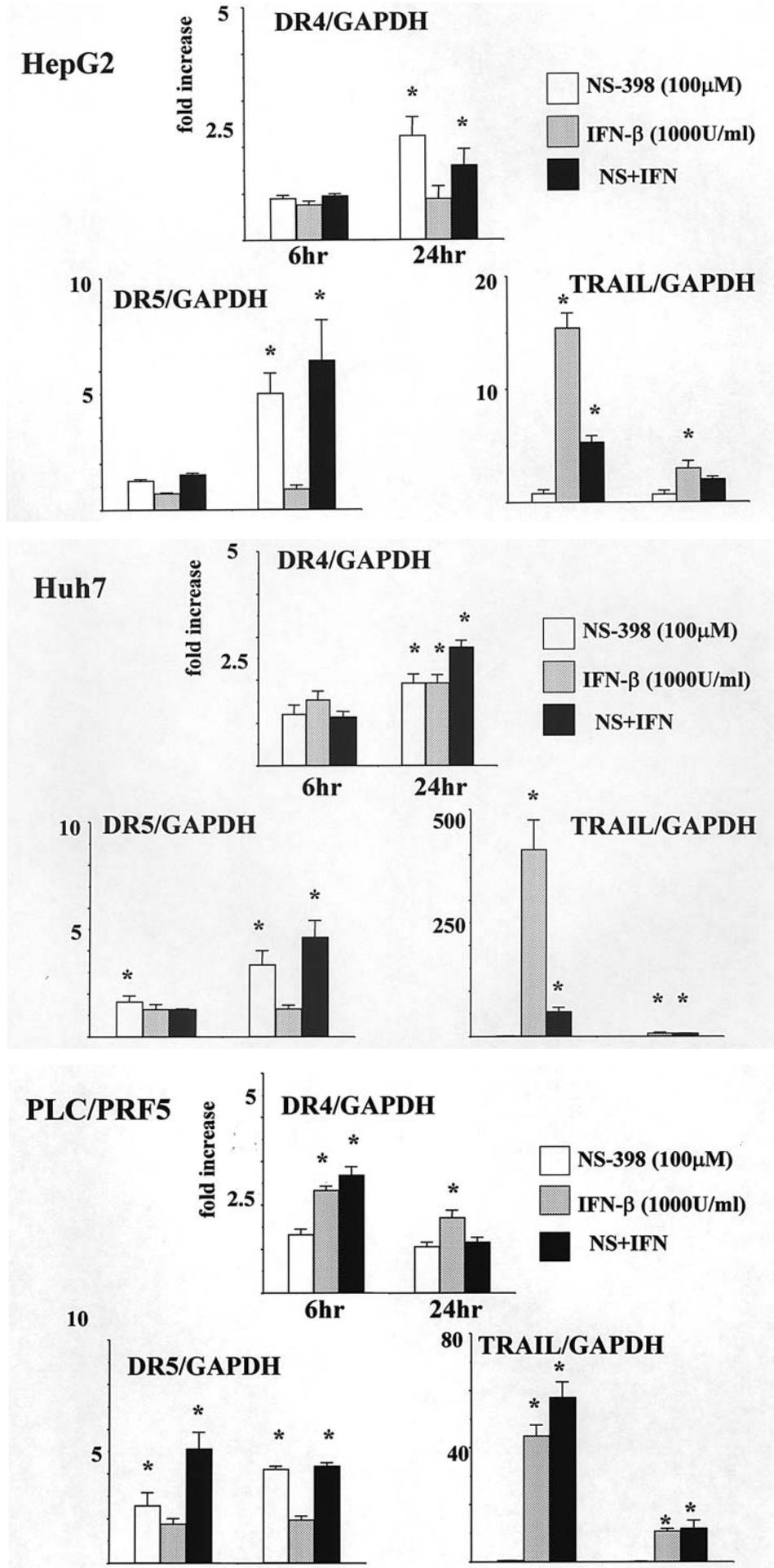
Cell lines and cell culture. Human hepatoma cell lines, HepG2, Huh7, and PLC/PRF/5 cells, were obtained from the Human Science Research Resources Bank (Osaka, Japan).

The 3 cell lines were cultured in DMEM with 1% penicillin/streptomycin and 10% heat-inactivated FBS at 37°C .

Cell growth assays. Cells (3×10^3 per well) were seeded in triplicate in 96-well microplates and allowed to attach. Twenty-four hours later, the medium was discarded and replaced with fresh medium containing various concentrations of NS-398 and/or IFN. The medium was changed every 48 h, and the number of viable cells at each time point was determined by MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instruction. The effect of soluble TRAIL, TRAIL neutralizing antibody, and PGE2 on cell growth was also evaluated.

Western blot analysis. Cells were washed twice with ice-cold PBS and lysed for 30 min on ice with lysis-buffer (30 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1 mmol/l PMSF, 1.5% Triton-X 100, and protease inhibitors). Samples containing 50 μ g protein were separated by SDS-PAGE and electrophoretically transferred to Hybond-P nitrocellulose membranes (Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA). After blocking for 60 min at room temperature (RT) with 5% non-fat milk in Tween-TBS, membranes were

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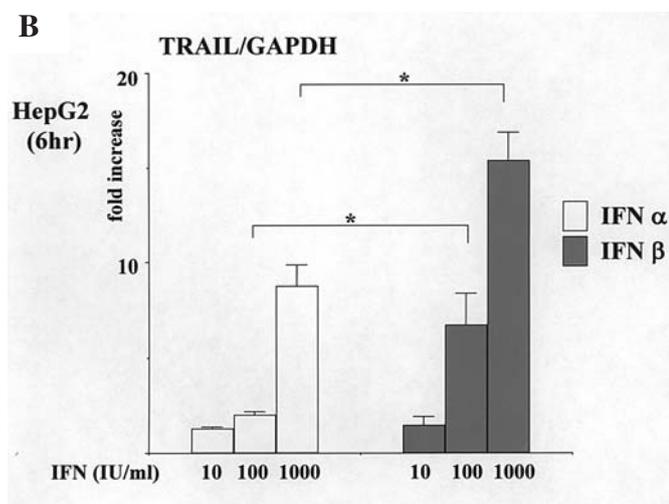
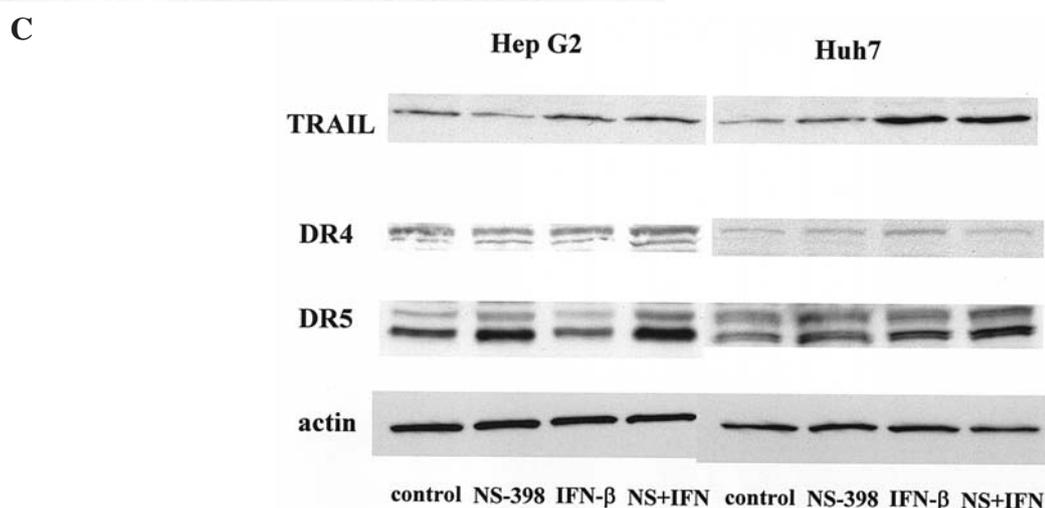


Figure 2. Transcription and expression of DR4, DR5, and TRAIL detected by real-time RT-PCR and Western blotting in hepatoma cell lines. A, Cells were treated with IFN- β (1000 IU/ml) and/or NS-398 (100 μ M) for 6 or 24 h. Expression levels of DR4, DR5, and TRAIL mRNA were determined by real-time RT-PCR as described in Materials and methods. The expression of each mRNA was normalized according to the expression levels of house-keeping GAPDH, and the increase of expression in comparison to that of control cells was calculated. Data represent mean \pm SD of 4 samples. * p <0.05 compared with control cells in each cell line. B, The difference in the effects of IFN- α (0-1000 IU/ml) and IFN- β (0-1000 IU/ml) on induction of TRAIL mRNA were evaluated. HepG2 cells were treated with IFN- α (0-1000 IU/ml) or IFN- β (0-1000 IU/ml) for 6 h. Data are represent mean \pm SD of 4 samples. * p <0.01 compared with the increase in IFN- α treated cells at the indicated concentrations. C, HepG2 and Huh7 cells were treated with IFN- β (1000 IU/ml) and/or NS-398 (100 μ M) for 24 h, and the expression of each protein was analyzed by Western blotting. The result shown is a representative example from 3 experiments.



incubated with the following antibodies: goat polyclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat polyclonal TRAIL (Santa Cruz Biotechnology), rabbit polyclonal anti-DR4 (Pro Science, Long Beach, CA, USA), goat polyclonal anti-DR5 (Alexis), and rabbit polyclonal anti-COX-2 (Cayman Chemical). After 3 washes for 10 min each, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 60 min at RT. Bands were reacted with the ECL detection reagent and captured on X-ray film.

Measurement of caspase activity. After treatment with NS-398 and/or IFN- β for the indicated time periods, activity of caspase-3 and -8 activity in these cells was assayed with an APOPCYTO colorimetric assay kit (MBL, Tokyo, Japan) according to the manufacturer's instruction.

Detection of apoptosis. Apoptotic cells were detected with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Briefly, after treatment with NS-398 and/or IFN- β for 48 h, the cells were washed 3 times with PBS and stained with 5 μ g/ml DAPI (Sigma Chemical Co.) for 30 min at 37°C and observed by fluorescence microscopy.

Real-time RT-PCR. Total cellular RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA),

according to the manufacturer's instruction. Real-time RT-PCR for DR4, DR5, TRAIL, and COX-2 was performed using the ABI PRISM 7700 instrument (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with gene-specific primers and the SYBR Green I protocol. A total of 1.0 μ g RNA was reverse-transcribed to complementary DNA (cDNA) in a total volume of 20 μ l, and 1 μ l of this mixture was used as a template for RT-PCR. The primers sequences were as follows: TRAIL: sense 5'-ACCAACGAGCTGAAGCAGAT-3', anti-sense 5'-TCCTTGATGATTCCCAGGAG-3'; DR4: sense 5'-CAGAACATCCTGGAGCCTGTAAC-3', anti-sense 5'-ATGTCCATTGCCTGATTCTTTGTG-3'; DR5 sense 5'-TGC AGCCGTAGTCTTGATTG-3', anti-sense 5'-GCACCAAG TCTGCAAAGTCA-3'; COX-2: sense 5'-TGAAACCCAC TCCAAACACA-3', anti-sense 5'-AACTGATGCGTGAAGT GCTG-3'; GAPDH: sense 5'-CAACTACATGGTTTACATG TTC-3', anti-sense 5'-GCCAGTGGACTCCACGAC-3'. Relative expression ratios normalized to that of GAPDH were calculated.

In vivo effect of NS-398 and IFN- β on HCC proliferation in nude mice. This experiment was approved and performed on the guideline of the Animal Experimentation Committee of the School of Medicine, Keio University. Female athymic nude mice (BALB/C, 5-6 week old, Clea Japan, Tokyo, Japan) were housed under controlled temperature and humidity

in a 12-h light-dark cycle. To create the xenograft model, cultured Huh7 cells (1×10^7 cells/mouse) were subcutaneously injected to the back of mice. Tumor size and body weight was measured three times weekly, and tumor volumes (mm^3) were evaluated as length \times (width) $^2 \times 1/2$. When the tumor volume reached $\sim 150 \text{ mm}^3$, the mice were randomly divided into four groups ($n=10$ each) and treatment was started. NS-398 was suspended in water with gum arabic and given orally with intubation at a dose of 15 mg/kg every day, and IFN- β was intraperitoneally injected at a dose of 5×10^4 IU/mice three times a week until day 14, and vehicle only was given as control. Four days after the cessation of treatment, on day 18, the mice were sacrificed and tumors were resected and fixed with 10% neutral-buffered formalin, and they were cut into 5- μm sections after embedding in paraffin. Apoptotic cell was evaluated by TUNEL method as described before (27) with some modifications. The number of TUNEL positive cells was counted in five randomly chosen non-overlapping areas of each section (magnification $\times 400$) and the percentage of positive cells compared with the total tumor cell number was calculated.

Statistical analysis. The data are expressed as mean \pm standard deviation (SD). Differences between the means of two groups were evaluated by the Kruskal-Wallis test or Mann-Whitney U test. A $p < 0.05$ was considered significant.

Results

COX-2 inhibitor amplified cell death of human hepatoma cells in addition to the effect of IFN- β . IFN- β alone decreased the viability of PLC/PRF/5 cells dose-dependently, but did not decrease the viability of Huh7 cells. Viability of HepG2 cells was inhibited by IFN- β alone only at a concentration of 2000 IU/ml. NS-398 alone caused a dose-dependent decrease in the viability of both Huh7 and PLC/PRF/5 cells, but decreased viability of HepG2 only at a concentration of 200 μM . On the other hand, the combination treatment synergistically decreased cell viability in a dose-dependent manner in all 3 cell lines (Fig. 1A).

Sequential changes in the viability of HepG2 cells are shown in Fig. 1B. At 24 h, there was no synergism between the effects of IFN- β and NS-398, but at later time-points, their effects were synergistically enhanced in a time-dependent manner. IFN- β alone resulted in 25% inhibition, NS-398 alone resulted in 15% inhibition, while the combination treatment caused 74.8% inhibition at 96 h.

We further examined the effect of IFN- α on the viability of these cell lines (Fig. 1C). In contrast to the result of IFN- β treatment, IFN- α did not significantly decrease cell viability in any of the 3 cell lines, whether it was used alone or in combination with NS-398.

Fig. 1D shows representative phase contrast and fluorescence photomicrographs with DAPI staining in PLC/PRF/5 cells. Combined treatment with IFN- β and NS-398 increased the number of cells with high fluorescence and nuclear condensation to a significantly greater extent than treatment with each agent alone. The morphometric analysis of the number of apoptotic cells in 5 randomly selected frames yielded results similar (data not shown) to those shown in Fig. 1A.

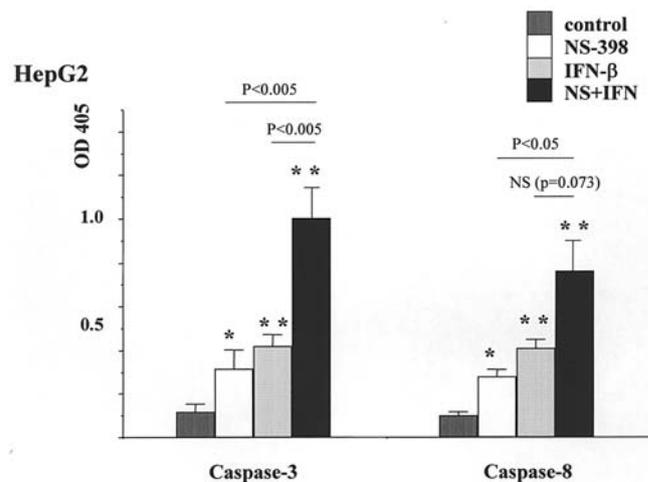


Figure 3. Effect of IFN- β and/or NS-398 on caspase-3 and -8 activity in HepG2 cells. HepG2 cells were treated with IFN- β (1000 IU/ml) and/or NS-398 (100 μM) for 24 h, followed by a measurement of the activity of caspase-3 and -8 using a colorimetric assay kit. Data represent mean \pm SD of 6 samples. * $p < 0.05$; ** $p < 0.005$ compared with control cells.

IFN- β up-regulates TRAIL expression and NS-398 up-regulates TRAIL receptor expression. Real-time RT-PCR showed that IFN- β (1000 IU/ml) remarkably increased the levels of TRAIL mRNA even at 6 h following the treatment in the 3 cell lines (Fig. 2A), and the expression level was about 400-fold higher than that of control in Huh7 cells. This up-regulation was dose-dependent and up-regulated levels of TRAIL were much lower with IFN- α treatment than with IFN- β (Fig. 2B). IFN- β did not alter the level of DR5 mRNA in any of the cell lines, while DR4 mRNA levels were slightly up-regulated in Huh7 and PLC/PRF/5 cells, but not in HepG2 cells. In contrast, treatment with NS-398 (100 μM) up-regulated the transcription of TRAIL receptors, and notably, increased DR5 mRNA levels up to 5-fold. TRAIL mRNA levels, however, were not changed by NS-398. Consequently, combination treatment induced the expression of both TRAIL ligand and TRAIL receptor.

A typical result of Western blotting is shown in Fig. 2C. NS-398 and IFN- β increased the expression of DR5 and TRAIL, respectively, and combination treatment amplified both expressions.

Caspase activation. Activation of caspase-8 was induced by either NS-398 or IFN- β alone, and even more so when they were given in combination. The caspase-8 activity of HepG2 at 24 h after treatment was about 6-fold higher than that of control cells, followed by the activation of caspase-3 (Fig. 3).

Response to exogenous TRAIL stimulation and blockade by the antibody and PGE2. As shown in Fig. 4, soluble TRAIL at a concentration of 100 ng/ml reduced the viability of HepG2 cells (83.5% vs. control). NS-398 enhanced this reduction to 68.3%, while IFN- β did not. The enhanced reduction of cell viability with NS-398 was also observed in Huh7 cells.

We then examined effect of the TRAIL neutralizing antibody (1 $\mu\text{g}/\text{ml}$) in HepG2 and Huh7 cells (Fig. 5). In HepG2 cells, 1 $\mu\text{g}/\text{ml}$ of the TRAIL neutralizing antibody partially rescued growth suppression induced by NS-398 (100 μM) and IFN- β (1000 IU/ml) from 56.2 to 73.7%. In

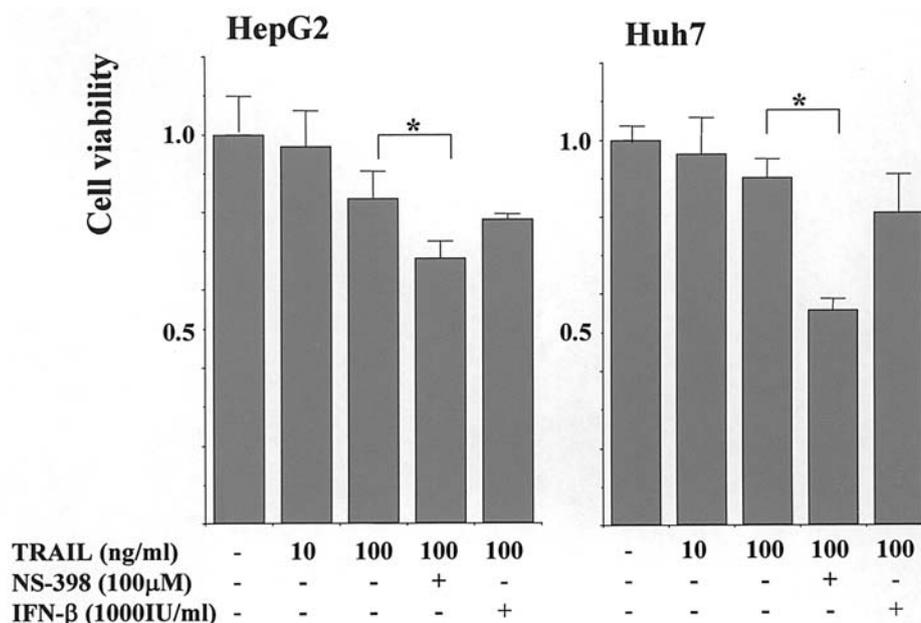


Figure 4. Effect of IFN- β and NS-398 on TRAIL-induced cytotoxicity in HepG2 and Huh7 cells. Cells were treated with recombinant TRAIL (10, 100 ng/ml) in combination with IFN- β (1000 IU/ml) or NS-398 (100 μ M) for 48 h. Cell viability was determined by MTS assay. Data represent mean \pm SD of 6 samples. * p <0.05 compared with soluble TRAIL (100 ng/ml)-treated cells.

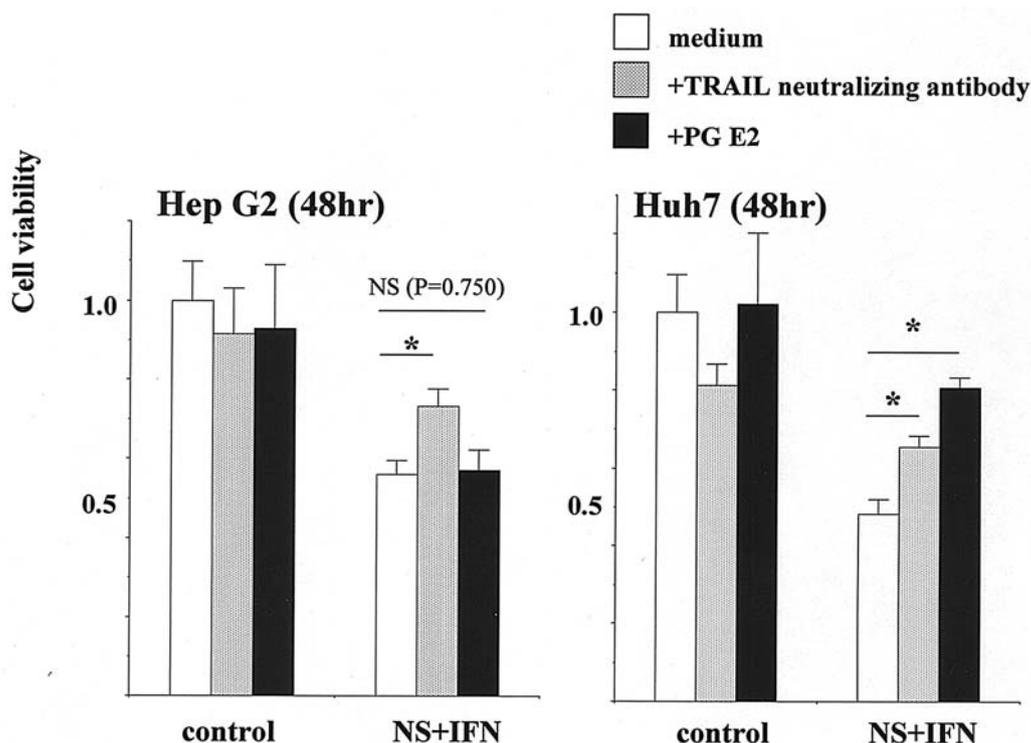


Figure 5. Effect of the anti-TRAIL neutralizing antibody and PGE2 on toxicity induced by IFN- β and/or NS-398 in HepG2 and Huh7 cells. Cells were treated with IFN- β (1000 IU/ml) and/or NS-398 (100 μ M) for 48 h in the absence or presence of the anti-TRAIL neutralizing antibody (1 μ g/ml) and PGE2 (10 μ M). Cell viability was determined by MTS assay. Data represent mean \pm SD of 6 samples. * p <0.001 compared with cells incubated in a culture medium.

Huh7 cells, the growth suppression was rescued from 48.3 to 65.5% in the presence of TRAIL neutralizing antibody. On the other hand, treatment with PGE2 changed the viability of Huh7 cells from 48.3 to 80.5%, while that of HepG2 was not significantly improved (from 56.2 to 57.1%).

IFN- β increases COX-2 expression. We examined the direct effect of IFN- β on COX-2 expression in Huh7 cells. Real-time RT-PCR showed that IFN- β remarkably increased COX-2 mRNA levels in a dose-dependent manner (Fig. 6A). Western blotting showed that IFN- β increased COX-2 protein levels

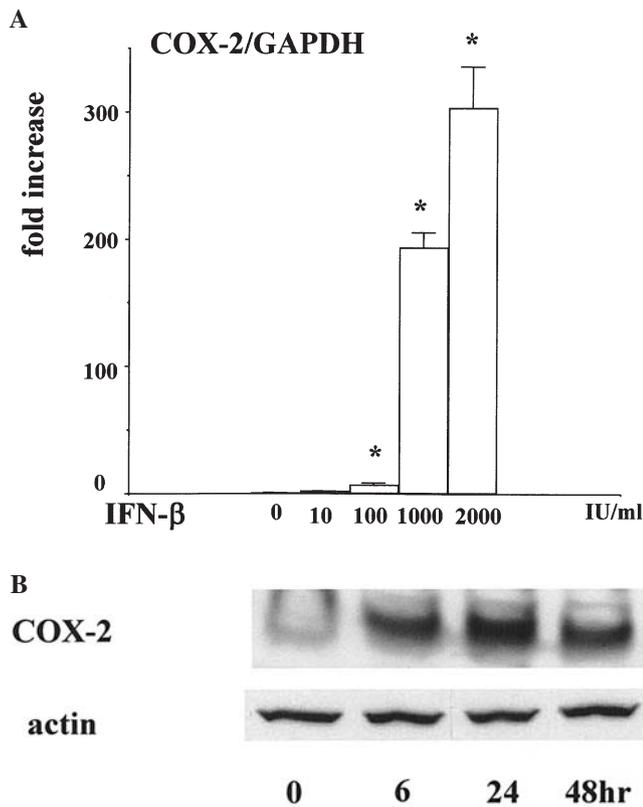


Figure 6. Effect of IFN- β on COX-2 expressions in Huh7 cells. A, Cells were treated with IFN- β (0-2000 IU/ml) for 24 h. Expression of COX-2 mRNA was examined by real-time RT-PCR. Data represent mean \pm SD of 4 samples. * p <0.05 compared with control cells. B, Cells were treated with IFN- β (1000 IU/ml) for 6, 24, and 48 h. COX-2 protein levels were examined by Western blotting. Results shown are a representative example from 3 experiments.

in a time-dependent manner, and the peak was detected at 24 h after stimulation (Fig. 6B).

In vivo effect of NS-398 and IFN- β on HCC proliferation in nude mice. Even single treatment with either NS-398 or IFN- β showed a significant inhibitory effect on HCC development compared with the control group (Fig. 7). Furthermore, tumor volumes in combined treated group became significantly smaller than other three groups on the 5th day and later. Growth inhibitory rate at the end of the experiment (day 18) in the NS-398 treated group, the IFN- β treated group, and the combination treatment group were 50, 46, and 86%, respectively. As shown in Fig. 8, the number of TUNEL-positive cells was significantly increased in the combination treatment group.

Discussion

In this study, we demonstrated that NS-398 and IFN- β synergistically suppress cell growth and induce apoptosis in human hepatoma cell lines. We found that NS-398 up-regulated the expression of DR, especially that of DR5, while IFN- β up-regulated the expression of TRAIL, and the combination of both agents induced apoptosis through caspase-8 and -3 activation. Furthermore, apoptosis induced by NS-398 and IFN- β was inhibited by the TRAIL neutralizing antibody in

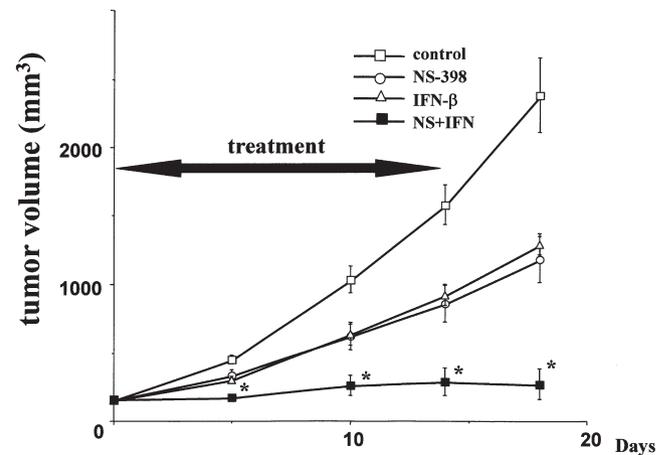


Figure 7. Effect of IFN- β and NS-398 on HCC development in nude mice. Administration of IFN- β and NS-398 was started when the tumor volume reached to \sim 150 mm³. Treatment group consisted of either control group, NS-398 treated group (15 mg/kg p.o., every day), IFN- β treated group (5×10^4 IU i.p., 3/week), and both NS-398 and IFN- β treated group. Tumor size was measured at the indicated time-point. Data represent mean \pm SD of 10 samples. * p <0.05 compared with other group.

both Hep G2 and Huh7 cells. The results demonstrated that these effects are mediated, at least partially, through the TRAIL pathway. At present, the mechanism by which NS-398 increases DR5 expression is unknown. However, this effect is most likely p53-independent, since DR5 induction was observed in both p53 wild (HepG2) and p53 mutant (PLC/PRF5 and Huh7) hepatoma cells.

In addition, we examined whether or not the synergism was COX-2 dependent, which has been an area of controversy (28). Several factors are thought to be involved in the mechanism by which COX-2 contributes to tumorigenesis, such as an increase in angiogenesis through the production of vascular endothelial growth factor (VEGF), an increase in invasiveness through the activation of matrix metalloproteinases (MMPs), and an increase in cell adhesion through up-regulation of b1 and α V β 3 integrin expression (29). Our results showed that exogenous PGE2 addition partially inhibited apoptosis induced by NS-398+IFN- β in Huh7 cells, but not in HepG2 cells, indicating that the COX-2 dependent pathway is also partially involved in this mechanism. Although we could not fully explain the difference in results between Huh7 and HepG2 cells, it is possible that COX-2 expression under basal conditions in each cell line is a contributing factor. Indeed, the expression level of COX-2 mRNA in Huh7 under basal condition was much higher than that in HepG2 (data not shown). We further investigated whether IFN directly affects the activity of COX-2 in hepatoma cell lines. Recent studies have demonstrated direct activation of the COX-2 gene by IFN- γ in mice macrophages through binding to the ISRE elements in the mouse COX-2 promoter which was highly preserved in the human COX-2 gene (30). Furthermore, COX-2 induction by type 1 IFN (IFN- α) was also reported in human small non-small cell lung cancer cells (31). As shown in Fig. 6, IFN- β up-regulated the amount of COX-2 protein and mRNA levels as early as 6 h, and its peak was detected 24 h following drug administration. These results suggested

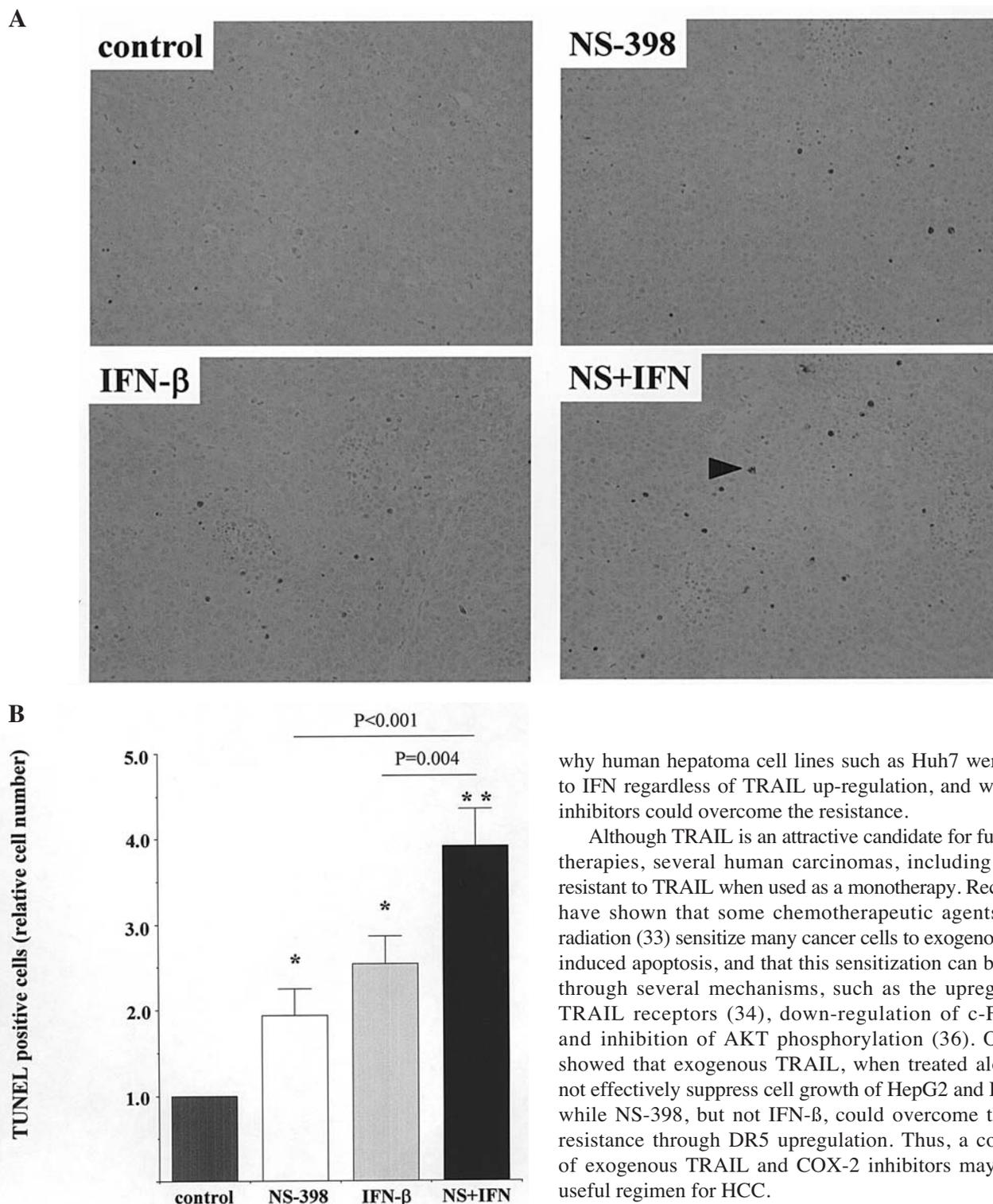


Figure 8. TUNEL staining in tumors derived from mice in each group. A, Representative photographs. Black arrow indicates the apoptotic cell in the tumor (x200). B, Semiquantitative analysis of TUNEL-immunopositive cells. For comparison of the different treatment groups, the control was arbitrarily set to one. Data represent mean \pm SD of 5 samples. *p<0.005 and **p<0.001 compared with control group.

the involvement of another mechanism in the synergism by NS-398 and IFN- β , at least in certain hepatoma cell lines, i.e., COX-2 inhibitors directly suppress the up-regulated COX-2 activity and subsequent PGE2 production downstream of IFN signaling. IFN-mediated COX-2 induction might also explain

why human hepatoma cell lines such as Huh7 were resistant to IFN regardless of TRAIL up-regulation, and why COX-2 inhibitors could overcome the resistance.

Although TRAIL is an attractive candidate for future cancer therapies, several human carcinomas, including HCC, are resistant to TRAIL when used as a monotherapy. Recent studies have shown that some chemotherapeutic agents (32) and radiation (33) sensitize many cancer cells to exogenous TRAIL-induced apoptosis, and that this sensitization can be achieved through several mechanisms, such as the upregulation of TRAIL receptors (34), down-regulation of c-FLIP (35), and inhibition of AKT phosphorylation (36). Our results showed that exogenous TRAIL, when treated alone, could not effectively suppress cell growth of HepG2 and Huh7 cells, while NS-398, but not IFN- β , could overcome the TRAIL resistance through DR5 upregulation. Thus, a combination of exogenous TRAIL and COX-2 inhibitors may also be a useful regimen for HCC.

IFN- α and IFN- β may have different anti-proliferative effects in many cancers and IFN- β has been reported to have greater apoptosis-inducing effects than IFN- α (35-37). In human hepatoma cell lines, IFN- β was shown to be more potent anti-proliferative than IFN- α , either when used alone or in combination with certain anticancer drugs (38). Our results showed that IFN- β had similar anti-proliferative effect as IFN- α when used alone, but IFN- α did not have a synergistic effect with NS-398 at the concentrations tested. We speculated that the extent of TRAIL production might contribute to the difference. The results of real-time RT-PCR (Fig. 2B) revealed that both IFN- α and IFN- β up-regulated TRAIL mRNA in a dose-dependent manner, and it is suggested

that IFN- β is a much stronger TRAIL inducer than IFN- α in HepG2 cells.

Several studies have suggested that combined administration of IFN- α with NSAIDs can improve virological response in chronic hepatitis C (39,40), although the opposite result has also been reported (41). Some of the proposed mechanisms for this synergism include increased STAT1 phosphorylation (42), and upregulation of serum 2'5'-oligoadenylate synthetase (43). Giambartolomei *et al* showed that maximal STAT1 phosphorylation was achieved when IFN- α was administered at a concentration of 300 IU/ml (42). In our study, the synergism between IFN- β + NS-398 was achieved in a dose-dependent manner, indicating that the synergistic effect on virological response and that on apoptosis are different mechanisms.

Our *in vivo* study showed that single treatment of either NS-398 or IFN- β reduced the growth of xenotransplanted HCCs and that combined treatment of NS-398 and IFN- β showed a more inhibitory effect. Immunohistochemical study with TUNEL staining revealed that apoptosis played at least a partial role in the growth inhibition. These results underline and strengthen the effect of combination treatment shown in the *in vitro* study. One limitation of this nude mouse tumor implant approach is that it does not allow us to examine the auto-immune response, so further study using another carcinogenic model is needed for future clinical use.

In conclusion, we demonstrated that IFN- β and NS-398 synergistically induce cell death of human hepatoma cells through the TRAIL/death receptor-mediated apoptotic pathway, and possibly through COX-2 inhibition. TRAIL-based cancer therapy is currently used clinically, and our results imply that TRAIL in combination with COX-2 inhibitors could also be an effective therapeutic regimen for the future treatment of HCC. This is the first report to show that the COX-2 inhibitor such as NS-398 can overcome IFN resistance in the treatment of human hepatoma both *in vitro* and *in vivo*, and this combination treatment may be an effective chemotherapeutic regimen for the treatment of HCC.

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