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Effect of interferon-γ on the susceptibility to Fas (CD95/APO-1)-mediated cell death in human hepatoma cells

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Abstract Many tumors, including hepatocellular carcinomas (HCCs), resist Fas-mediated cell death, which is one of the effector mechanisms in the host's anti-tumor response; however, this resistance can be abolished by interferon-γ (IFN-γ). IFN-γ may sensitize Fas-mediated cell death in several ways, but the exact mechanism in HCCs is uncertain. In this study, we thoroughly investigated the effect of IFN-γ on the susceptibility of one human normal liver cell line and 12 HCC cell lines to Fas-mediated cell death. We also investigated the effect of IFN-γ on the expression of various apoptosis-related genes such as the Fas/TNF-related genes, the *bcl-2* family, and the caspase family of genes. Although most cell lines showed considerable constitutive expression of Fas, all tested cell lines resisted Fas-mediated cell death without IFN-γ. When cells were pretreated with IFN-γ, only three cell lines were made significantly susceptible to Fas-mediated cell death (SNU-354, SNU-387 and SNU-423); the other 10 cell lines were not affected. IFN-γ increased the mRNA expression of Fas, TRAIL and caspase-1, and surface Fas was also increased. The strongly sensitized cell lines (SNU-354, SNU-387 and SNU-423) showed a particularly potent increment in surface Fas after IFN-γ treatment (increase in surface Fas >1.7-fold). This result enabled us to conclude that a potent increment of surface Fas expression is a major sensitizing mechanism of IFN-γ. We conclude that IFN-γ cannot play a sensitizing role in most HCC cell lines and that IFN-γ makes HCC cells susceptible to Fas-mediated cell death through a marked up-regulation of surface Fas in some HCC cells.

Key words IFN-γ · Fas · Cell death · Hepatoma

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Introduction

Fas (CD95/APO-1) belongs to the tumor necrosis factor (TNF) receptor family, and Fas-bearing cells undergo apoptosis by ligation with Fas ligand (FasL) [11, 22]. Since FasL is mainly expressed on the activated T cells and Fas-mediated cell death is one of the main effector mechanisms of cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells [1, 14], the sensitivity to Fas-mediated cell death in tumor cells may represent the susceptibility of tumors to the anti-tumor activity of the host.

Human hepatocellular carcinoma (HCC) cells have been known to be resistant to Fas-mediated cell death [21]. Because normal hepatocytes express Fas abundantly and the liver is one of the most vulnerable organs to Fas stimuli [6, 8, 9], the acquisition of resistance to Fas-mediated cell death may be a prerequisite for hepatocarcinogenesis. In human HCCs, loss or down-regulation of Fas expression is a common phenomenon and this is regarded as a mechanism responsible for resistance to Fas-mediated cell death [10, 19, 26, 29]. Moreover, Fas-expressing HCCs showed better clinical outcome, such as higher disease-free survival rates, than Fas-negative HCCs [20]. The expression of soluble Fas, which acts as a competitive inhibitor in the interaction between FasL and membrane-bound Fas, is also regarded as a cause of resistance to Fas-mediated cell death [13, 20]. Recently, the expression of CD40 [30] or the formation of the procaspase 3/p21 complex, which is a possible caspase-3 inhibitor [31], or alternatively the expression of IAP family gene such as IAP-like protein

(ILP) [31] have been suggested as other causes of resistance to Fas-mediated cell death in human HCC cells.

Interferon-γ (IFN-γ) is a type II interferon, and is mainly secreted by T and NK cells [5]. It is well known that IFN-γ makes tumor cells sensitive to various apoptotic stimuli [7, 16, 23, 28, 33, 34]. Several effects of IFN-γ have been suggested as possible mechanisms in sensitizing Fas-mediated cell death. These previous reports have suggested the up-regulation of Fas [7, 16, 28, 34], down-regulation of Bcl-2 or Bcl-x_L [16, 28], up-regulation of Bax or Bak [16, 23], and up-regulation of caspase-1 or other caspases [23, 33]. However, these investigations were performed on restricted cell lines, and their results were occasionally controversial. In human HCCs, it was reported that tumor cells showed resistance to Fas-mediated cell death [21] and this resistance could be abolished by IFN-γ treatment [35].

Since FasL is expressed on activated CTLs and NK cells as effector molecules and IFN-γ is mainly secreted by them, these two molecules probably co-exist in the lymphocyte-infiltrated tumor micro-environment and it is meaningful to elucidate the influence of IFN-γ on Fas-mediated cell death. In the present study, we thoroughly investigated the effect of IFN-γ on the susceptibility to Fas-mediated cell death in one human normal liver-derived cell line and 12 HCC-derived cell lines. We also investigated the effect of IFN-γ on the expression of various apoptosis-related genes to elucidate the mode of action of IFN-γ in sensitizing Fas-mediated cell death.

Materials and methods

Cell lines and cell culture

Chang liver (ATCC CCL 13), SK-HEP-1 (ATCC HTB 52), Hep G2 (ATCC HB 8065), Hep 3B (ATCC HB 8064) and PLC/PRF/5 (ATCC CRL 8024) were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). Hep G2.2.15 cells, stable transfectants of the HBV genome into Hep G2 [25], were also included. These cell lines were grown in MEM containing 10% fetal calf serum (Gibco BRL, Grand Island, N.Y.). SNU-182, SNU-354, SNU-387, SNU-398, SNU-423, SNU-449 and SNU-475 were obtained from the Korean Cell Line Bank (Seoul, Korea) [24]. These cell lines were grown in RPMI 1640 containing 10% fetal calf serum. All media contained 100 U/ml of penicillin and 100 μg/ml of streptomycin.

RNase protection assay

Total RNA was isolated from cells with a RNeasy kit (Qiagen, Santa Claris, Calif.). The RNase protection assay (RPA) was performed with a RiboQuant multi-probe RPA kit (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions. ^{32}P-labeled antisense riboprobes compatible with apoptosis-related genes and internal control (L32 and GAPDH) were synthesized with hAPO-1, hAPO-2 and hAPO-3 Human Apoptosis template set (Pharmingen), 2.75 mM ATP, GTP, CTP, 100 μCi [α-^{32}P]-UTP (3000 Ci/mmol; NEN, Boston, Mass.) and 20 U T7 RNA polymerase. ^{32}P-labeled antisense riboprobes were hybridized with 10 μg of total RNA at 56 °C for 16 h. After hybridization, 20 ng of RNase A and 50 U of RNase T1 were added to digest unhybridized RNA. Duplex RNA hybrids were loaded onto 6% denaturing polyacrylamide gels containing 8 M urea and autoradiography was performed. Band densities were measured with an imaging densitometer (Bio-Rad, Hercules, Calif.) [26].

Immunofluorescence staining and flow cytometry

HCC cells were treated with or without 250 U/ml of IFN-γ for 36 h; adherent cells were then detached with 0.125% trypsin and 0.5 mM EDTA. Cells were washed with PBS and re-suspended in RPMI 1640 containing 1% fetal calf serum and incubated with anti-Fas monoclonal antibody, DX2 (Calbiochem, La Jolla, Calif.), [12] or anti-ICAM-1 monoclonal antibody, 84H10 (Immunotech, Marseille, France), at 4 °C for 30 min. Cells were washed twice with PBS containing 0.5% BSA. FITC-conjugated goat anti-mouse IgG (Becton Dickinson, Lincoln Park, N.J.) was added and incubated at 4 °C for 30 min. Cells were washed again in PBS containing 0.5% BSA and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed using a FACStar (Becton Dickinson) and data was analyzed with a WinMDI program. For quantitative analysis, the ratio (F/F_0) of the mean fluorescence intensities in the presence (F) and absence (F_0) of anti-Fas antibody was calculated [26].

Measurement of cell death

HCC cells were pre-incubated with or without 250 U/ml of IFN-γ for 36 h. Medium was replaced with fresh complete medium with or without 250 ng/ml of agonistic IgM anti-Fas monoclonal antibody, CH11 (MBL, Watertown, Mass.), and an LDH assay was performed after 36 h [15]. One hundred microliters of culture media was transferred into a 96-well plate, the remaining cells were lysed with Triton X-100, and 50 μl of cell lysate was also transferred into a 96-well plate. NADH (Sigma, St. Louis, Mo.) and pyruvate were then added to each well. Absorbance kinetics were measured on a plate reader at a wavelength of 340 nm. Cell death percentage was calculated as: % of cell death = [(LDH activity in 100 μl of culture media – LDH activity in 100 μl of fresh media)/(LDH activity in 50 μl of cell lysate – LDH activity in 50 μl of fresh media)] × 50.

Results

Effect of IFN-γ on the Fas-mediated cell death of HCC cell lines

HCC cells were pretreated with IFN-γ and Fas-mediated cell death was evaluated. Of the 13 cell lines, three HCC cell lines (SNU-354, SNU-387 and SNU-423) became markedly susceptible to Fas-mediated cell death after IFN-γ pretreatment; however, the other 10 cell lines showed only weak sensitization to Fas-mediated cell death after the same IFN-γ pretreatment (Fig. 1).

IFN-γ increased the mRNA expression of Fas

To investigate the effect of IFN-γ on mRNA expression of Fas/TNF-related genes, RPA was performed. In seven cell lines (Chang liver, Hep G2, SNU-354, SNU-387, SNU-423, SNU-449 and SNU-475), Fas transcripts were increased by the IFN-γ treatment (Fig. 2A). The extent of increase in the Fas transcripts was determined by calculating the ratio of the density of the Fas band and the density of the GAPDH band (Fig. 2B). The changes in mRNA expression level of the other Fas-related genes were also recorded after IFN-γ treatment

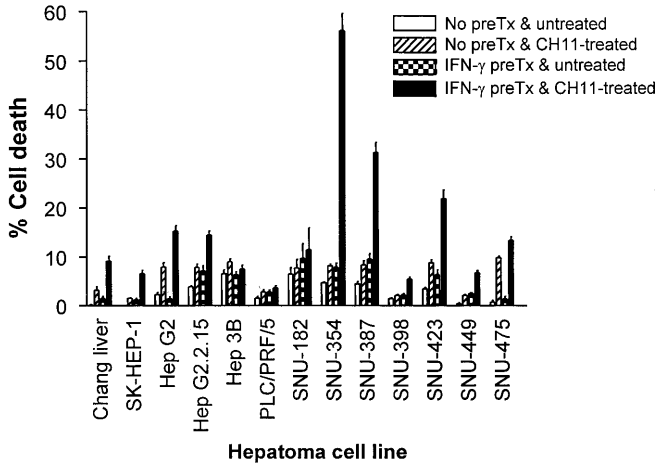


Fig. 1 The effect of IFN-γ on susceptibility to Fas-mediated cell death in HCC cell lines. HCC cells were pretreated with IFN-γ (250 U/ml) for 36 h and then treated with the agonistic anti-Fas monoclonal antibody, CH11, for another 36 h. The extent of cell death was measured by LDH assay. Each bar represents mean $\pm$ standard error of four independent experiments. SNU-354, SNU-387 and SNU-423 became markedly susceptible to Fas-mediated cell death after IFN-γ pretreatment

in a number of HCC cell lines. SNU-449 showed increased FADD transcripts, and SNU-398 showed increased FLICE and FADD and decreased FAF and FAP transcripts. An increased tendency to express TRAIL mRNA was also observed in several cell lines after IFN-γ treatment (Fig. 2A).

IFN-γ had no effect on the mRNA expression level of *bcl-2* family genes

To investigate the effect of IFN-γ on the expression of *bcl-2* family genes, RPA was performed. Although many tumor cells express Bcl-2 for apoptotic resistance, all HCC cell lines tested showed no detectable *bcl-2* transcripts and no change by IFN-γ treatment (Fig. 3). Furthermore, no changes in the mRNA levels of any of the tested genes belonging to the *bcl-2* gene family were detected.

IFN-γ induced the mRNA expression of caspase-1

We investigated whether IFN-γ affected mRNA expression of caspase family genes. All cell lines except PLC/PRF/5 showed an increased mRNA expression of caspase-1 (Fig. 4). Even cell lines which had no basal expression of caspase-1 showed its induced expression. There were no detectable changes in the transcript levels of the other caspases tested.

Surface expression of Fas after IFN-γ treatment

We investigated whether Fas surface expression was also increased by IFN-γ treatment. Chang liver, SNU-354, SNU-387, SNU-398, SNU-423 and SNU-449 showed not only constitutive surface expression of Fas but also increased expression after IFN-γ treatment (Fig. 5). SK-HEP-1, Hep G2, Hep G2.2.15 and SNU-475 also showed constitutive surface expression of Fas, but expression was minimally increased by IFN-γ treatment. SNU-182 also expressed basal Fas minimally and barely increased the expression of Fas after IFN-γ treatment. Hep 3B and PLC/PRF/5 expressed surface Fas neither constitutively nor inducibly after IFN-γ treatment.

Change in surface expression of Fas antigen

To quantify the extent of surface Fas increase caused by IFN-γ treatment, we calculated the fold increase of surface Fas by measuring the mean fluorescence intensities of flow cytometric histograms (Fig. 6). HCC cell lines SNU-354, SNU-387, SNU-423 and SNU-449 showed marked increases in surface Fas levels (increase in surface Fas >1.7-fold). Among these cell lines, three (SNU-354, SNU-387 and SNU-423) became potently susceptible to Fas-mediated cell death (Fig. 1), although SNU-449 was still resistant to Fas-mediated cell death after IFN-γ pretreatment. These results suggest that a marked increase of surface Fas is a major mechanism of IFN-γ in the sensitization to Fas-mediated cell death. However, there was no correlation at all between the actual surface level of Fas and the susceptibility to Fas-mediated cell death, and it could be speculated that the surface level of Fas is not an indicator of the susceptibility to Fas-mediated cell death.

HCC cells had no defect in the signaling of IFN-γ stimuli

Some HCC cell lines showed increased surface Fas expression after IFN-γ treatment; the other cell lines, on the other hand, showed no change in surface Fas expression. Therefore, we speculated that HCC cell lines showing unresponsiveness to IFN-γ treatment might have no receptor for IFN-γ or have disrupted IFN-γ signaling. To rule out this possibility, we investigated the surface change of the IFN-γ-responsive molecule, ICAM-1, after IFN-γ treatment. HCC cell lines showing no or very weak increases in surface Fas by IFN-γ – Hep G2, Hep G2.2.15, Hep 3B, SNU-182 and SNU-475 – responded well to IFN-γ treatment in terms of their surface expression of ICAM-1 (Fig. 7). On the other hand, cell lines showing strong increases in surface Fas after IFN-γ treatment – SNU-387 and SNU-398 – did not respond to IFN-γ treatment as assessed by ICAM-1 surface expression. These results suggest that the extent of regulation in Fas expression by IFN-γ is not associated with the disruption of IFN-γ receptor or signaling. This conclusion can also be supported by the observation that IFN-γ increased caspase-1 mRNA in most tested cell lines.

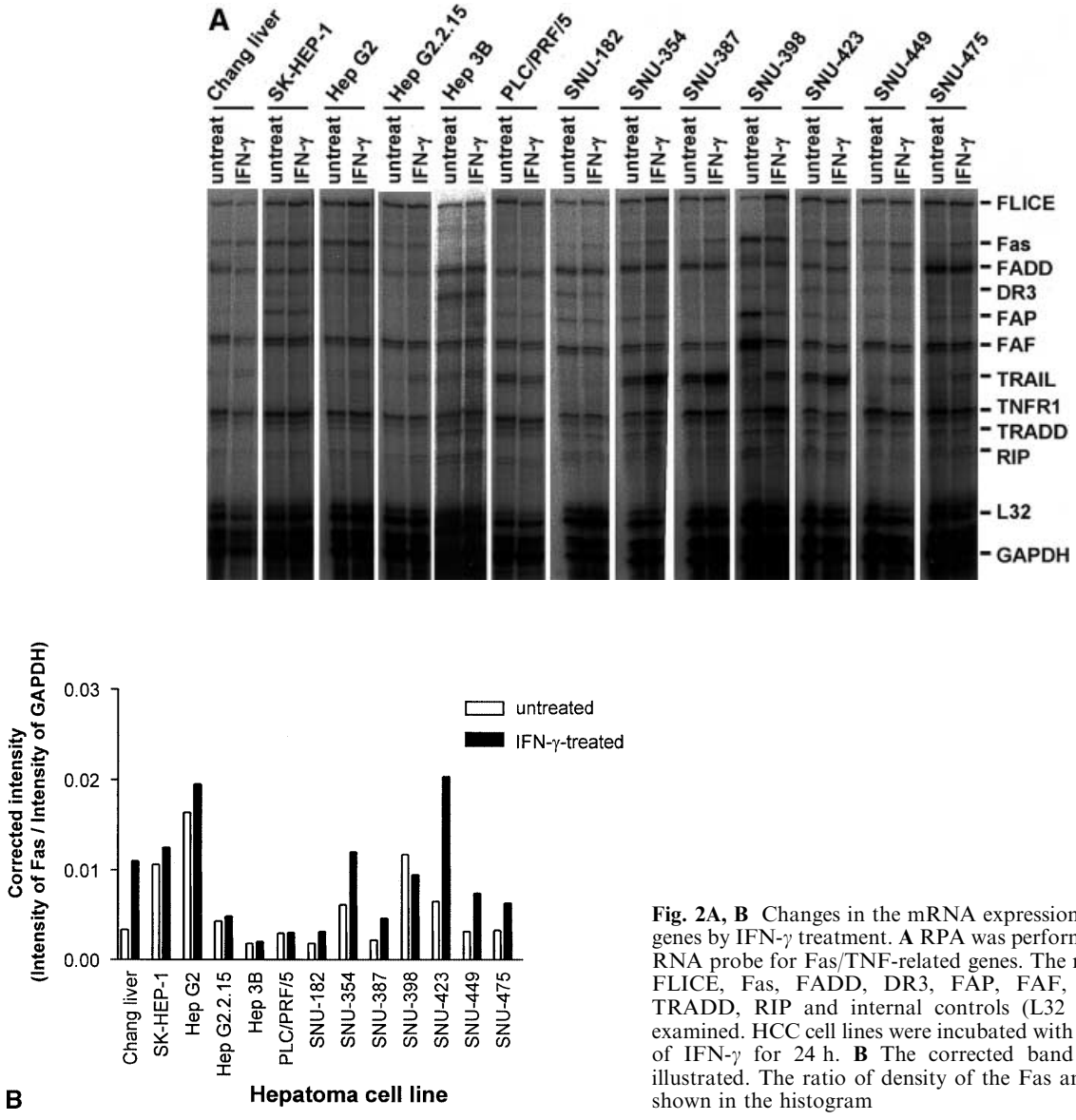


Fig. 2A, B Changes in the mRNA expression of Fas/TNF-related genes by IFN-γ treatment. **A** RPA was performed with an antisense RNA probe for Fas/TNF-related genes. The mRNA expression of FLICE, Fas, FADD, DR3, FAP, FAF, TRAIL, TNFR 1, TRADD, RIP and internal controls (L32 and GAPDH) were examined. HCC cell lines were incubated with or without 250 U/ml of IFN-γ for 24 h. **B** The corrected band intensity of Fas is illustrated. The ratio of density of the Fas and GAPDH bands is shown in the histogram

Discussion

IFN-γ acts as a sensitizer in Fas-mediated cell death [7, 16, 23, 28, 33–35]. In the present study, we thoroughly investigated the effect of IFN-γ on the susceptibility to Fas-mediated cell death in one human normal liver-derived cell line and 12 HCC-derived cell lines. IFN-γ increased the expression of Fas mRNA and surface Fas in the majority of HCC cell lines. When the extent of cell death was assessed, in a manner reported previously [21], all HCC cell lines were found to show resistance to Fas-mediated cell death without IFN-γ treatment, although some cell lines constitutively highly expressed surface Fas. IFN-γ markedly made cells susceptible to Fas-mediated cell death in only three cell lines. The other 10 cell lines remained unsusceptible to Fas-mediated cell death even though active changes occurred in the expression of Fas or caspase-1. These results suggested that most human HCC cells resist Fas-mediated cell death even though they are exposed to FasL and IFN-γ simultaneously.

Among the four cell lines which showed a marked increase in surface Fas when induced by IFN-γ (increase in surface Fas >1.7-fold), three cell lines, the exception being SNU-449, became markedly susceptible to Fas-mediated cell death by IFN-γ. Therefore, in HCC cell lines which became susceptible to Fas-mediated cell death by IFN-γ (SNU-354, SNU-387 and SNU-423), a marked increased in the surface expression of Fas could be considered as a major mechanism of IFN-γ sensitization to Fas-mediated cell death. Nevertheless, the actual surface level of Fas was not an indicator of susceptibility to Fas-mediated cell death in human HCC cells. Chang liver, SK-HEP-1, and Hep G2 highly expressed Fas on their cell surfaces; however,

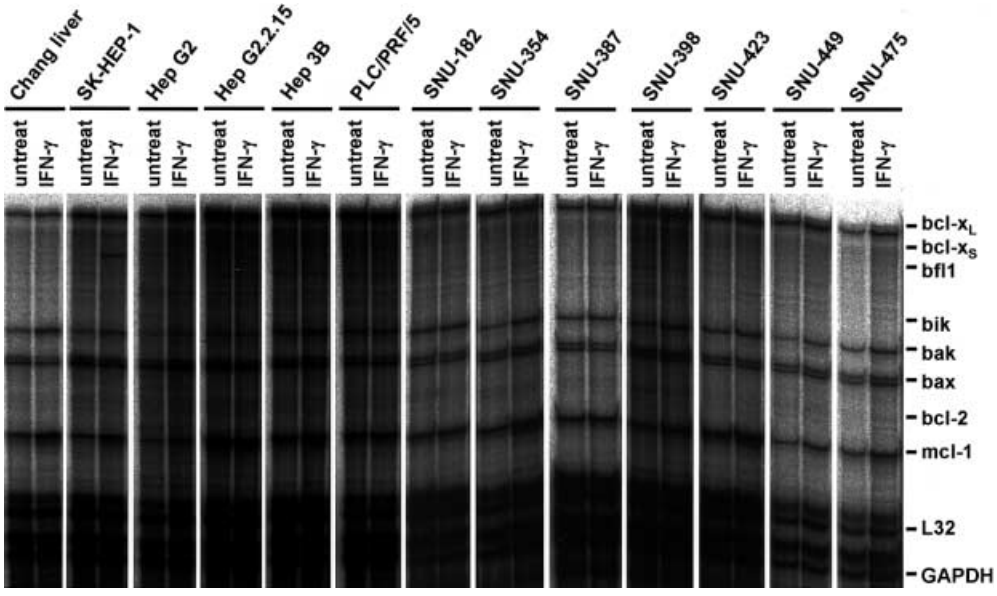
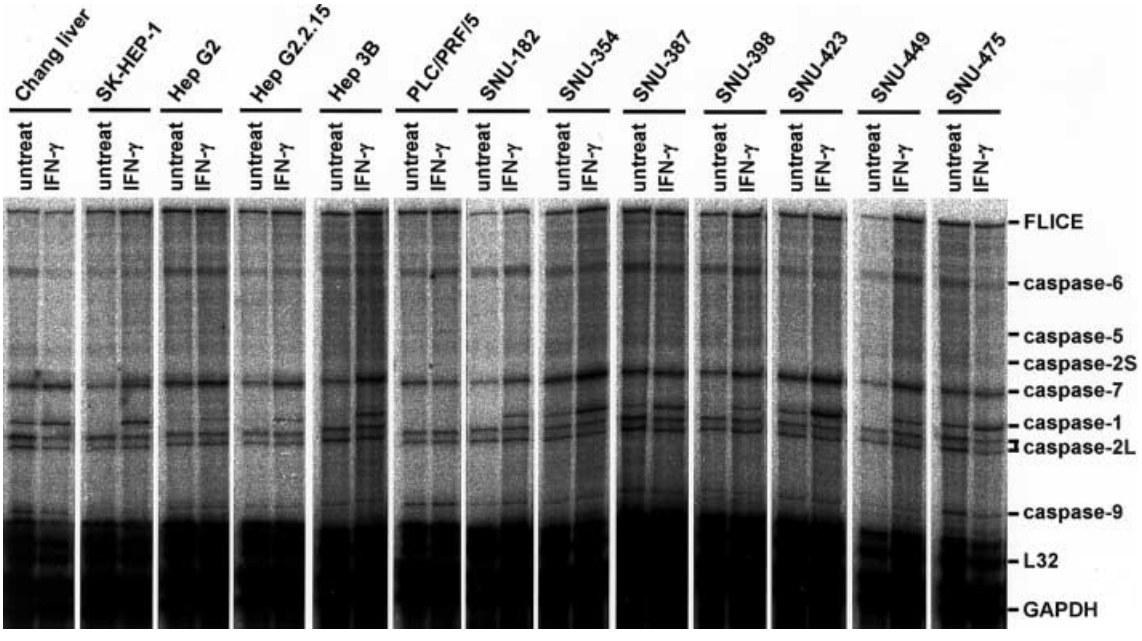


Fig. 3 Expression of *bcl-2* family genes in HCC cell lines. The mRNA expression of the *bcl-2* family genes *bcl-x$_L$*, *bcl-x$_S$*, *bfl1*, *bik*, *bak*, *bax*, *bcl-2*, and *mcl-1* and of the internal controls (L32 and GAPDH) was analyzed by RPA before and after IFN-γ treatment. HCC cell lines were incubated with or without 250 U/ml of IFN-γ for 24 h

these cell lines resist Fas-mediated cell death. In these cell lines, other apoptosis-inhibiting mechanisms may play a role in the resistance to Fas-mediated cell death. It was also reported that the Fas system might not play a major role in the cell death of HCCs, which means that HCC cells resist Fas-mediated cell death despite Fas expression [17]. Among the Fas/TNF-related genes, TRAIL was also increased by IFN-γ treatment; however, the significance of TRAIL up-regulation is not clear.

In addition to the increased Fas expression, increased levels of transcripts of caspase-1 were also induced by IFN-γ treatment. Up-regulation of caspase-1 by IFN-γ and its role in apoptosis was reported in U937 leukemia cells and HT-29 colon cancer cells [33, 34]. Moreover, it was reported that the activation of the STAT signaling pathway by IFN-γ could induce apoptosis through the induction of caspase-1 expression [2], and IFN-γ induced apoptosis in human erythroid progenitor cells through up-regulation of Fas and up-regulation and subsequent activation of caspase-1, -3, and -8 [3, 4]. It was also reported that STAT1 null cells did not express caspase-1, -2, and -3, and that STAT1 null cells were resistant to TNF-α-induced cell death

Fig. 4 Changes in the mRNA expression of caspase family genes by IFN-γ treatment. RPA was performed with an antisense RNA probe to determine the mRNA expression of caspase-1, -2, -5, -6, -7, -8, and -9 and the internal controls L32 and GAPDH. HCC cell lines were incubated with or without 250 U/ml of IFN-γ for 24 h



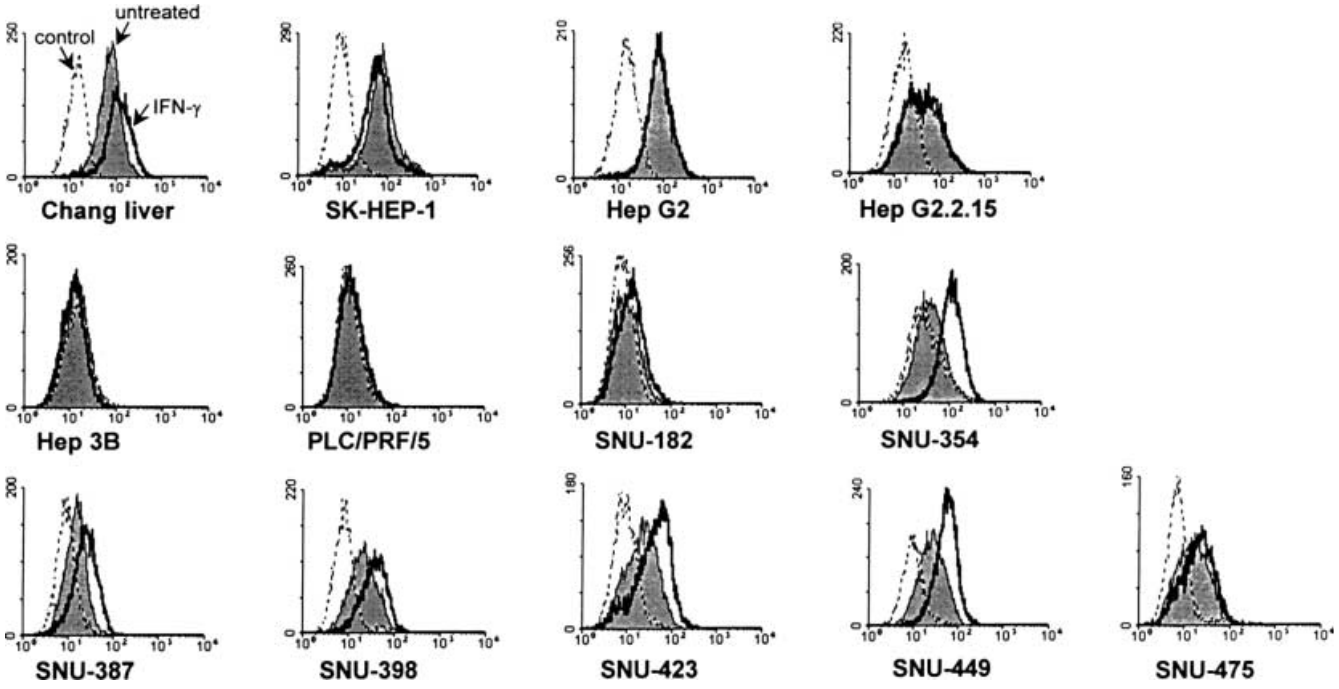


Fig. 5 Increased surface expression of Fas induced by IFN-γ treatment. HCC cell lines were treated with IFN-γ (250 U/ml) for 36 h, cell surface Fas was stained with DX2 anti-Fas antibody, and fluorescence intensity was measured by flow cytometry. In each cell line, the *dashed line* represents the negative control with no anti-Fas antibody, the *filled area* represents the basal level of Fas without IFN-γ treatment, and the *solid line* represents the level of Fas with IFN-γ treatment

[18]. These findings imply that IFN-γ can regulate the expression of caspase-1 through the action of STAT1 and that caspase-1 is actively involved in the apoptotic process. In the present study, however, many cell lines which showed caspase-1 induction after IFN-γ treatment did not become susceptible to Fas-mediated cell death after IFN-γ treatment. This result implies that the induction of caspase-1 expression might not play a major role in the IFN-γ-mediated sensitization to Fas-mediated cell death.

We also investigated changes in the transcript levels of *bcl-2* family genes after IFN-γ treatment. No change in transcript level of any member of the *bcl-2* family genes tested was detected. In colon cancer cell lines, changes of the mRNA level of *bcl-2* family genes have been considered to be one of the mechanisms responsible for sensitization to Fas-mediated apoptosis by IFN-γ treatment. These previous results included the down-regulation of Bcl-2 and up-regulation of Bax in COLO 201 colon cancer cells [16] and the up-regulation of Bak in HT-29 colon cancer cells [23]. In our study using HCC cell lines, these changes were not observed. In particular, no HCC cell line tested showed detectable expression of *bcl-2*, although it was previously reported that over-expression of Bcl-2 is able to protect HCC cells from Fas-mediated cell death [32], as has been said of other tumors over-expressing Bcl-2. We conclude that HCC cells do not utilize over-expressed Bcl-2 as a component of the mechanism of resistance to Fas-mediated cell death.

The acquisition of resistance to Fas-mediated cell death in the malignant transformation of hepatocytes is considered a key element of carcinogenesis. Normal hepatocytes express Fas abundantly, and therefore they are vulnerable to Fas stimuli [6, 8, 9]. Furthermore, in many HBV-associated HCCs, carcinoma is accompanied with chronic active hepatitis, during which infiltrated lymphocytes usually express FasL

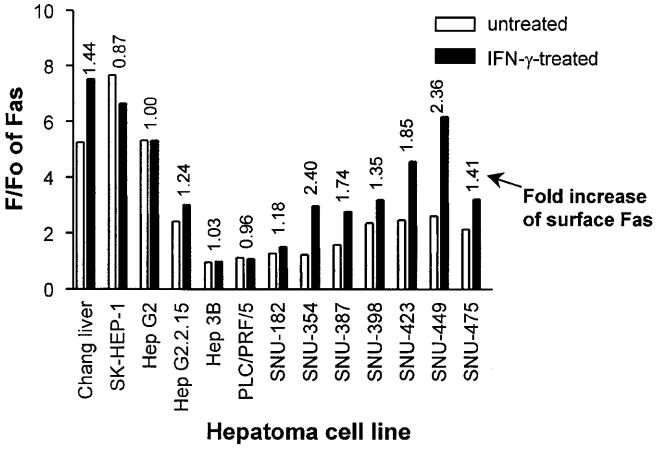


Fig. 6 Fold increase of surface Fas. Intensity of Fas surface expression is illustrated. F/F_0 was calculated as the ratio of the mean fluorescence intensities obtained from the two respective histograms in the presence (F) and absence (F_0) of anti-Fas antibody. Fold increase of surface Fas was calculated as the ratio of F/F_0 in the presence and absence of IFN-γ. SNU-354, SNU-387, SNU-423 and SNU-449 showed marked increases in surface Fas level, induced by IFN-γ (increase in surface Fas >1.7-fold)

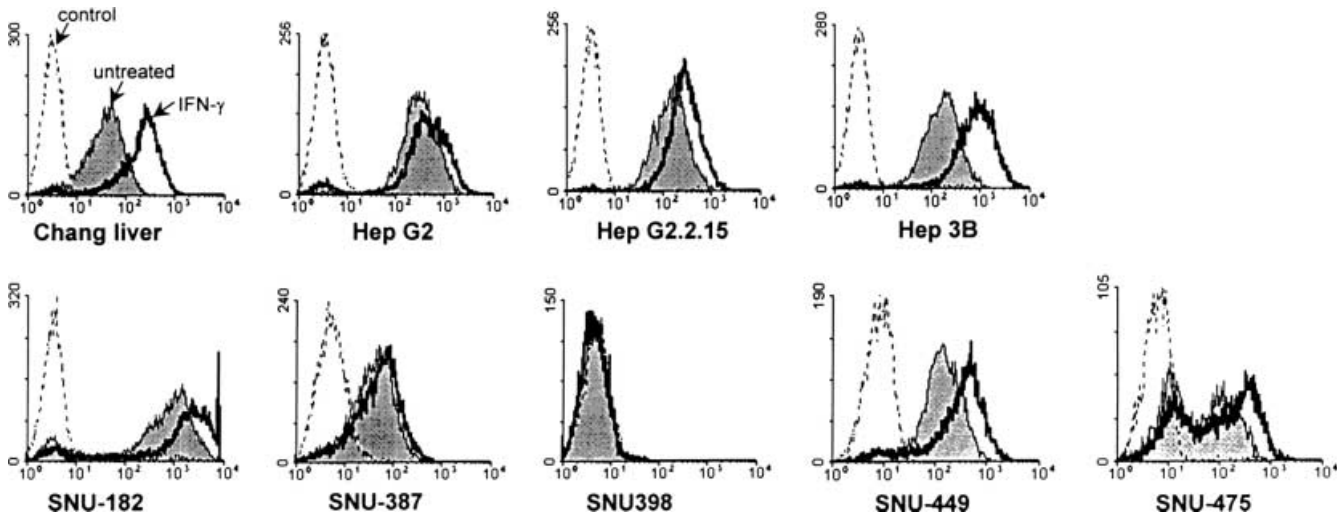


Fig. 7 Increased surface expression of ICAM-1 by IFN-γ treatment. HCC cell lines were treated with IFN-γ (250 U/ml) for 36 h, and then cell surface ICAM-1 was stained with anti-ICAM-1 antibody and the fluorescence intensity measured by flow cytometry. In each cell line, the *dashed line* represents the negative control, with no anti-ICAM-1 antibody, the *filled area* represents the basal level of ICAM-1 without IFN-γ treatment, and the *solid line* represents the level of ICAM-1 with IFN-γ treatment

[19]. For the successful development of carcinoma in this environment, Fas-resistant cells should be naturally selected and the finally formed HCCs should be resistant to Fas-mediated cell death. Furthermore, it has been recently reported that some HCCs express FasL and that FasL-expressing HCC cells can counterattack the anti-tumor immune reactions of the host [27, 29]. If HCC cells express FasL for this benefit, then resistance to Fas-mediated cell death is a prerequisite.

Previously, we investigated the possibility of resistance to Fas-mediated cell death with the same panel of HCC cell lines and proposed several possible mechanisms of resistance [26]. In the present study, we observed that many HCC cells resist Fas-mediated cell death even after treatment with IFN-γ, which might be produced by CTLs and NK cells in the tumor microenvironment and sensitize many tumor cells to Fas-mediated cell death. In three potently sensitized HCC cell lines, a marked increment of surface Fas expression was considered to bear a major responsibility for sensitization to Fas-mediated cell death by IFN-γ.

In conclusion, IFN-γ cannot play an apoptotic sensitizing role in most HCC cell lines. However, IFN-γ makes HCC cells susceptible to Fas-mediated cell death through the potent up-regulation of surface Fas in some HCC cells.

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