IFN-α–Induced Apoptosis in Hepatocellular Carcinoma Involves Promyelocytic Leukemia Protein and TRAIL Independently of p53

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Abstract

IFNs are pleiotropic cytokines that have been shown to be important regulators of cell growth. IFN-α has recently been recognized to harbor therapeutic potential in prevention and treatment of hepatocellular carcinoma (HCC). However, HCC cells respond differentially to IFN treatment, the mechanism of which is largely unknown. To address this issue, we analyzed the effect of IFN-α on different liver tumor cell lines. We found that growth inhibiting effects of IFN-α in hepatoma cells require PML-NB induction and, moreover, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) expression on the mRNA and protein level. RNAi silencing of PML down-regulates TRAIL expression in hepatoma cells and correspondingly blocks IFN-α–induced apoptosis. In addition, PML-deficient primary hepatocytes fail to up-regulate TRAIL upon IFN-α-treatment in contrast to their wild-type counterparts. These data identify TRAIL as a novel downstream transcriptional target of PML-mediated apoptosis in hepatomas and suggest that PML and TRAIL play important roles in IFN-regulated apoptosis in HCC. Furthermore, the mechanism is independent of the p53 status of the tumor cells. In summary, our results identify central molecules mediating IFN-α induced apoptosis in liver tumors, shed light on the differential response of hepatoma cells to IFN exposure and, thus, may contribute to an efficient application of this substance in the treatment of liver cancer. [Cancer Res 2009;69(3):855–62]

Introduction

Liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Hepatocellular carcinoma (HCC), which accounts for 80% to 90% of primary liver tumors, is characterized by a very poor prognosis and is associated with high mortality (1). However, current available therapeutic modalities for HCC are largely inadequate. Thus far, cytotoxic chemotherapy has proven ineffective, and until recently, no first-line therapy existed for advanced HCC. In the recent SHARP trial, the kinase inhibitor sorafenib has shown promising success in treatment of HCC and is currently emerging as first-line therapy for advanced HCC (2). However, despite major efforts to improve diagnosis and treatment of HCC, therapeutic options in particular for advanced tumor stages remain limited. Thus, the need for novel therapeutic agents and strategies is obvious. IFNs have been used with varying effectiveness as proapoptotic agents in the treatment of malignancies, as e.g., myeloma (3), renal (4), or bladder cancer (5), and there is increasing evidence of effectiveness of IFNs in the treatment and prevention of HCC. Recent studies show that IFNs, in combination with chemotherapeutics (6) or agents such as proteasome inhibitors (7, 8), may prove to be clinically effective. It has been reported that IFN therapy, even after curative treatment of HCC, could prevent HCC recurrence and improve survival. Moreover, pegylated IFN-α has been shown to effectively treat advanced HCC, even more so in combination with 5-fluorouracil (6). Still, the precise mechanisms by which IFNs exert antitumor activity remain unclear.

One of the IFN target genes is the death ligand tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 9) that has been identified as an important mediator of IFN-induced apoptosis in leukemia (10), human myeloma (3), and bladder cancer (11). TRAIL triggers apoptosis upon binding of its receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), recruiting adapter proteins and activate caspases upon activation (12). TRAIL has been shown to effectively induce apoptosis in several types of transformed cells, including HCC but not in most normal cells. Therefore, TRAIL is discussed to have valuable therapeutic potential in the treatment of HCC (13).

Another IFN target gene involved in apoptosis regulation is the promyelocytic leukemia (PML) gene (14). PML localizes to subnuclear speckles, so called PML nuclear bodies (NB) that increase in size and number upon stimulation with IFNs. A large number of proteins with diverse functions have been found to localize to PML NBs, and their central role in multiple cellular processes such as proliferation, apoptosis, and regulation of transcription is well-established (15, 16). Studies in PML *knockout* mice showed that PML deficiency leads to impaired induction of apoptosis (17). Moreover, comprehensive studies have shown that the PML protein is frequently lost in human cancers of various histologic origins (18). However, the exact molecular function of PML and PML NBs is still largely unclear.

Thus far, a functional role for PML in HCC has not yet been defined. Our data show that IFN exposure leads to induction of PML NBs in hepatoma cells. Furthermore, we show that PML is indispensable for IFN-mediated TRAIL induction and concomitant apoptosis induction in hepatoma cells. This accounts for cells expressing either functional or mutated forms of the tumor suppressor p53 and does, thus, not require a functional p53 status of the tumor cell. Subsequently, the regulation of IFN-induced cell

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death is impaired in a subset of TRAIL-resistant cells. In conclusion, IFN-α–mediated cell death in HCC seems to require PML-mediated TRAIL-induction and is independent of the p53-status of the tumor cell.

Materials and Methods

Cell lines. Hep3B, Huh7, Huh6, HepG2, Chang, and CEM cells were obtained from the American Type Culture Collection. The liver cell lines were maintained in DMEM and CEM T-cells in RPMI 1640 (Life Technologies Bethesda Research Laboratories) supplemented with 10% heat-inactivated FCS (Life Technologies Bethesda Research Laboratories), 10 mmol/L HEPES (Life Technologies Bethesda Research Laboratories), 5 mmol/L L-glutamine (Life Technologies Bethesda Research Laboratories), and 100 μg/mL gentamicin (Life Technologies Bethesda Research Laboratories) in 5% CO_2. Before stimulation, the cells were cultured for 24 h in DMEM without FCS, followed by treatment with IFN-α and as indicated.

Isolation and culture of primary mouse hepatocytes. Primary mouse hepatocytes (PMH) were isolated from healthy liver tissue obtained from mice with a two-step perfusion technique as described (19). Before stimulation, the cells were cultured for 24 h in DMEM without FCS, followed by treatment with IFN-α and as indicated.

Immunofluorescence stainings. Cells were grown in 12-well plates on coverslips and treated with IFN as indicated, washed once with PBS and fixed for 5 min at −20°C with methanol/acetone (1:1). The following procedure was described previously (20). After being dried, cells were blocked for 30 min at room temperature in PBS containing 5% (v/v) goat serum. Cells were then incubated for 60 min with the primary antibody (rabbit polyclonal PML antibody H-238 from Santa Cruz) at room temperature, subsequently washed 5 times (5 min each) in PBS before incubation for 45 min with an fluorochrome-conjugated secondary antibody (Texas-red–coupled goat anti-rabbit antibody from The Jackson Laboratory). Chromosomal DNA was stained with 4′, 6-diamidino-2-phenylindole (DAPI). Stained cells were mounted on glass slides and examined with an epifluorescence microscope (Axioplan-2; Zeiss). Images were processed using the Adope Photoshop 6.0 software. PML NB fluorescence was quantified by laser scanning cytometrie (CompuCyte). Three thousand cells were measured per sample.

Western blotting. Cells were harvested, lysed, and lysates processed as previously described (21). The TRAIL antigen was detected with a polyclonal rabbit-antibody obtained from Novus Biologicals. β-Actin and PML antibodies (H-238 and E-15) were purchased from Santa Cruz Biotech.

Reverse transcription-PCR. RNA was isolated using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. After 10 min denaturation at 70°C, 1 μg of RNA was incubated with oligo$(dT)_{12\text{-}18}$ primer (Roche), deoxynucleotide triphosphates (Invitrogen), and M-MLVT RT (2U; Invitrogen) at 42°C for 1 h to generate cDNA. The cDNA was used as a template for PCR amplification of the following gene products: TRAIL (5′-ggt cca tgt cta tca agt gct c-3′ and 5′-gac gaa gag agt agt aac agc-3′; 603 bp), β-actin (5′-gtg ggg cgc ccc agg cac ca-3′ and 5′-ctc ctt aat gtc acg cac gat ttc-3′; 484 bp). Five microliters of aliquots were amplified in a DNA thermocycler (Perkin-Elmer Gene Amp PCRSystem 9700) with 0.5U of Taq DNA polymerase (Sigma) in a 50 μL reaction. Thirty-five reaction cycles for TRAIL and 25 cycles for β-actin were performed. Each cycle consisted of a denaturation step, 94°C for 30s, an annealing step, 55°C for 30s, and an elongation step, 72°C for 30s. The reaction was completed with a 72°C elongation step for 10 min. PCR products were analyzed on 1.5% to 2% agarose gels and visualized by ethidium bromide staining.

Quantitative real-time PCR. Quantitative PCR was performed using a Lightcycler System according to the manufacturer's instructions (Roche Diagnostics) using the following primers: TRAIL (5′-ggt cca tgt cta tca agt gct c-3′ and 5′-gac gaa gag agt agt aac agc-3′; 603 bp),β-actin (5′-gtg ggg cgc ccc agg cac ca-3′ and 5′-ctc ctt aat gtc acg cac gat ttc-3′; 484 bp).

RNA interference. RNA interference (RNAi) was performed as previously described (20) using an oligonucleotide targeting sequence inserted into the pSUPER vector to knock down PML. In the control experiments, empty pSUPER vector was used.

Determination of cell death. As a direct measurement of cell death, DNA fragmentation was quantified by determination of the percentage of cells with subdiploid DNA content, essentially as described (22). LZ-TRAIL (*Killer*TRAIL) was obtained from Axxora.

Annexin V-staining was performed according to the manufacturer's instructions (BD Biosciences). In brief, cells were treated with siRNA or rescue constructs as indicated, exposed to 500IU IFN-α or left untreated and harvested after 36 h. Cells were washed twice with cold PBS (4°C), resuspended in binding buffer containing 10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2, and incubated with 5 μL FITC-conjugated Annexin V for 15 min in the dark at room temperature. Externalization of phosphatidylserine was identified by flow cytometry (FACScan; BD Biosciences).

For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a total of 5×10^4 cells were seeded in 96-well plates (Costar) after transfection with siRNA or rescue plasmid as indicated and treated with or without apoptotic stimulus (500IU IFN-α) in 150 μL medium at 37°C. After 20 h, 30 μL of an MTT solution [5 mg/mL MTT (Sigma Co.), in PBS] was added for additional 4 h, the medium was then removed and cells were lysed with 95% isopropanol/5% acetic acid (v/v). Absorption was determined at 560 nm. The percentage of viable cells was calculated as follows: 100 (absorption measured − absorption lysed cells)/(absorption medium − absorption lysed cells).

Flow cytometry. Cells were rinsed twice with PBS 36 h after stimulation, detached from culture dishes with PBS containing 20 mmol/L EDTA, washed, and resuspended in PBS supplemented with 5% FCS, essentially as described previously (23). Approximately 5×10^5 cells were used per sample. Incubations with primary and secondary antibodies were performed for 30 min at 4°C, followed by 2 washing steps with PBS/5% FCS after each incubation. Analysis was done with a Becton Dickinson fluorescence-activated cell sorting (FACS) flow cytometer and Cellquest software.

Cells were incubated in 50 μL of PBS/5% FCS with TRAIL-R2-Fc for TRAIL staining or, as the respective control, in the presence of huIgG (Southern Biotechnology Associates) at a concentration of 10 μg/mL. Biotinylated secondary goat anti-mouse or goat anti-human antibodies (Southern Biotechnology Associates) and Streptavidin-PE (Pharmingen) were used in a final volume of 50 μL at a dilution of 1:200.

Cell-mediated cytotoxicity assay. Cell-mediated lysis was quantitated using standard chromium-51 release assay (24). In brief, hepatoma cells were stimulated or not with 500 IU/mL IFN-α (Sigma) for 30 to 40 h, subsequently washed twice with PBS, detached with 2 mmol/L EDTA, and counted. CEM cells were labeled with 100 mCi/mL ^{51}Cr for 1 h in culture medium. Effector cells and target cells were coincubated for 16 h. Spontaneous release was determined by incubating target cells alone, total release by directly counting labeled cells. Percent cytotoxicity was calculated as follows: % specific lysis = (experimental cpm − spontaneous cpm/total cpm −spontaneous cpm) × 100. Duplicate measurements of four step titrations of effector cells were used for all experiments.

To block the various death ligands, effector cells were incubated with TRAIL-R2-Fc fusion proteins for 30 min before addition of target cells at a concentration of 10 μg/mL. Human IgG1 was used as an isotype-matched control. Mean values of % specific lysis ± SD. were calculated from triplicate samples.

Results

IFN-α induces cell death in various hepatoma cell lines independently of p53. IFN-α was reported to act negatively on cell growth in hepatoma cells (25, 26). The exact mechanism of action by which it facilitates cell death in hepatomas is currently unclear.

To address this issue, we used a panel of hepatoma cell lines. We could confirm apoptosis induction upon IFN-α administration in HepG2, Huh6, Huh7, and Chang liver cells by analysis of subdiploid DNA-content. In contrast, the amount of apoptosis in Hep3B cells

was significantly lower than in the IFN-sensitive hepatoma cell lines. IFN started to be effective at concentrations as low as 500 U/mL in all other cell lines tested but did not induce apoptosis in Hep3B cells even at the highest concentration of 2,000 U/mL (Fig. 1*A*). Cell death induction was confirmed by detection of Annexin V externalization as a rather early event in apoptosis after application of 1,000 U/mL IFN-α for different periods of time (Fig. 1*B*).

Sensitivity to IFN-α is not due to functional p53 as Huh7 cells, which express an unfunctional mutant of p53 (27, 28), do undergo apoptosis upon IFN-α exposure, whereas Hep3B cells, which are deficient for p53 (27, 28), do not.

In conclusion, hepatoma cells respond differentially to IFN-α most likely by a p53 independent mechanism.

PML is induced upon IFN-α treatment in human hepatoma cells. The tumor suppressor PML has been described as important mediator of apoptosis (16) and as IFN target gene (14). Therefore, we aimed to investigate a potential role in IFN-induced apoptosis of human hepatoma cells.

Immunofluorescence analysis of the IFN-treated cell lines revealed a marked increase in both the number and size of PML NBs appearing as typical discrete speckled structures within the nuclei of all hepatoma cells tested (Fig. 2*A*). Semiquantitative fluorescence analysis of PML induction revealed its up-regulation in all hepatocarcinoma cell lines tested (Fig. 2*B*). To test the influence of IFN-α on PML induction on the transcriptional level, we used quantitative PCR and showed that PML mRNA was rapidly induced in all cell lines used after treatment with IFN-α (Fig. 2*C*).

Taken together, all cell lines showed PML induction upon IFN treatment, independent of their p53 status. The data suggest correlation of apoptosis with strong PML mRNA and protein induction. Noteworthy, Hep3B cells did also respond with an increase in PML expression on the mRNA and protein level upon IFN-α stimulation but did not undergo apoptosis upon IFN-α treatment.

IFN-α increases TRAIL expression in hepatomas. TRAIL is an IFN target gene (9). However, the underlying regulatory mechanism has not been further evaluated thus far. PML is assigned to be involved in transcriptional regulation (15). To evaluate a role of PML in IFN-induced apoptosis of hepatoma cells, we addressed its influence on TRAIL expression.

FACS analysis revealed that surface expression of TRAIL was induced upon IFN-α exposure in all hepatoma lines used (Fig. 3*A*). To test a potential role of IFN-αin TRAIL induction on the transcriptional level, we investigated the influence of IFN-α on TRAIL mRNA levels. Quantitative PCR of RNA isolated from all hepatoma cell lines used after treatment with IFN-α for various times showed that TRAIL mRNA was rapidly induced in all cell lines after treatment with IFN-α (Fig. 3*B*). Thus, IFN-α may influence the expression of TRAIL at various levels, either by inducing TRAIL-regulating molecules or by directly influencing TRAIL expression on the transcriptional or translational level.

As normal cells and a number of tumor cell lines show resistance toward TRAIL-induced apoptosis (29), we sought to define TRAIL sensitivity of the cell lines used. To this end, hepatoma cells were incubated with Leuzin Zipper (LZ)-TRAIL (a recombinant highly active form of TRAIL) for 24 h. We observed that Huh6, Huh7, HepG2, and Chang cells died upon exposure to LZ-TRAIL, whereas Hep3B cells did not (Fig. 3*C*). The latter observation was not unexpected, as Hep3B cells have been described previously to be TRAIL resistant (7). Assuming that IFN-α–induced TRAIL expression may importantly be involved in cell death regulation in most hepatocarcinomas, resistance to TRAIL would explain insensitivity to IFN-α–induced cell death of certain cell lines such as Hep3B.

To confirm a direct implication of TRAIL in the IFN-α response, we investigated whether it is possible to interfere with IFN-α–induced apoptosis in TRAIL-sensitive hepatoma cells by using death receptor-Fc-proteins and, thus, blocking TRAIL in IFN-treated sensitive human hepatoma cells. Treatment of the IFN-responsive cell lines with TRAIL-R2-Fc resulted in significantly impaired apoptosis of the cells (Fig. 3*D*).

These studies show that TRAIL is a central mediator of the IFN- response of hepatocarcinoma cells and, moreover, that TRAIL-induction induced by IFN-α goes in line with PML protein induction.

RNAi silencing of PML impairs IFN-induced TRAIL expression and apoptosis. We sought to further confirm the importance of PML in IFN-induced apoptosis of hepatoma cells by RNAi through expression of a short hairpin RNA (pSUPER-PML), which specifically targets PML. The constructs have been described previously (20).

In contrast to the control pSUPER vector, cotransfection of the shPML construct strongly reduced expression of overexpressed PML in hepatoma cells upon IFN-α treatment. In addition, PML suppression potently inhibited TRAIL induction as shown by Western Blot analysis (Fig. 4*A*). As well, FACS analysis revealed that IFN-treated control cells transfected with the pSUPER vector alone

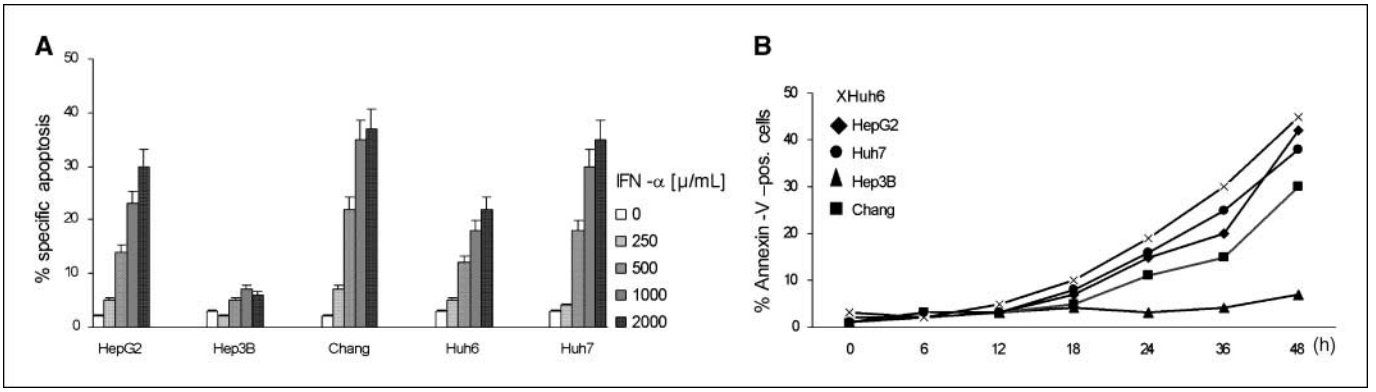

Figure 1. IFN-α induces cell death in hepatoma cell lines. HepG2, Hep3B, Chang, Huh6, and Huh7 cells were exposed to different doses of IFN-α and harvested after 36 h. Apoptosis was quantified by analyzing the percentage of cells with subdiploid DNA content, as described by Nicoletti and colleagues, (ref. 22; *A*). Hepatoma cells were exposed to 1,000 U/mL IFN-α for different time points and incubated with 5 μL FITC-conjugated Annexin V for 15 min in the dark at room temperature. Externalization of phosphatidylserine was identified by flow cytometry (FACScan; BD Biosciences; *B*). *Columns,* mean of five experiments performed; *bars,* SD. *Annexin V–pos,* Annexin V–positive.

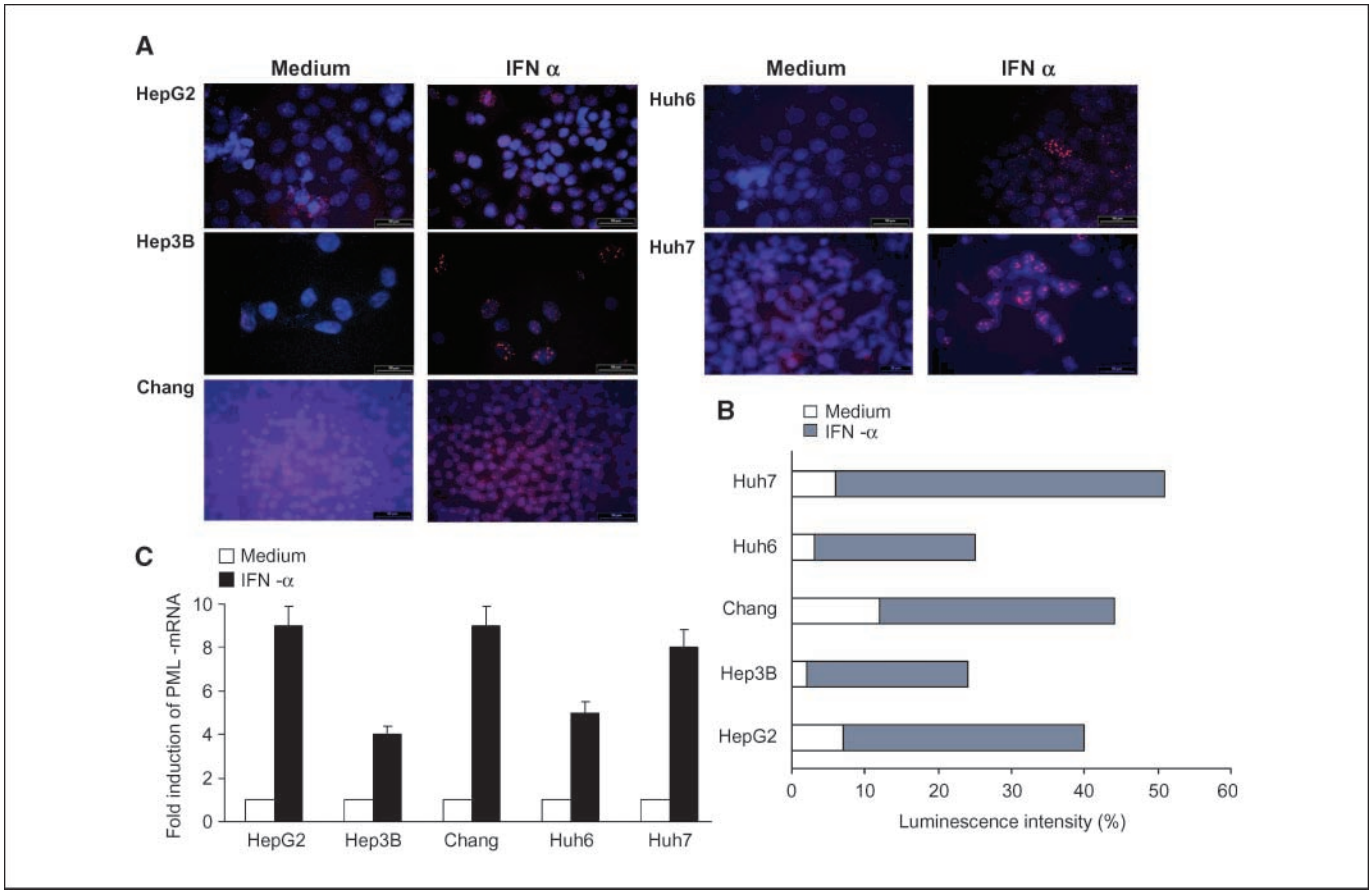

Figure 2. IFN-α induces PML expression in hepatoma cells. PML protein was detected by indirect immunofluorescence staining in hepatoma cells after treatment with 1,000 U/mL IFN-α for 36 h or not. Nuclear DNA (*blue*) was visualized by DAPI staining (*A*). Quantification was done by LSC analysis, which was performed as described in Materials and Methods. Three thousand cells per sample were scanned (*B*). Hepatoma cells were incubated with 1,000 U/mL IFN-α or medium and RNA was extracted after 12 h. mRNA levels were determined by quantitative PCR using β-actin for normalization (*C*). *Columns,* mean of five experiments performed; *bars,* SD.

exhibited a significant induction of TRAIL, which was markedly reduced in the shPML-treated cells (Fig. 4*B*). As a consequence of the reduction in PML expression, IFN-induced cell death is markedly decreased as determined by FACS-based DNA fragmentation analysis (Fig. 4*C*). IFN induced up to 30% to 40% cell death in cells treated with the pSUPER vector but to a much lesser extent (10%) in the shPML group when compared with untreated controls.

To examine if IFN-α–induced TRAIL on the surface of hepatoma cells is functional, we determined whether IFN-α–treated hepatoma cells were able to kill TRAIL-sensitive CEM target cells. We observed that ~45% of CEM cells were lysed by IFN-α–stimulated hepatoma cells but not by unstimulated cells (Fig. 4*D*). In the presence of the TRAIL-R2-Fc, which is capable of blocking TRAIL, but not with a control (huIgG1) antibody, specific lysis of CEM cells induced by IFN-α–treated hepatoma cells was substantially inhibited, demonstrating specificity of the TRAIL effect. Upon PML silencing in the respective hepatoma cell line, specific lysis of coincubated CEM cells was inhibited to a comparable amount as upon blocking of TRAIL (Fig. 4*D*).

These data show that IFN-α–induced TRAIL on hepatoma cells is functional and, thus, capable of inducing apoptosis in surrounding TRAIL-sensitive cells. Moreover, the results clarify a central role for PML in IFN-α–induced cell death, which is mediated by TRAIL.

PML is essential for the IFN-dependent regulation of TRAIL. To further confirm the significance of PML for IFN-α–dependent TRAIL regulation and cell death in the liver, we used primary hepatocytes from PML-deficient mice. PMH were cultivated for 48 h after preparation and thereafter treated with IFN-α for 24 hours. In line with results in hepatoma cells, we observed an increase in TRAIL expression on the cell surface of wild-type (WT) PMH by FACS analysis (Fig. 5*A*) and TRAIL mRNA levels upon incubation with IFN-α (Fig. 5*B*). In contrast, PML-deficient PMH did not show TRAIL up-regulation upon IFN-α treatment.

After coincubation of IFN-α–treated or non–IFN-α–treated PMH with CEM cells as targets cells, the CEM cells underwent apoptosis only upon contact with IFN-α–treated effectors, as determined by subdiploid DNA content analysis (Fig. 5*C*). To test for specificity of this effect, we preincubated the PMH effector cells in the presence or absence of TRAIL-R2-Fc or control IgG before stimulation with IFN-α and subsequent coincubation with CEM target cells. Lysis of CEM target cells was almost abolished in the presence of TRAIL-R2-Fc, whereas preincubation of IFN-α–stimulated cells with control IgG did not result in apoptosis inhibition. These results indicate the involvement of IFN-α–induced TRAIL in lysis of neighboring TRAIL-sensitive cells.

Coincubation of CEM target cells with PML-deficient PMHs, if pretreated with IFN-α or not, did not result in apoptosis of the targets. These data further confirm that PML is crucial for IFN-α–induced TRAIL-mediated apoptosis in liver pathology.

Discussion

IFN-α has been used with varying potency in the treatment of several malignancies (30). Recent studies show that IFNs may be a valuable tool in the supportive treatment of advanced HCC (6, 31). The underlying molecular mechanism by which IFN-α exerts antitumor activity is not completely understood. Some studies suggest a role in influencing angiogenesis and tumor cell viability (26), and it has been shown that the death ligand TRAIL is a critical IFN-induced apoptosis mediator in this scenario (32). However, mechanisms and elements involved in IFN-mediated cell death of hepatoma cells have not been elucidated thus far. Our data presented in this study suggest that (*a*) TRAIL is significantly involved in IFN-α–induced apoptosis in hepatocarcinoma, (*b*) PML is a major contributor in IFN-induced apoptosis by mediating the regulation of TRAIL in liver tumor cells, and (*c*) IFN-α–induced apoptosis in hepatomas is independent of p53.

As a model system, we used different liver cell lines with variable p53-status, as well as primary mouse liver cells (PMH). IFN-α exposure of the cell lines led to TRAIL expression and consecutively to apoptosis, meaning IFN responsiveness, in four of five hepatoma cell lines used. However, despite increased TRAIL expression, Hep3B cells are not in functional terms IFN responsive, as these cells do not undergo apoptosis upon IFN-α exposure. Interestingly, recombinant TRAIL kills only the IFN responsive (HepG2, Huh6, Huh7, and Chang) and not the nonresponsive (Hep3B) cells with equal efficiency. However, Hep3B cells have been previously described to be TRAIL resistant (7). These data suggest that the failure of IFN to induce apoptosis in certain lines is due to a lack in TRAIL sensitivity.

To confirm the involvement of TRAIL in this context, we used a death receptor–Fc fusion protein to block the respective ligand. Addition of TRAIL-R2-Fc could significantly impair IFN-α–induced apoptosis in the four IFN-sensitive cell lines. These results indicate significant contribution of the TRAIL receptor/ligand system to IFN-α–induced apoptosis in hepatomas and show that IFN-induced cell death of the tumor cells corresponds to TRAIL sensitivity of the respective cell line. Our results are in line with the fact that normal cells and a number of tumor cell lines show resistance toward TRAIL-induced apoptosis (29) despite expressing TRAIL-R1 and TRAIL-R2 on the cell surface. The molecular basis for this differential sensitivity is not clear, despite some studies show that cotreatment with chemotherapeutic agents, proteasome inhibitors, or irradiation resulted in sensitization of TRAIL-resistant tumor cell lines (33, 34). The mechanisms deciding on TRAIL resistance versus sensitivity are diverse and cell type specific (29). We tested whether IFN-α also exhibits a TRAIL-sensitizing effect in primarily

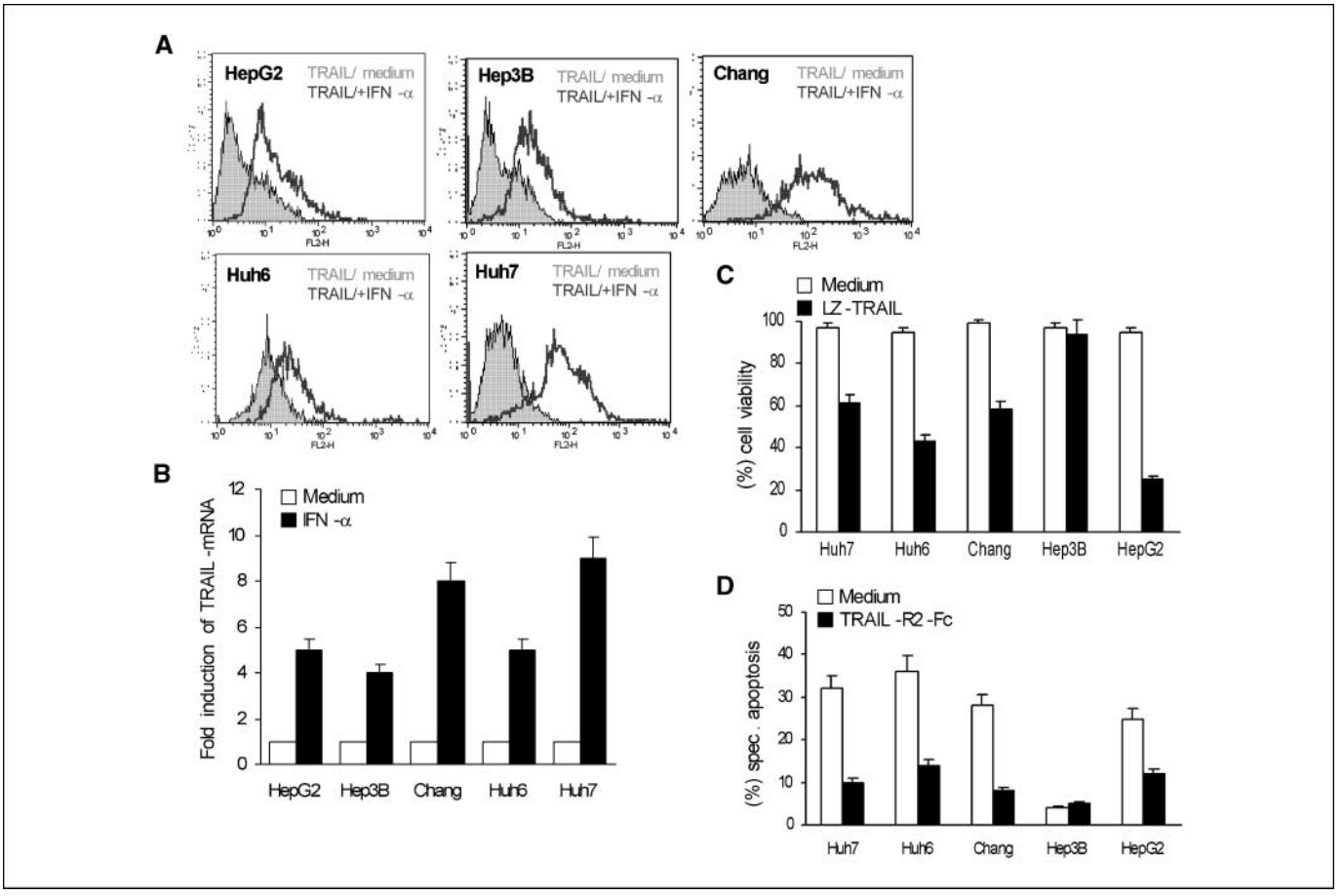

Figure 3. TRAIL expression upon IFN-α stimulation correlates with PML expression and cell death. FACS staining of TRAIL with TRAIL-R2-Fc or huIgG1 on hepatoma cells, treated with or without IFN-α for 36 h, as indicated (*A*). Hepatoma cells were incubated with 1,000 U/mL or medium, and TRAIL-mRNA was extracted after 12 h. mRNA levels were determined by quantitative PCR using β-actin for normalization (*B*). Hepatoma cell lines were exposed to 100 ng/mL LZ-TRAIL for 24 h and cell viability was determined by MTT assay (*C*). Cells were preincubated with 10 μg/mL receptor-Fc-protein before exposure to 1,000 U/mL IFN-α for 36 h, and apoptosis was quantified by analyzing the percentage of cells with subdiploid DNA content, as described by Nicoletti and colleagues (ref. 22; *D*). *Columns,* mean of five experiments performed; *bars,* SD.

TRAIL-resistant hepatoma cell lines but could not detect any IFN-α–mediated increase in TRAIL-induced apoptosis in any of the cell lines tested as well as in PMH (data not shown).

In contrast to unstimulated hepatoma cells, those cells stimulated with IFN-α showed a significantly higher capability to lyse TRAIL-sensitive CEM T-cells. Upon blocking of TRAIL on hepatoma cells with TRAIL-R2-Fc, lysis of CEM cells was almost completely impaired. This finding is especially interesting in the context of recent findings that tumor-infiltrating T cells are TRAIL sensitive (35). Thus, functional expression of TRAIL on HCCs may not only cause the autocrine suicide of hepatoma cells but may also negatively affect the immune response by inducing apoptosis in invading TRAIL-sensitive immune cells, most importantly in T cells. This effect would be especially harmful when the tumor cells have acquired a TRAIL resistant phenotype.

Furthermore, IFN-α–induced TRAIL expression on tumor cells that have acquired TRAIL resistance may even support tumor progression. When expressed on normal tissue, TRAIL has been shown to impair tumor development and metastasis because the absence of TRAIL has been shown to result in an increase in metastasis in the liver (36). Many types of tumor cells develop resistance to IFN-α–induced growth inhibition (37–39). TRAIL resistance as well as IFN-α resistance may be acquired after long-term exposure or selective outgrowth of cells with such a resistance, providing a growth advantage in the course of tumor growth and dedifferentiation.

IFN-α is responsible for the regulation of several hundred genes involved in diverse cellular processes (40). These genes include PML (14), and PML NBs have on their part been suggested to be sites of transcriptional regulation. Our study evaluates a role of PML in IFN-induced apoptosis in liver tumors and shows IFN-induced PML induction that causes increase of TRAIL expression. To confirm a role for PML in IFN-induced apoptosis in hepatomas, RNAi was used to specifically down-regulate PML expression. Treatment of IFN-responsive hepatoma cells with shPML led to a partial inhibition of the growth-inhibitory effect of IFN-α and correlated with the reduction of PML protein levels. Furthermore, PML-deficient primary hepatocytes (PMH) from PML *knockout* mice did not show any TRAIL expression upon IFN-α treatment in contrast to WT hepatocytes. These show an up-regulation of TRAIL mRNA and protein levels upon IFN-α.

TRAIL is functional on PMH, as IFN-stimulated PMH kill surrounding TRAIL-sensitive CEM cells, which can be inhibited by blocking TRAIL with TRAIL-R2-Fc. Importantly, these results indicate that reduced or deficient PML expression results in down-regulation of both TRAIL mRNA and protein levels. Therefore, differential PML and subsequent TRAIL induction seems to determine the response phenotype to IFN.

To understand its role in the IFN response of liver tumors, a major interest of future studies will therefore be the definition of transcription factors that regulate activation of TRAIL in interplay with PML. Those factors may well be responsible for the differential

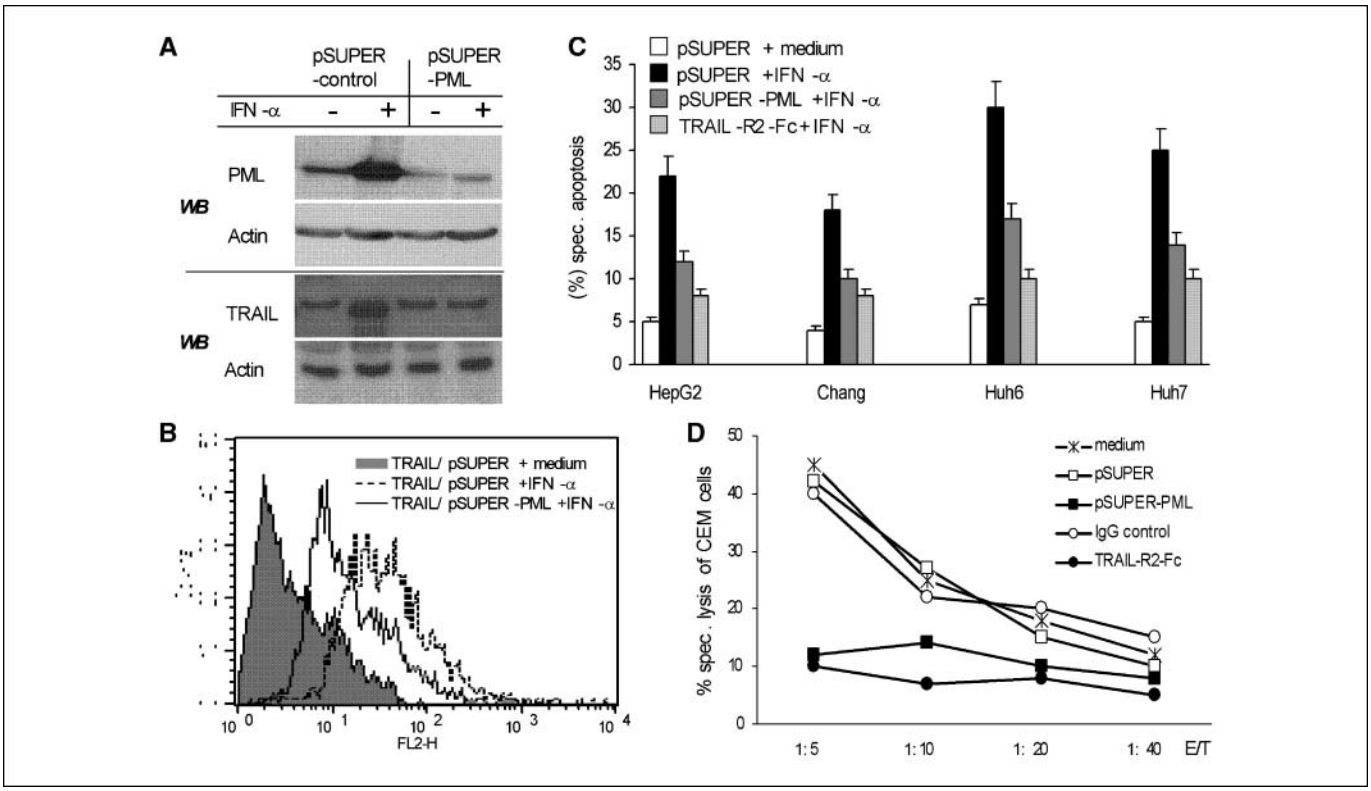

Figure 4. Knockdown of PML expression impairs IFN-α–induced TRAIL expression and cell death. HepG2 cells were transfected with pSUPER (*control*) or pSUPER-PML as indicated and treated with IFN-α or not. Cell lysates were analyzed by immunoblotting (*A*) or total cells by FACS (*B*) with the antibodies indicated. Cells were treated as in *A*, and cell death was quantified by analyzing the percentage of cells with subdiploid DNA content, as described by Nicoletti and colleagues (22). To block TRAIL, cells were pretreated with or without TRAIL-R2-Fc for 30 min and then incubated for 36 h in the presence or absence of IFN-α (*C*). HepG2 cells were transfected with pSUPER-PML, treated with or without 1,000 U/mL IFN-α for 24 h, washed twice with PBS, and coincubated with ^{51}Cr-labeled CEM cells as targets in a standard chromium release assay. Mean values of duplicate samples from four step titrations at effector/target (*E/T*) ratios as indicated of one of three representative experiments are shown. To block death ligands, cells were pretreated with or without TRAIL-R2-Fc for 30 min and then incubated for 36 h in the presence or absence of IFN-α. Data represent mean values of triplicate samples (*D*). *WB,* Western blotting.

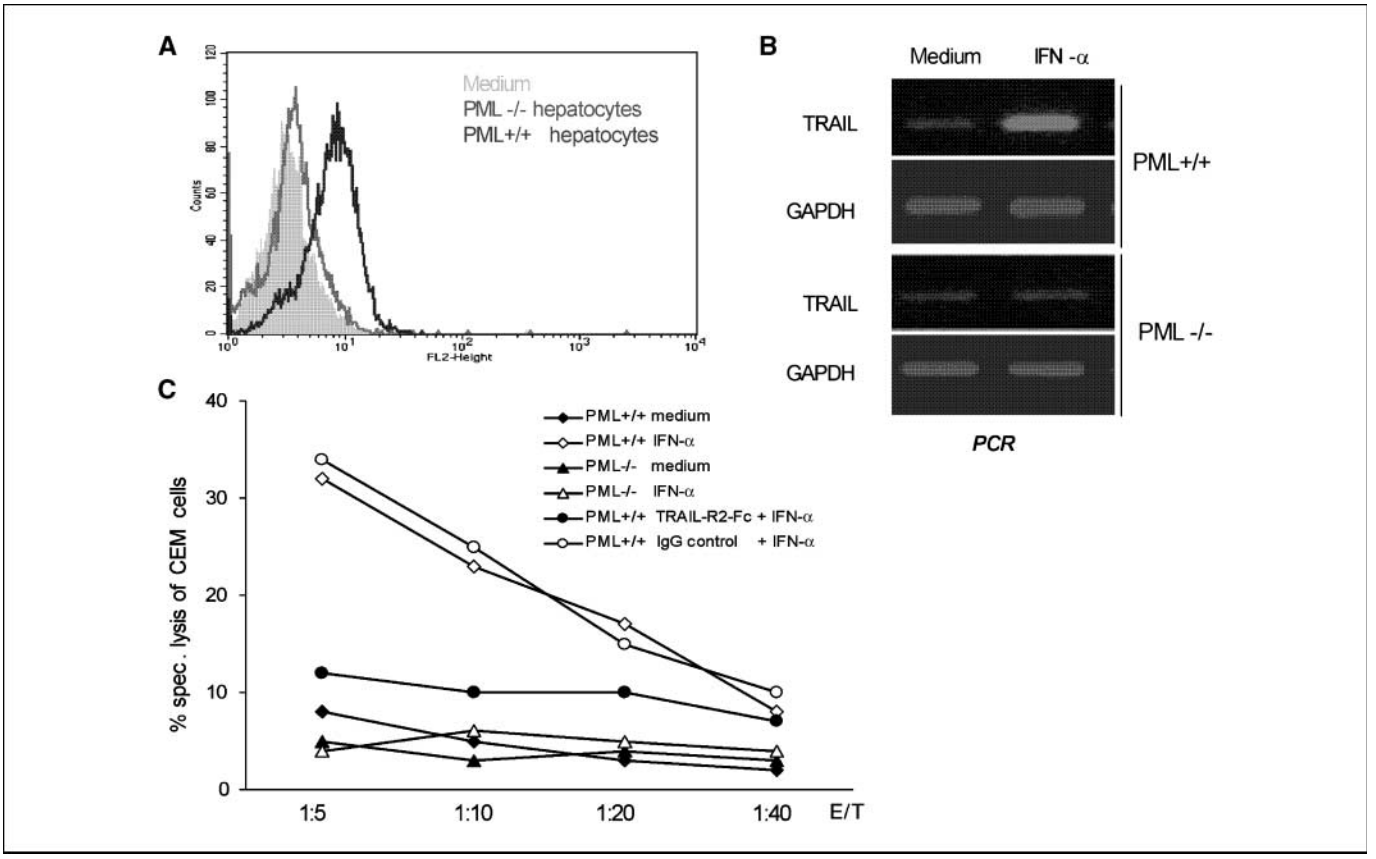

Figure 5. IFN-α fails to induce TRAIL expression in PML−/− mouse hepatocytes. PMH were treated with 1,000 U/mL for 36 h and surface expression of TRAIL was determined by specific staining with TRAIL-R2-Fc and FACS analysis (*A*). Cells were harvested after 24 h of IFN-α (1,000 U/mL) treatment for total RNA preparation and PCR (*B*). PMH were plated, treated with or without IFN-α for 24 h, washed five times with PBS, and covered with four step titrations of CEM cells or with medium. The supernatant was harvested at different time points and apoptotic CEM cells determined by subdiploid DNA content analysis. Where indicated, PMH effector cells, treated with or without IFN-α for 24 h and washed thoroughly, were incubated in the presence or absence of TRAIL receptor–Fc fusion proteins with CEM target cells (*C*). *Columns,* mean of five experiments performed; *bars,* SD. The data shown are representative for results in five separate preparations of PMH obtained from five different mice. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

IFN responsiveness of hepatoma cells and liver tumors, respectively. However, our herein-presented data rule out a role for p53 for this mechanism, which has been shown to act as important PML-binding partner in other scenarios. We observe IFN-α sensitivity in Huh-cell lines with functional (Huh6) as well as nonfunctional (Huh7) p53 status. As well, HepG2 cells show IFN sensitivity, which is not compromised after knockdown of p53 expression (data not shown). In addition, gene array analyses of HCC tissue as well as cell lines studying IFN gene regulation do show differential IFN sensitivity and gene cluster, but these features are not correlated to p53 expression (40).[4] In conclusion, these data suggest that IFN-α–induced PML-mediated TRAIL regulation and apoptosis is independent of p53.

Furthermore, preliminary data of TRAIL promoter sequence analyses in different liver cancer cell lines reveal variations in those sites, which correspond to response elements involved in IFN signaling. These results suggest that relevant sites may harbor mutations that may contribute to the differential response of HCCs to IFN-mediated effects.

Revealing mechanisms of IFN responsiveness of HCCs will contribute important knowledge to establish better therapeutic strategies for liver tumors and will, thus, enable a more efficient application of IFN-α in clinical cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[4] Unpublished data of own ongoing studies.

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