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Analysis of IFN-γ-Induced Cell Cycle Arrest and Cell Death in Hepatocytes[1]

Arihiro Kano,* Yoshifumi Watanabe,*,[2] Naoki Takeda,† Shin-ichi Aizawa,† and Toshihiro Akaike*

*Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama 226; and †Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860

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The mechanism by which IFN-γ induces cell cycle arrest and cell death in primary cultured hepatocytes was examined. The cell death exhibits apoptotic characters such as the appearance of apoptotic bodies and DNA fragmentation. IFN-γ induced cell cycle arrest at the initial stage, followed by cell death. A protein synthesis inhibitor, cycloheximide, significantly inhibited cell death, implying that IFN-γ induces *de novo* proteins involved in the death of hepatocytes. One of the most important apoptosis-related proteins, p53, was induced by IFN-γ in hepatocytes in a dose- and time-dependent manner. Northern blot analysis demonstrated that IFN-γ enhanced p53 mRNA expression as well as $p21^{WAF1/Cip1/sdi1}$ mRNA expression, which is mediated by the increased expression of the p53 protein. Interestingly, IFN-γ also induced cell death in p53-deficient hepatocytes. The cell death occurred rather earlier in p53-deficient cells than in normal hepatocytes. However, the cell death was not accompanied by apoptotic bodies. Therefore, IFN-γ-induced hepatocyte cell death is p53-independent, and p53 may contribute to the apoptotic characters. In conclusion, IFN-γ is supposed to cause cell cycle arrest by inducing p53 and $p21^{WAF1/Cip1/Sdi1}$, and it was demonstrated that IFN-γ induces p53-independent cell death in primary cultured hepatocytes.

Key words: apoptosis, cell cycle, hepatocytes, IFN-γ, p53.

Hepatitis is an inflammatory liver disease with various causes (viral infection, bacterial infection, alcohol, drug injury, *etc.*). Although there are many unsolved problems related to the underlying mechanism, it is clear that hepatocytes are the major target cells damaged in hepatitis. However, it is not clear what kind of molecules and what kind of regulation are critically involved in the hepatic damage. It has been reported that tumor necrosis factor α (TNFα), interleukin 1 and 6 (IL-1 and IL-6), and interferon-γ (IFN-γ) are important mediators of the inflammation. Increased production of these inflammatory cytokines is often detected in cases of hepatitis (*1, 2*) or in a liver infected with bacteria (*3*), and they are considered to play important roles in the onset of hepatitis (*4*). Of these cytokines, evidence is accumulating that IFN-γ is critically involved in immune-mediated hepatitis (*5-9*). However, the mechanism by which IFN-γ induces hepatic cell death remains substantially unclear.

Cell death is classified into two distinct types, namely, necrosis and apoptosis. Apoptosis is a typical form of programmed cell death that eliminates unwanted cells in the development of the immune system, organ formation, and embryogenesis (*10*). The characteristic features of apoptosis are the condensation and fragmentation of nuclear chromatin, accompanied by the compaction of cellular organelles, dilatation of the endoplasmic reticulum, and a marked reduction in cell volume (*10*). The tumor suppressor gene, p53, plays a central role in apoptosis and the increased expression or activation of this gene induces apoptosis in a number of cell types (*11-16*). In hepatocytes, of the cytokines involved in hepatitis, both TNFα (*17*) and IFN-γ (*18*) induce apoptosis, which is thought to be one of the mechanisms in the pathogenesis of hepatitis. However, the role of p53 in hepatic apoptosis has not been examined.

In this study, we examined the role of p53 in hepatic apoptosis induced by IFN-γ. We found that IFN-γ induced p53 expression in hepatocytes and that the IFN-γ-induced p53 was transcriptionally active since p53 in IFN-γ-treated hepatocytes also induced the $p21^{WAF1/Cip1/sdi1}$ gene. However, IFN-γ-induced cell death in p53-deficient hepatocytes without the typical characteristics of apoptosis. Therefore, IFN-γ induces p53-independent cell death in primary cultured hepatocytes.

MATERIALS AND METHODS

Reagents and Animals—Recombinant mouse IFN-γ was purchased from Genzyme (Cambridge, MA), and recombinant human insulin from Wako Pure Chemical Industries (Osaka). Hybridomas (PAb122) producing anti-mouse p53 antibodies were obtained from the American Type Culture

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[2] To whom correspondence should be addressed. Tel: +81-045-924-5801, Fax: +81-045-924-5815, E-mail: ywatanab@bio.titech.ac.jp

Collection (Rockville, MD). The antibodies were used after purification by protein G-Sepharose chromatography. The female ICR, C57BL/6 mice employed in the experiments in this study were purchased from Charles River Japan (Kanagawa). p53-deficient mice were established as described (*19*). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

Cell Preparation—Parenchymal hepatocytes were prepared as previously described (*20*). Briefly, a liver was perfused with a 0.0125% collagenase solution. After the liver had been excised, parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at $50\times g$ for 90 s. The dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia). The viable parenchymal hepatocytes were suspended in Williams'E medium containing antibiotics and then plated at a density of 1×10^4 cells/well in flat-bottomed 96-well plates (Sumitomo Bakelite, Tokyo) pre-coated with collagen. The hepatocytes were incubated at 37°C for 3 h in order for them to adhere to the collagen-coated plates and then the medium was changed to a new one containing 5% FCS, 10 ng/ml EGF (epidermal growth factor), and 10^{-7} M insulin before the experiments.

Analysis of DNA Synthesis and Cell Death—The DNA synthesis by treated hepatocytes was evaluated as the incorporation of 5-bromo-2'-deoxy-uridine (BrdU) (*21*) with a BrdU Labeling and Detection Kit III (Boehringer Mannheim Biochemica, Mannheim, Germany), following the manufacturer's instructions. Briefly, BrdU was added to treated hepatocytes on a 96 well flat-bottomed microtiter plate, and then the cells were incubated at 37°C. The cells were then washed and fixed with HCl-ethanol at −20°C for 40 min. The cells were treated with nuclease and then reacted with peroxidase conjugated monoclonal anti-BrdU antibodies. The substrate (ABTS) for peroxidase was added after washing, and the mixture was incubated at room temperature for 5 min with an enhancer. The absorbance of each well at 415 nm was measured using a micro plate reader, MTP-120 (Corona Electronic, Ibaragi).

For the estimation of cell death, the activity of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis and one of the commonly used hallmarks of cellular cytotoxicity (*22*), was measured in the supernatants of the samples using a CytoTox 96™ Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) (*23*), following the manufacturer's instructions. The percentage of lysis was calculated using the formula:

$$\%\ \text{LDH release} = 100\times(\text{Experimental release} - \text{Effector spontaneous release} - \text{Target spontaneous release})/(\text{Target maximum release} - \text{Target spontaneous release})$$

Maximum release was obtained on complete solubilization of hepatocytes with 0.1% Triton X-100.

Immunoprecipitation and Western Blotting—Cells were harvested with lysis buffer (10 mM Tris-HCl, pH 8.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 100 mM NaF, 0.2 mM Na_3VO_4, and 0.5 mM PMSF). The sample solution was incubated with protein G-Sepharose bearing anti-mouse p53 antibodies for 1 h at 4°C after preincubation with protein G-Sepharose. The Sepharose was pelleted by brief centrifugation, and then washed with the same buffer three times. The bound protein was extracted with SDS loading buffer and then subjected to SDS-PAGE on a 7.5-20% gradient gel. Proteins were then electroblotted onto PVDF

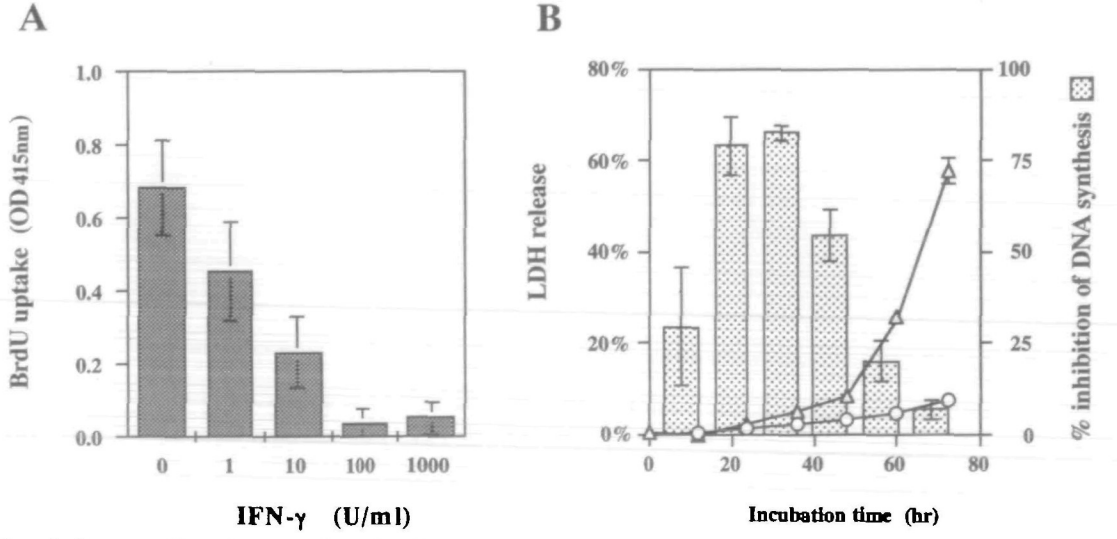


Fig. 1. **IFN-γ induces cell cycle arrest and cell death in primary cultured murine hepatocytes.** Hepatocytes were cultured on a collagen-coated plate in the presence of EGF (10 ng/ml) and serum (5%). (A) Cells were cultured with IFN-γ at various concentrations for 24 h, BrdU incorporation between 24 to 48 h after stimulation was then measured. (B) Hepatocytes were cultured with IFN-γ (100 U/ml) in the presence of EGF (10 ng/ml) and serum (5%) for the indicated times, and then the LDH-activities in the supernatants and DNA synthesis for 12 h at determined times were measured as described under "MATERIALS AND METHODS." Percent inhibition of DNA synthesis was calculated as follows: % inhibition = 100 × (BrdU incorporation of non-treated control − BrdU incorporation of IFN-γ-treated sample)/(BrdU incorporation of non-stimulated control). Circles, non-treated control; triangles, IFN-γ-treated. Data represent means ± SD.

membranes (Amersham, Arlington Heights, IL) in cold transfer buffer for 3 h at 60 V. The filters were incubated with the first antibodies for 1 h at room temperature after blocking with 3% skim milk, washed with TBS, and then incubated with the peroxidase-labeled second antibodies for 1 h. Bands were detected with an ECL kit (Amersham, Illinois).

RNA Extraction and Northern Blotting—Total RNA was extracted with TRI zol (Gibco BRL, Grand Island, NY), subjected to electrophoresis, and then transferred to a Hybond nylon membrane (Amersham). The genes for p53 and p21 were prepared by RT-PCR using primers (sense for p53: TGTGTAATAGCTCCTGCATGG, anti-sense for p53: TCTTGGTCTTCAGGTAGCTGG, sense for p21: TCCAATCCTGGTGATGTCC, anti-sense for p21: ACACCAGAGTGCAAGACAGC). The PCR products were inserted into the pGEM vector plasmid (Promega). RNA probes were prepared using a Gene Images RNA labeling kit, following the manufacturer's instructions (Amersham). After hybridization, the blots were washed many times at room temperature in 1×SSC plus 0.1% SDS, and then 0.1×SSC plus 0.1% SDS, and the bands were detected using a Gene Images CDP-Star detection kit (Amersham).

RESULTS

IFN-γ Inhibited DNA Synthesis and Induced Cell Death in Primary Cultured Mouse Hepatocytes—DNA synthesis of primary cultured hepatocytes was triggered by the addition of EGF and serum 3 h after plating. The DNA synthesis was inhibited by IFN-γ in a dose-dependent manner (Fig. 1A). In addition, IFN-γ also induced cell death in hepatocytes regardless of the presence of EGF and serum (Fig. 1B). Interestingly, as shown in Fig. 1B, LDH release was not detected in the supernatants within 40 h after the addition of IFN-γ, while the DNA synthesis was decreased between 24-36 h. Therefore, the inhibition by IFN-γ of DNA synthesis occurred before IFN-γ-induced cell death. The cell death was accompanied by the typical characters of apoptosis such as the appearance of apoptotic bodies and DNA fragmentation (Fig. 2). These data demon-

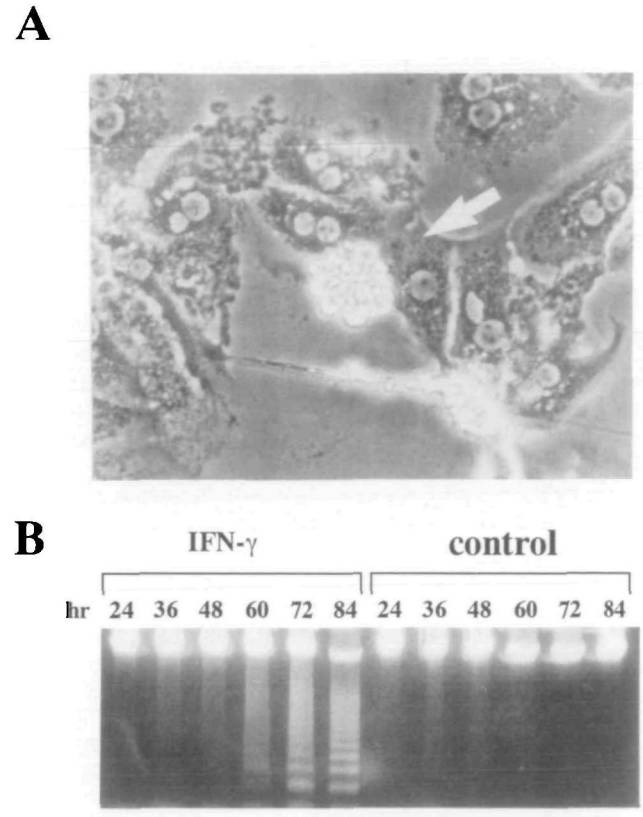


Fig. 2. **Cell death induced by IFN-γ in primary hepatocytes is accompanied by apoptotic characteristics.** (A) Primary hepatocytes were cultured with IFN-γ (100 U/ml) for 48 h. Morphological changes were observed under a microscope. The arrow in the figure indicates a typical apoptotic body of a hepatocyte. Magnification: ×200. (B) Hepatocytes were cultured with IFN-γ (100 U/ml) for the indicated times, and then incubated with the lysis buffer (10 μg/ml proteinase K, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% SDS) for 15 h at 37°C. Chromosomal DNA was obtained by phenol/chloroform (1 : 1) extraction and ethanol precipitation. After RNase treatment for 1 h at 37°C, the same amount of DNA from each sample was subjected to electrophoresis on a 1.0% agarose gel containing 0.1 mg/ml ethidium bromide.

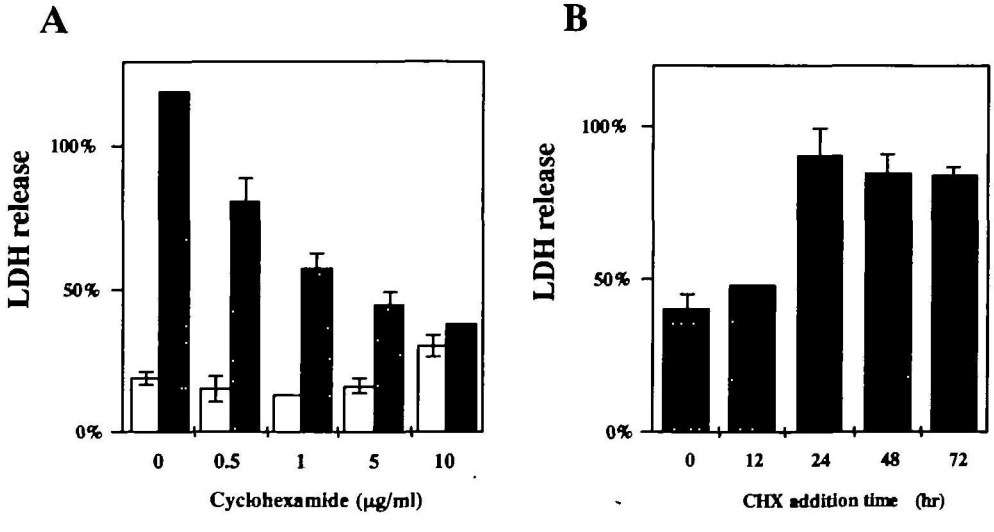


Fig. 3. **Cycloheximide inhibits IFN-γ-induced cell death in hepatocytes.** Hepatocytes were cultured with IFN-γ (100 U/ml) in the presence of CHX. The LDH activities in the supernatants were measured after the incubation. (A) Dose-dependent inhibition by CHX of IFN-γ-induced apoptosis in hepatocytes. Cells were cultured for 72 h with IFN-γ and CHX at various concentrations. Open bars, non-treated hepatocytes. Filled bars, IFN-γ-treated hepatocytes. (B) Time-course analysis of the inhibitory effect of CHX on IFN-γ-induced apoptosis in hepatocytes. CHX (5 μg/ml) was added at the indicated times following the addition of IFN-γ. The supernatants were collected at the end of the culture (72 h), and then the LDH activities in them were measured. Data represent means±SD.

strate that IFN-γ-induced apoptosis in primary cultured hepatocytes.

Many cases of apoptosis are known to be accompanied by *de novo* protein synthesis (*24*). The apoptosis induced by IFN-γ was also inhibited by the addition of a protein synthesis inhibitor, cycloheximide (CHX) (Fig. 3). CHX inhibited LDH release from IFN-γ stimulated hepatocytes dose-dependently (Fig. 3A). However, the inhibition occurred only when CHX was added within 24 h after IFN-γ (Fig. 3B). These data suggest that cell death regulating protein is induced by IFN-γ within 24 h after stimulation.

IFN-γ Enhanced the Expression of Transcriptionally Active p53 in Primary Cultured Hepatocytes—The data above indicate that IFN-γ induces an apoptosis-related protein in hepatocytes within 24 h. There are various apoptosis-related proteins, such as ICE, Bcl-2, Bax, and MDM-2. After several trials, we found that IFN-γ significantly enhanced p53 protein expression in primary hepatocytes (Fig. 4). IFN-γ-enhanced p53 protein expression in a dose-dependent manner (Fig. 4A). Kinetic analysis revealed that the p53 protein expression was strikingly enhanced by IFN-γ within 24 h after stimulation, and interestingly had disappeared 48 h after in both the whole lysate and nuclei (Fig. 4, B and C). Therefore, the expression of p53 was only transiently induced by IFN-γ in the cytosol and the protein was accumulated in nuclei. Since nuclear translocation of the p53 protein is essential for its function (*25*), the induced p53 in nuclei is thought to be active. On the other hand, p53 mRNA expression was persistently induced by IFN-γ until 48 h after stimulation while a DNA damaging reagent, 5-fluorouracil (5-FU), did not induce an increase of p53 mRNA expression (Fig. 5). To determine whether or not the increased p53 is functional as a transactivator, p53-dependent cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1/sdi1}$ (*26–28*), mRNA expression was examined. As shown in Fig. 6, p21 mRNA expression was also significantly enhanced by IFN-γ as well as 5-FU, which did not increase p53 mRNA expression.

Effect of IFN-γ on p53-Deficient Hepatocytes—To investigate the substantial roles of p53 induced by IFN-γ in hepatocytes, we employed hepatocytes from p53-deficient mice in the following experiments. Surprisingly, IFN-γ induced cell death in p53-deficient hepatocytes as well as in $p53^{(+/+)}$ cells (Fig. 7). However, IFN-γ did not induce cell cycle arrest in p53-deficient hepatocytes. Interestingly, despite that IFN-γ-induced cell death indicated by LDH release was not inhibited in p53-deficient hepatocytes, typical apoptotic bodies were not observed in the same cells (Fig. 7B).

As expected, IFN-γ did not induce p21 mRNA expres-

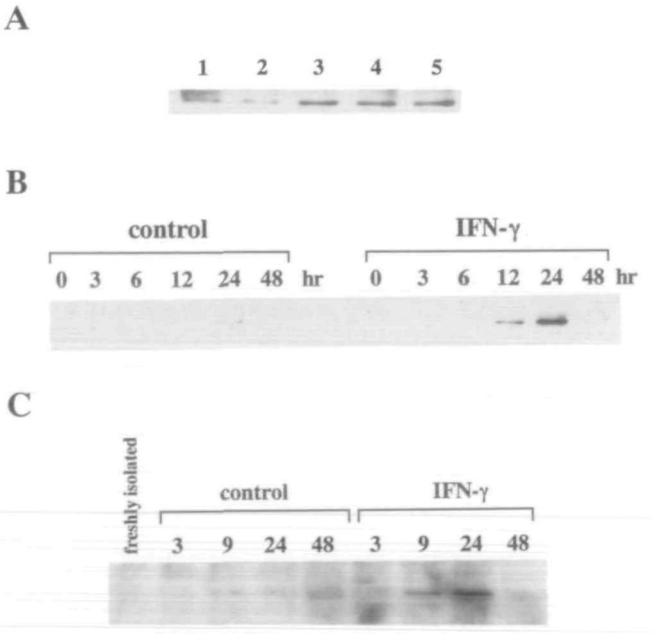


Fig. 4. **p53 protein expression in IFN-γ-treated hepatocytes.** Hepatocytes were cultured with IFN-γ, and then the p53 protein expression in the cells was examined. (A) Hepatocytes were cultured with IFN-γ at various concentrations for 24 h, and then immunoprecipitation of p53 was carried out. (B) Hepatocytes treated with IFN-γ (100 U/ml) were harvested at the indicated times and then subjected to immunoprecipitation of p53. Control: non-treated hepatocytes, IFN-γ: IFN-γ-treated hepatocytes. (C) Hepatocytes were treated with IFN-γ (100 U/ml) for the indicated times, and then the nuclei were extracted. The nuclei were subjected to Western blotting for p53. Control: non-treated hepatocytes, IFN-γ: IFN-γ-treated hepatocytes.

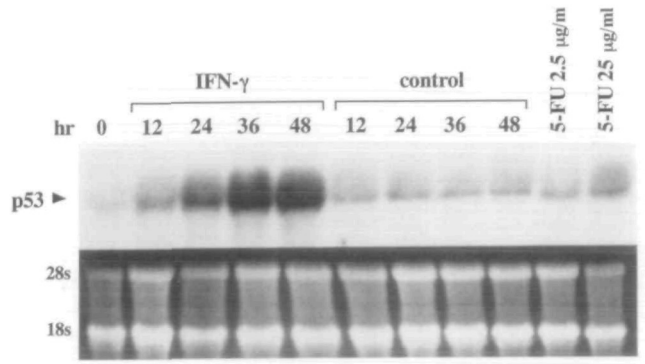


Fig. 5. **Analysis of p53 mRNA expression in IFN-γ-treated hepatocytes.** Hepatocytes were cultured with IFN-γ (100 U/ml) in the presence of EGF (10 ng/ml) and serum (5%). RNA was extracted from non-treated (control) or IFN-γ-treated hepatocytes at the indicated times and then subjected to Northern blot analysis. Hepatocytes were also treated with 5-FU for 24 h, and then the extracted RNA was examined in the same way.

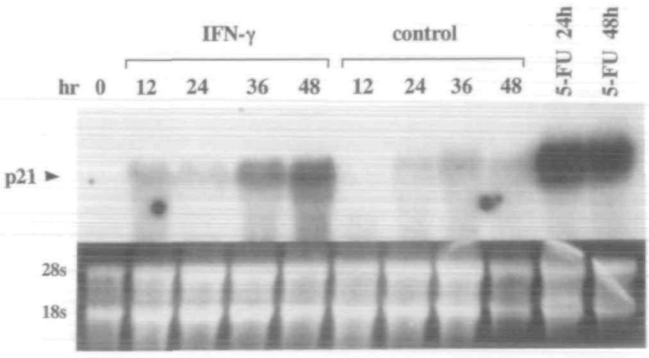


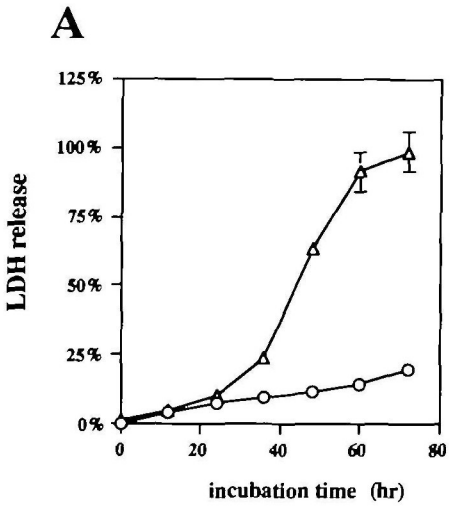
Fig. 6. **IFN-γ induces $p21^{WAF1/Cip1/sdi1}$ mRNA expression in hepatocytes.** Total RNA was extracted from non-treated (control) or IFN-γ (100 U/ml)-treated hepatocytes after various times, and then subjected to Northern blot analysis using a RNA probe for p21. Hepatocytes were also treated with a DNA damaging reagent, 5-FU (25 μg/ml).

sion in p53-deficient mice after 24 h (Fig. 8), while in $p53^{(+/+)}$ hepatocytes p21 mRNA was strongly induced. However, slight expression of p21 mRNA was detected within 24 h after the treatment with IFN-γ. The expression is thought to comprise p53-independent induction of p21 by IFN-γ. In addition, 5-FU did not induce p21 mRNA in p53-deficient hepatocytes, implying that the induction of p21 mRNA by 5-FU in Fig. 6 was mediated by the increase in p53 protein expression with 5-FU because 5-FU did not increase p53 mRNA expression.

DISCUSSION

In this study, we demonstrated that IFN-γ induced p53-independent cell death in murine primary cultured hepatocytes. Despite that the involvement of IFN-γ in the pathogenesis of hepatitis has long been suggested, this is the first report that this cytokine directly induces tumor suppressor genes such as p53 and p21, cell cycle arrest, and apoptosis in hepatocytes.

The effect of IFN-γ is dependent on the cell type(s). In some cell types, IFN-γ has an antiproliferative effect or induces apoptosis (*29-31*), while it inhibits apoptosis in another cell type (*32*). Our previous study and present one demonstrated that IFN-γ is cytotoxic for hepatocytes. We found that IFN-γ induces transcriptionally active p53 molecules in hepatocytes because IFN-γ-induced p53 molecules also induced a CDK inhibitor, $p21^{WAF1/Cip1/Sdi1}$, mRNA in control hepatocytes, while IFN-γ did not induce p21 mRNA in p53-deficient hepatocytes 24 h after the treatment. The induced p21 molecules are considered to arrest the cell cycle of hepatocytes in the G_1 phase. There have been a number of studies on the involvement of p53 in apoptosis (*12, 14, 16, 33, 34*). Therefore, we thought at first that IFN-γ-induced p53 is a key factor in IFN-γ-induced apoptosis of hepatocytes. However, surprisingly, the apoptosis is independent of IFN-γ-induced p53 because this cytokine induced cell death in p53-deficient hepatocytes. In addition, the cell death in p53-deficient hepatocytes occurred rather earlier than in control hepatocytes. IFN-γ induced p53 mRNA constantly in hepatocytes while the protein expression was transiently detected within 24 h, and had disappeared after 48 h. Despite the transient increased expression of the p53 protein, cell death in normal hepatocytes required more than 48 h after the stimulation with IFN-γ in the presence of serum. On the other hand, in p53-deficient hepatocytes IFN-γ caused LDH release 24 h after the stimulation. Some investigators have reported that tumor suppressor genes such as p21



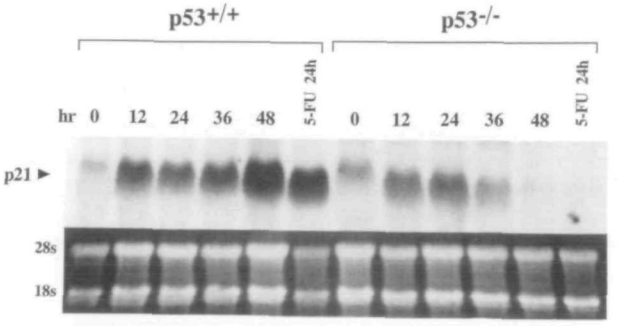


Fig. 8. **Expression of $p21^{WAF1/Cip1/sdi1}$ mRNA in p53-deficient hepatocytes treated with IFN-γ.** Hepatocytes from p53-deficient mice were cultured with IFN-γ (100 U/ml), and then total RNA was extracted from the cells and subjected to Northern blot analysis of p21 mRNA expression as described under "MATERIALS AND METHODS."

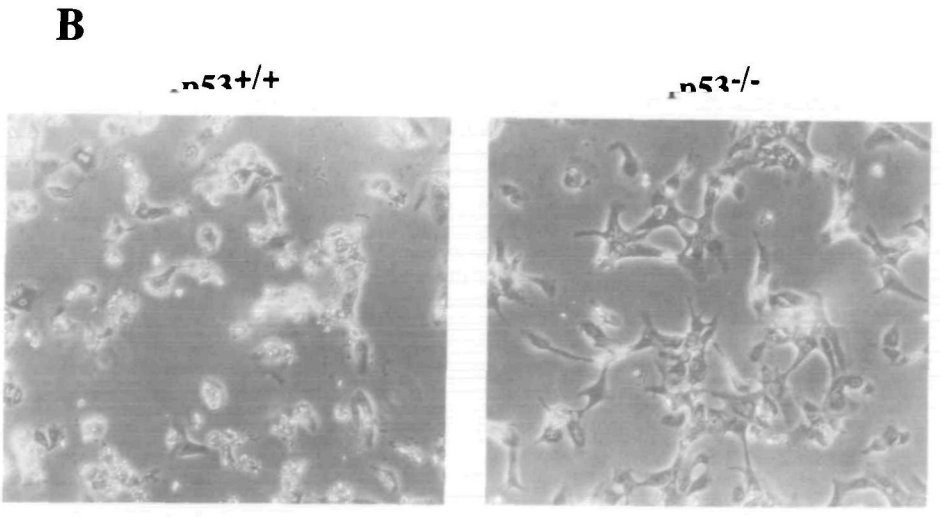


Fig. 7. **Effect of IFN-γ on p53-deficient hepatocytes.** Primary hepatocytes from p53-deficient mice were treated with IFN-γ, and then the experiments were performed. (A) IFN-γ induces cell death in p53-deficient hepatocytes. p53-deficient hepatocytes were cultured with IFN-γ (100 U/ml) for the indicated times. The LDH activities in the supernatants were then measured. Open circles, non-treated p53-deficient hepatocytes. Open triangles, IFN-γ-treated p53-deficient hepatocytes. Data represent means±SD. (B) Morphological comparison of IFN-γ-treated $p53^{(+/+)}$ hepatocytes (left) and IFN-γ-treated p53-deficient hepatocytes (right) 48 h after the addition of IFN-γ. Magnification: ×40.

inhibits apoptosis (*35-37*). Therefore, the p21 expression induced by IFN-γ may inhibit the apoptosis within 24 h, inducing cell cycle arrest prior to the apoptosis. In fact, the inhibition by IFN-γ of DNA synthesis paralleled the transient expression of the p53 protein. Then, after the disappearance of p53, cell death started to occur. The mechanism underlying the disappearance of the p53 protein despite the abundant expression of p53 mRNA is unknown. However, it has been reported that the p53 protein inhibits its translation by binding mRNA for negative feedback (*38*). The same mechanism may contribute to the regulation of the p53 protein in IFN-γ-stimulated hepatocytes.

Intriguingly, typical apoptotic characters such as apoptotic bodies were not detected in p53-deficient hepatocytes despite that IFN-γ-induced cell death in the same cells, implying that p53 plays a major role in inducing apoptotic characters rather than cell death. Therefore, cell death and the induction of apoptotic characters are thought to be regulated by distinct pathways.

At the initial stage within 24 h after the stimulation of p53-deficient hepatocytes, slight expression of p21 was detected, indicating that the expression was due to a p53-independent mechanism. Chin *et al.* have also reported that IFN-γ induces p21 expression in a p53-independent manner in different cell lines (*39*).

The mechanism by which p53 is induced by IFN-γ in hepatocytes is unknown. In most cases, the p53 protein is induced by DNA damage (*13*). However, there are some distinct pathways for the induction or activation of p53. For example, some investigators reported that NO induces p53 expression and apoptosis in some cell types (*40, 41*). However, a NO synthase inhibitor (N^G-monomethyl-L-arginine) did not inhibit IFN-γ-induced apoptosis in hepatocytes (data not shown). In addition, it has also been reported that IFN-γ alone does not induce NO in hepatocytes (*42*). Therefore, it is unlikely that NO is involved in the mechanism. The transduction signal of IFN-γ that regulates the expression of tumor suppressor genes such as p53 and p21 remains to be studied.

The apoptosis induced by IFN-γ in hepatocytes was effectively blocked by a protein synthesis inhibitor, CHX, demonstrating that IFN-γ induces *de novo* synthesized molecules regulating apoptosis in hepatocytes. This indicates that the hepatic cell death induced by IFN-γ is one type of programmed cell death. The group of Kimchi have identified some molecules, such as cathepsin D (*43*), thioredoxin (*29*), and DAP-3 (*44*), involved in IFN-γ-induced apoptosis in HeLa cells. The possibility that these molecules are involved in IFN-γ-induced apoptosis of hepatocytes is under investigation.

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