IFN-alpha prevents the growth of pre-neoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat

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Interferon (IFN)-alpha treatment is a common therapy for chronic viral hepatitis and contributes to preventing hepatocarcinogenesis. However, it is not clear whether IFN-alpha directly inhibits the clonal expansion of pre-neoplastic hepatocytes. To clarify the mechanism by which IFN-alpha prevents hepatocarcinogenesis, we examined the effect of IFN-alpha in a chemically induced hepatocarcinogenesis model initiated by diethylnitrosamine (DEN) and promoted by 2-acetylaminofluorene (2-AAF) and partial hepatectomy, in which hepatocellular carcinoma (HCC) arises through pre-neoplastic foci without inflammation or fibrosis. The protocols of IFN-alpha administration were started simultaneously with chemical initiation and lasted for either 4 or 40 weeks. The pre-neoplastic foci and neoplastic HCC were evaluated at 4 or 40 weeks after chemical initiation, respectively. The effects of IFN-alpha were assessed by the expression of tumor-related genes and cell cycle-related genes in the pre-neoplastic foci, using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). As a result of IFN-alpha treatment, the numbers and average volume of pre-neoplastic foci were reduced. The proliferating cell nuclear antigen index and the expression of G1 cyclins were also reduced in the pre-neoplastic foci in the IFN-treated group. The expression of p21, which is an inhibitor of cyclin-kinase complexes, was higher in the foci of the IFN-treated group, while p53 expression was not altered in this group, compared with the control group. IFN-alpha also suppressed the tumor development at 40 weeks after initiation. And in the long-term IFN-alpha-treated group, both the tumor numbers and average tumor size were markedly more reduced than those in the short-term-treated group. Therefore, it was demonstrated that longer treatment with IFN-alpha was more effective, compared with shorter treatment. In conclusion, it was shown that IFN-alpha directly prevented and delayed hepatocarcinogenesis through the suppression of pre-neoplastic cell proliferation and that it may partially depend on p21 induction through a p53-independent pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. In hepatitis B virus (HBV) or hepatitis C virus (HCV) carriers, persistent virus infection seems to be associated with hepatocarcinogenesis (1, 2). Interferon (IFN)-alpha can eradicate the hepatitis virus and is clinically useful for treating persistent hepatitis. Recently, some clinical studies have shown that IFN-alpha treatment plays a preventive role in hepatocarcinogenesis, even if complete viral eradication is not achieved (3–5). While IFN-alpha is demonstrated to be a multifunctional cytokine preventing viral replication and inhibiting cell proliferation (6), the antitumorigenic effects of IFN-alpha have yet to be clarified.

The Solt and Farber model is a well-known animal model of hepatocarcinogenesis that is not influenced by hepatitis virus, inflammation, fibrosis or other environmental factors (7). In this model, initiation by diethylnitrosamine (DEN) induces pre-neoplastic hepatocytes in rats, whereas treatment with 2-acetylaminofluorene (2-AAF) and partial hepatectomy promotes clonal expansion of these pre-neoplastic cells. 2-AAF forms adducts with DNA, resulting in structural distortion, and consequently blocks DNA replication. The clonal expansion of pre-neoplastic cells during 2-AAF treatment has been suggested to be due to the failure of nuclear expression of p53 (8–11), a factor that plays an important role in maintaining the integrity of the genome.

p53 is one of the most important tumor suppressor genes and is inactivated in many kinds of human cancer, including HCC (12, 13). p21 protein, a downstream signal from p53, has been found in various abnormal conditions especially cancers in humans and rats (15–17). p53 and subsequent induction of p21 both induce the G1 cell cycle arrest at the event of DNA damage (18). G1 cyclins (cyclin D and E), in complex with CDKs, are major players driving cell cycle from G1 to the S phase. p21 directly binds to cyclin–CDK complexes and prevents CDK-dependent phosphorylation, subsequently inactivating the retinoblastoma protein (14). p21 is also known to inhibit proliferating cell nuclear antigen (PCNA)-dependent DNA replication (19, 20). Therefore, when a cell is crippled and unable to express p53 and/or p21, cell cycle regulation becomes disturbed. This event sequentially leads to an unregulated cell proliferation, one of the critical steps toward carcinogenesis. Actually, tumor cells usually have accumulated alterations of the genes especially those involved in the cell cycles (21). In human HCCs, abnormalities of p21 and p53 have been reported (22, 23).

Abbreviations: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; GST-P, glutathione S-transferase placenta; HCC, hepatocellular carcinoma; IFN, interferon; LCM, Laser capture micro-dissection; PCNA, proliferating cell nuclear antigen.
To investigate the antitumorigenic effect of IFN-alpha in vivo, we examined the direct effect of IFN-alpha on hepatocarcinogenesis using the Solt and Farber model, focusing on genes affecting the cell cycle, such as a tumor suppressor gene, p53, a cell cycle regulating gene, p21 and cell cycle-related factors, G1 cyclins.

Materials and methods

Animal model and study design
Male Fischer 344 rats weighing ~180 g were obtained from Charles River Japan. IFN-alpha was kindly provided by the Sumitomo Pharmaceutical Co. Osaka, Japan). A modification of the protocol for chemical hepatocarcinogenesis originally described by Solt and Farber (7) was used. According to this protocol, rats were given 200 mg/kg of DEN (purchased from Tokyo-kasei) intraperitoneally. Two weeks after the DEN shots, the rats were fed via gastric tubes with 10 mg/kg of 2-AAF (purchased from Wako Pure Chemical Co., Osaka, Japan) every day for 2 weeks. A partial hepatectomy was performed a week after starting 2-AAF treatment.

To examine the development of pre-neoplastic foci (experiment 1) and neoplastic HCC (experiment 2), rats were killed 4 and 40 weeks after DEN injection, respectively. The effects of IFN-alpha on pre-neoplastic foci and HCC were investigated separately. In experiment 1, in the IFN-treated group, 10 MU/body of IFN-alpha was administered intraperitoneally every day for 4 weeks from the day of DEN initiation. In experiment 2, during the 4 weeks after DEN treatment, IFN-alpha administration was performed as in experiment 1. To examine whether IFN-alpha suppresses the carcinogenic initiation or the clonal expansion of initiated hepatocytes, the IFN-treated group was divided into two subgroups in experiment 2. After 4 weeks of DEN treatment, the rats were divided into two groups in which IFN-alpha treatment was continued twice a week (IFN-1 group) or was stopped (IFN-2 group) (Figure 1).

In both experiments, five to eight were used in each group. After the rat was killed, the liver was sliced at 5 μm thickness using a razor blade and was fixed in 10% buffered formalin. One slice evenly sampled from each liver lobe was also frozen and embedded with OCT compound. These frozen tissue blocks were cut with cryostat into 10 μm sections for mRNA extraction.

Immunohistochemical staining was done with a Large Volume DAKO LSAB Kit with peroxidase (DAKO, Copenhagen, Denmark), based on the manufacturer’s instructions. Formalin-fixed sections were deparaffinized in 390 M.Nakaji et al.

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Immunohistochemical staining was done with a Large Volume DAKO LSAB Kit with peroxidase (DAKO, Copenhagen, Denmark), based on the manufacturer’s instructions. Formalin-fixed sections were deparaffinized with xylene and treated with 3% hydrogen peroxide to eliminate endogenous enzyme reactions. After the treatment with blocking solution (bovine serum albumin), the solutions were replaced with either the primary antibody or normal serum. The primary antibodies are rabbit polyclonal anti-glutathione S-transferase placenta (GST-P) antibody (MBL Co., Nagoya, Japan), mouse monoclonal anti-PCNA antibody (PC-10; DAKO, Glostrup, Denmark), rabbit polyclonal anti-p21 antibody (M-19; Santa Cruz, CA), rabbit polyclonal anti-p53 antibody (FL393; Santa Cruz) and rabbit polyclonal anti-IFN-alpha/beta receptor antibody (C-18; Santa Cruz). After the incubation, the solutions were replaced with the biotinylated secondary antibody. Then, after incubation again, a streptavidin-horseradish peroxidase conjugate was added and the signals were visualized with 0.03% diaminobenzidine. The counterstaining was done with Mayer’s hematoxylin.

The numbers of GST-P-positive pre-neoplastic foci were counted on images captured with a CCD camera on a Macintosh computer. All GST-P-positive foci over 0.2 mm in diameter were counted using an image analyzer (Mac SCOPE, Mitani, Japan). The numbers of pre-neoplastic foci per unit volume and average volume of foci were estimated on the stereological study according to the method of Campbell et al. (24). The PCNA and p21 labeling indices were determined as the percentage of positively stained nuclei by counting 1000-2000 cells in the pre-neoplastic foci.

RT–PCR for p53, p21, cyclin D1 and cyclin E in pre-neoplastic foci

The mRNA levels of genes affecting cell cycle—p53, p21, cyclin D1 and cyclin E—were measured in surrounding normal hepatocytes as well as pre-neoplastic hepatocytes in each rat. The Laser capture micro-dissection (LCM) technique made it possible to collect surrounding hepatocytes and pre-neoplastic hepatocytes separately. Navigated LCM was performed with a PixCell II apparatus (Arcturus Engineering, Mountain View, CA) on frozen sections stained with hematoxylin and eosin. LCM parameters were as follows: 80 mW of the laser power, 1.5 ms of the laser pulse duration and 7.5 μm of the laser spot diameter. We captured approximately the same number of pre-neoplastic and normal hepatocytes in each animal. After LCM, total RNA was extracted from the collected cells, using an RNeasy kit (Qiagen, Valencia, CA). Total RNA was eluted into 30 μl of nuclease-free water, and was transcribed in reverse into cDNA, using an Oligo-dT primer (Promega, Madison, WI). Forty cycles of PCR amplification were performed using gene-specific primers. PCR amplifications were performed in a total volume of 50 μl, containing 8 μl of the cDNA solution, 1 U of Ex-Taq polymerase (Takara Shuzo Co., Shiga, Japan), each primer at 0.5 μM, and each deoxynucleotide triphosphate at 0.25 mM in diluted 1x PCR buffer. The primer sets, optimum annealing temperatures and size of amplification products are shown in Table I. PCR products were separated by electrophoresis on an ethidium bromide-stained 2% agarose gel. We quantified the result of RT–PCR amplified fragments using densitometer. The mean band densities were determined using NIH Image 1. Schematic diagram of the experimental design using the modified Solt and Farber protocol and IFN-alpha treatment schedule. [ ], 2-AAF; PH, partial hepatectomy; W, week; shading, IFN-alpha (10 MU/body/day).
Image software and the ratio of the mean densities of the bands for each gene and beta actin were calculated.

In experiment 2, the nodules with diameters $>1$ mm were counted and histologically examined. Each nodule was analyzed with hematoxylin and eosin staining and classified according to the published criteria (25). Tumor volumes were estimated using the following equation (26): 

$$\text{volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times \frac{1}{2}.$$

**Statistical analysis**

Values are represented as mean ± SD. The data were analyzed by the unpaired t test, and the $P$ value $< 0.05$ among samples was considered to be significantly different.

**Results**

**Liver weight and laboratory tests**

The average ratio of the liver weight against the total body was $3.09 \pm 0.46$ (control group) and $2.75 \pm 0.15\%$ (IFN group) in experiment 1, and $3.53 \pm 0.20$ (control group), $3.07 \pm 0.13$ (IFN-1 group) and $3.54 \pm 0.89\%$ (IFN-2 group), in experiment 2. In both experiments, the relative liver weight was not affected by IFN-alpha treatments. Aspartate and alanine transaminase, alkaline phosphatase, LDH and GGT activities did not show significant differences between the control group and IFN-treated group in experiment 1, nor among the control group, IFN-1 group and IFN-2 group in experiment 2 (data not shown).

**Immunohistochemical analysis for pre-neoplastic foci**

At the fourth week, pre-neoplastic lesions could be detected as clonal expansions of altered hepatocytes (Figure 2A). It was clearly distinguished from surrounding normal hepatocytes by immunoreactivity for GST-P, a marker for neoplastic and pre-neoplastic cells (27) (Figure 2B). The expression level of IFN-alpha/beta receptor was higher in the pre-neoplastic foci than

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Nucleotide sequence</th>
<th>Orientation</th>
<th>Length (bp)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>5'-TCCTCCCCACATCTTATCC-3'</td>
<td>Sense</td>
<td>261</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>5'-GACAAACAGAACCTCAA-3'</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>5'-CTAAGGAGAAGATGGGGAA-3'</td>
<td>Sense</td>
<td>232</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>5'-TGAGGCTTTGTGCTTCTT-3'</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5'-AAGTGCGTGTGGCGGTAGC-3'</td>
<td>Sense</td>
<td>422</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5'-TGAGGCCCCTGAAAGAG-3'</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin E</td>
<td>5'-CTGTCAGCTGAGTAGGAGAAG-3'</td>
<td>Sense</td>
<td>150</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>5'-AGGGTGCTACTGTGACCCTGGA-3'</td>
<td>Antisense</td>
<td></td>
<td></td>
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<tr>
<td>Beta actin</td>
<td>5'-TCATCACCATGGCAATGAG-3'</td>
<td>Sense</td>
<td>154</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5'-CAGTGTGTGGCGTACAGGT-3'</td>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Representative sections of preneoplastic foci at 4 weeks after DEN treatment. Clonal expansion of altered hepatocytes stained with H&E (A), anti-GST-P (B) and anti-IFN-alpha/beta receptor (C) are shown (original magnification ×100).
in normal surrounding hepatocytes (Figure 2C). Quantitative data for GST-P-positive foci are summarized in Figure 3. The estimated numbers of foci per cm\(^3\) in the liver were significantly reduced in the IFN-treated group compared with the control group (Figure 3A). The average size of individual pre-neoplastic foci also had a tendency to be diminished in the IFN-treated group than in control group, whereas statistically the difference was not significant (Figure 3B).

Because IFN-alpha has been known to have an anti-proliferative effect, we suggested that the proliferating...
cells were reduced by IFN-alpha treatment. As shown in Figure 4A and B, PCNA-positive cells were detected more prominently in pre-neoplastic foci than in surrounding normal hepatocytes. The PCNA-positive rate (PCNA index) in pre-neoplastic foci of the IFN-treated group (28.74 ± 4.486%) was significantly lower than that of the control group (35.86 ± 3.726%) (Figure 5A).

Previous studies have indicated that pre-neoplastic rat hepatocytes in vivo fail to induce nuclear p53 protein and fail to block replication in response to genotoxic compounds (28,29). Consistent with these reports, p53 expression was found in the nuclei of surrounding hepatocytes, although it was not detected in pre-neoplastic foci of either the control group or IFN-treated group (Figure 4C and D). Then, we examined the expression of p21, which is a critical downstream effector of p53. Similar to p53 expression, p21 was detected predominantly in the surrounding normal hepatocytes, although some pre-neoplastic hepatocytes also had p21 expression in their nuclei (Figure 4E and F). The p21-positive rate (p21 index) in pre-neoplastic foci was significantly higher in the IFN group (31.80 ± 6.327%) than in the control group (23.42 ± 8.362%) (Figure 5B).

**mRNA expression for p53, p21, cyclin D and E in pre-neoplastic foci**

Next, we also investigated the mRNA levels of several oncogenic and cell cycle-related genes, whether IFN-alpha suppressed these genes. Representative visualizations of the RT-PCR amplified fragments corresponding to p53, p21, cyclin D1, cyclin E and beta actin are shown in Figure 6A. The results of densitometric analyses for each gene are shown in Table II. p53 mRNA expression in pre-neoplastic foci was significantly lower than that in surrounding hepatocytes, although no significant difference was found between the control group and IFN-treated group. p21 expression in pre-neoplastic foci was also lower than that in surrounding hepatocytes. However, when compared among the different groups’ pre-neoplastic foci, p21 expression was significantly higher in the IFN-treated group than in the control one. Both cyclin D1 and cyclin E were expressed at higher levels in the pre-neoplastic foci than in the surrounding hepatocytes, and at lower levels in IFN-treated group than in control group.

**Macroscopic and microscopic findings for HCC**

In experiment 2, all of the rats in each group had 1 to 10 tumors in their livers. The tumor cell had either clear, eosinophilic or hyper basophilic cytoplasm, and all had enlarged and hyper chromatic nuclei (Figure 7A). These appearances show the HCC with various grades of differentiation. All hepatocytes including HCC expressed IFN alpha/beta receptor and the extent of the expressions was less in the HCCs than in the surrounding hepatocytes (Figure 7B). The numbers and the average volume of the tumors, both of which were assessed by GST-P expression (Figure 7C) were significantly lower in IFN-1 group than in the control group (Figure 8A and B). The tumor numbers in IFN-2 group were also somewhat reduced, compared with that in the control group, although the significant difference was not reached. Therefore, it is concluded that the tumor-suppressive effect was more evident in the IFN-1 group than in IFN-2 group. Histologically, all of the tumors in the IFN-1 group were well differentiated whereas 37.5% of the tumors in the IFN-2 group and 48.1% in the control group were moderately to poorly differentiated HCC, respectively (Table II).

### Table II. Evaluation of HCC

<table>
<thead>
<tr>
<th>Liver tumors</th>
<th>Number/animal</th>
<th>Estimated volume (mm³/liver)</th>
<th>Differentiation of HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Well (%)</td>
</tr>
<tr>
<td>Control</td>
<td>6.8 ± 2.2</td>
<td>584.1 ± 491.6</td>
<td>14 (51.9)</td>
</tr>
<tr>
<td>IFN-1</td>
<td>3.0 ± 1.6</td>
<td>56.3 ± 39.9</td>
<td>12 (100)</td>
</tr>
<tr>
<td>IFN-2</td>
<td>4.0 ± 1.6</td>
<td>249.5 ± 181.2</td>
<td>10 (62.5)</td>
</tr>
</tbody>
</table>

Fig. 5. Analysis of PCNA-positive rate and p21-positive rate in preneoplastic foci. The PCNA and p21 labeling indices were determined as the percentage of positively stained nuclei by counting 1000–2000 cells in the preneoplastic foci. The PCNA index in the IFN-alpha treated group was significantly lower than that in the control group (P < 0.01) (A), whereas the p21-positive rate in the IFN-treated group was significantly higher than that in the control group (P < 0.05) (B).
Fig. 6. Visualization of the RT–PCR amplified fragments corresponding to p53, p21, cyclin D1, cyclin E and beta actin from representatives of each group of rats (N, normal hepatocyte; F, preneoplastic foci) (A). Densitometric analyses of the ratio of expression of various mRNAs to beta actin mRNA expression; data are presented as mean ± SD (n = 4–7) (B).
Discussion

In the rat carcinogenic model using DEN, it is known that p53 is not accumulated in the induced GST-P-positive pre-neoplastic cells. It was shown previously that those cells do not harbor the mutations and that instead they have adaptive epigenetic changes (28–30). Furthermore, it was reported that expression of p21 was lower in pre-neoplastic cells than in normal hepatocytes. These phenomena rather suggested the essential role of p21 in regulating the proliferation of pre-neoplastic hepatocytes.

In human carcinogenesis, the roles of hepatic viruses are central and pivotal. In chronic HBV infection, HBx antigen has been shown to bind to and functionally inactivate the
tumor suppressor gene p53 (31). Furthermore, HBx antigen transcriptionally down-regulates the expression of the translation initiation factor sui 1, as well as the senescence factor, such as p21, either of which could inhibit hepatocyte proliferation (32). HCV also plays an important role in suppressing p53 function. HCV core protein transcriptionally represses the p53 promoter activity (33). It also represses the p21 promoter activity through pathways involving either the p53 gene or factors other than p53 (34). These reports suggested that the inactivations of p53 and/or p21 are important points in human hepatocarcinogenesis.

It has already been reported that IFN-alpha inhibits the growth of pre-neoplastic foci by inducing apoptosis. However, this was only based on a short-term experiment, which only analyzed the early phase of hepatocarcinogenesis (35). Therefore, we think that the experiment covering the whole period of carcinogenesis is necessary. Based on this thought, we conducted a study of the anti-proliferative effect of IFN-alpha through a longer period of HCC induction. We could demonstrate that IFN-alpha treatment suppressed not only the formation of pre-neoplastic foci but also the development to HCC. Also in our shorter-term experiment (experiment 1), we could show a reduction in the number and volume of pre-neoplastic foci. Next we demonstrated that the rate of PCNA-positive cells in pre-neoplastic foci was significantly reduced in the IFN-alpha-treated group rather than the control group. It was also shown that the induction of p21 was related to suppression of the pre-neoplastic cell proliferation. These results were consistent with several studies having shown that the antitumorogenic action of IFN-alpha in vitro is exerted through suppression of cell cycle as well as cytotoxic effects (36–38). In the lymphoid cell line, studies have reported that p21 is a central effecter for transducing the signal(s) from IFN towards the pathway(s) for suppressing cellular proliferation (39,40). However, there is little detailed information about the mechanism of the in vivo antitumorogenic effect of IFN-alpha. Our results here showed that IFN-alpha treatment suppresses the proliferation of pre-neoplastic cells, accompanied with the up-regulation of p21 expression, but without alteration of p53 expression.

It has been discovered already that the expression of p21 is regulated not only p53-dependently but also p53-independently, including pathways involving transforming growth factor beta, oxidative stress, c-jun, interferon regulatory factor 1 (IRF-1) and irradiation (41–43). Therefore, we think that IFN-alpha could induce p21 via p53-independent pathway, perhaps through IRF-1. In human HCC cell line, there was a report suggesting that the IFN-alpha could induce IRF-1 expression (44). It was also shown that the p21 promoter has several binding sites for IRF-1. Therefore, we suspected the consequential pathway for suppressing G1-cyclins started from IFN-alpha to IRF-1 to p21 to further downstream.

We showed that long-term treatment with IFN-alpha was more effective in reducing both tumor number and the volume. Among tumors induced, well-differentiated cancer was predominantly seen in the IFN-alpha-treated rats. In contrast, the poorly differentiated phenotype was detected only in non-IFN-alpha-treated rats. In general, it is known that human HCC is generated from pre-neoplastic lesions (adenomatous hyperplasia) that progresses to well to moderate to poorly differentiated cancer by recurrent de-differentiation of the cancer cells (45). Therefore, it could be thought that IFN-alpha inhibits the de-differentiation process during cancer development.

Since, we did not examine the effect of IFN-alpha on already existing neoplastic cells in this study, we do not know if IFN-alpha affects apoptosis induction in tumor cells. Regarding the quantitative expression of IFN-alpha/beta receptor, the pre-neoplastic cells had higher levels of the receptor than the surrounding normal hepatocytes. In contrast, neoplastic cells had lower levels than the surrounding cells. These phenomena suggest that IFN-alpha has greater influence in the early stage of the tumor development than in the later stages.

Thus, the administration of IFN-alpha before the cancerous stage may be useful in preventing HCC development. IFN-alpha treatment for patients with chronic hepatitis may be beneficial in directly preventing HCC.

References

9. Sillins,I., Finnberg,N., Ståhl,A., Hoégberg,J. and Stenius,U. (2001) p53 and Bcl-2 expression (44). It was also shown that the p21 promoter has several binding sites for IRF-1. Therefore, we suspected the consequential pathway for suppressing G1-cyclins started from IFN-alpha to IRF-1 to p21 to further downstream.

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