

## Anti-tumor effect of pegylated interferon in the rat hepatocarcinogenesis model

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**Abstract.** Interferon (IFN) is a multifunctional cytokine which works as a suppressor of hepatocarcinogenesis. Pegylated interferon (PEG-IFN) is a modified form of IFN with different pharmacokinetics. We evaluated the anti-tumor effect of PEG-IFN using a rat hepatocarcinogenesis model. Male Fisher Rats were treated using the Solt and Faber model to induce liver cancer. IFN and PEG-IFN were administered from chemical initiation, and pre-neoplastic foci and neoplastic hepatocellular carcinoma (HCC) were examined at 4 and 40 weeks after chemical initiation, respectively. Apoptosis-related molecules such as p53 and Fas-L, proliferating cell nuclear antigen (PCNA), and oxidative stress-related molecules such as 8-hydroxy-deoxyguanosine (8-OHdG) and thioredoxin (TRX) were assessed by immunohistochemical analysis and reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of Notch-1, a molecule related to the regenerative and oncogenic processes was also examined. The generation of foci and HCC were significantly suppressed in IFN and PEG-IFN groups compared with the control group. Whereas PCNA and Notch-1 were strongly expressed in the foci and HCC, Fas-L was mainly detected in the surrounding hepatocytes. 8-OHdG and TRX were also detected in the foci. Although PCNA and Notch-1 were down-regulated in IFN- and PEG-IFN-treated groups, Fas-L was up-regulated in those groups. The activation of Notch-1 signaling and the accumulation of oxidative stress in the pre-neoplastic

foci might be associated with the progression of HCC in the DEN-induced hepatocarcinogenesis model. The inhibitory effect of the PEG-IFN and IFN on hepatocarcinogenesis was almost the same, and this might be induced by the Fas-related apoptosis in the surrounding tissues.

### Introduction

The liver is an organ with a marked capacity to regenerate, and liver cancer is usually caused by its repeated tissue destruction and regeneration. Hepatocellular carcinoma (HCC) is usually generated through dysplastic nodules which are premalignant lesions in chronic liver diseases (1). The Solt and Farber model is an animal model of hepatocarcinogenesis that is not influenced by the hepatitis virus, inflammation, fibrosis, or other environmental factors (2). In this model, initiation by diethylnitrosamine (DEN) induces pre-neoplastic hepatocytes in rats, whereas treatment with 2-acetylaminofluorene (2-AAF) and partial hepatectomy promotes the clonal expansion of these pre-neoplastic cells. The carcinogenic step in this model is similar to that in human hepatocarcinogenesis. Therefore, the effect of chemotherapeutic reagents on pre-malignant lesions shows their potential in the treatment of human HCC. As yet, chemotherapy for HCC has not been clinically established, but some reports have shown that the combination of chemotherapeutic reagents and interferon  $\alpha$  had an inhibitory effect on the growth of liver cancers (3).

Interferon (IFN)- $\alpha$  is a multifunctional cytokine with immunomodulatory, anti-viral, and anti-proliferative properties. IFN- $\alpha$  is widely used in the treatment of human hematologic and solid malignancies (4,5). For the liver, it was reported that IFN- $\alpha$  had an inhibitory effect on hepatocarcinogenesis (6). Pegylated interferon (PEG-IFN) is a modified form of recombinant human IFN- $\alpha$  with sustained absorption and prolonged half-life after administration. The protein kinase profile which can be achieved by PEG-IFN has been improved from conventional IFN (7). PEG-IFN is a core drug for the treatment of chronic viral hepatitis, because of improved compliance and toxicity (8). PEG-IFN also has a strong anti-tumor effect and is now used for the prevention of several cancer types (9-11).

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Previously, we reported the inhibitory effect of IFN- $\alpha$  on hepatocarcinogenesis using this model (12). It was also reported that IFN- $\alpha$  induced apoptosis in the pre-neoplastic liver of rats (13). To evaluate the anti-tumor effect of PEG-IFN on hepatocarcinogenesis, we examined the generation of pre-neoplastic foci and neoplastic hepatocellular carcinoma and compared PEG-IFN with conventional IFN.

## Material and methods

**Animal model.** 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) and PEG-IFN was provided by the Chugai Pharmaceutical Co. (Tokyo, Japan). A modification of the protocol for chemical hepatocarcinogenesis originally described by Solt *et al* (2) was used. According to this protocol, rats were given 200 mg/kg of DEN (purchased from Tokyo-kasei, Japan) intraperitoneally. Two weeks after the DEN shots, the rats were fed via gastric tubes with 10 mg/kg of 2-AAF (purchased from the Wako Pure Chemical Co., Osaka, Japan) every day for 2 weeks. A partial hepatectomy was performed a week after starting 2-AAF treatment. Each dose of IFN and PEG-IFN was 0.5 MU/kg and 10  $\mu$ g/kg, and in total IFN and PEG-IFN were administered at 17 MU/body/40 weeks and 104  $\mu$ g/body/40 weeks, respectively. To examine the development of pre-neoplastic foci and neoplastic HCC, rats were sacrificed 4 and 40 weeks after DEN injection, respectively. The effects of IFN- $\alpha$  and PEG-IFN on pre-neoplastic foci and HCC were investigated separately (Fig. 1). At 40 weeks, nodules with diameters of 21 mm were counted and histologically examined. Each nodule was analyzed via hematoxylin and eosin staining and classified according to the published criteria (14).

**Immunohistochemical analysis.** In both experiments, five to eight rats were used in each group. After each rat was sacrificed, the liver was sliced at 5 mm thickness using a razor blade and fixed in 10% buffered formalin. Immunohistochemical staining was conducted with a Dako Envision Plus 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 treatment with blocking solution (bovine serum albumin), the solutions were replaced with either the primary antibody or normal serum.

For the primary antibodies: anti-glutathione S-transferase placenta (GST-P) antibody was purchased from MBL Co. (Nagoya, Japan), anti-proliferating cell nuclear antigen (PCNA) antibody was from Dako (Glostrup, Denmark), anti-thioredoxin (TRX) antibody was from Redox Bioscience Co. (Kyoto, Japan), anti-8-hydroxydeoxyguanosine (8-OHdG) antibody was from the Japan Institute for The Control of Aging, Nikken SEIL Co. (Shizuoka, Japan), anti-Notch-1 antibody, anti-Fas-L antibody and anti-p53 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation, the solutions were replaced with horseradish peroxidase-conjugated secondary antibody. Then, after

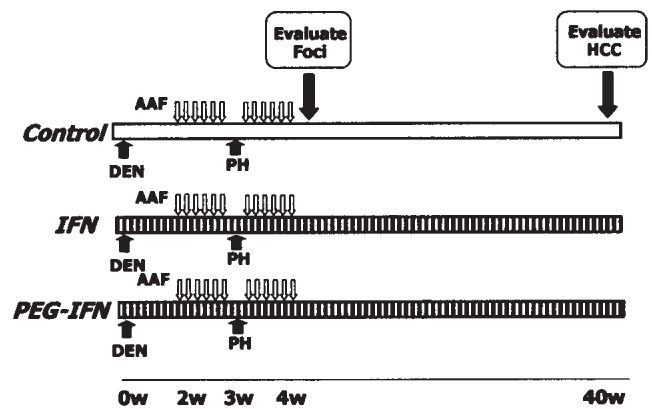


Figure 1. Schematic diagram of the experimental design.

further incubation, the signals were visualized with 0.03% diaminobenzidine. Counterstaining was done with Mayer's hematoxylin.

**Reverse transcription-polymerase chain reaction analysis.** The mRNA levels of genes were analyzed by reverse transcription-polymerase chain reaction analysis (RT-PCR). Total tissue RNA was extracted from the frozen tissues with Isogen (Nippongene, Toyama, Japan) according to the manufacturer's instructions, and reverse transcribed using an Oligo-dT primer (Promega, Madison, WI, USA) for the first-strand cDNA synthesis. PCR amplification was performed using gene-specific primers. The selected forward (F) and reverse (R) primers were as follows: Fas-L F: 5'-ATA GCC AAC CCC AGC ACA CC-3', Fas-L R: 5'-AAG TAC AAC CCA GCC TCA TT-3', TRX F: 5'-CCG CAA CAG CCA AAA TGG TGA-3', TRX R: 5'-AGC ATG ATT AGG CAA ACT CCG TAA-3', p53 F: 5'-TCC TCC CCA ACA TCT TAT CC-3', p53 R: 5'-GCA CAA ACA CGA ACC TCA AA-3', Notch-1 F: 5'-CAC CCA TGA CCA CTA CCC AGT T-3', Notch-1 R: 5'-CCT CGG ACC AAT CAG AGA TGT T-3', Hes-1 F: 5'-CGA CAC CGG ACA AAC CAA A-3', Hes-1 R: 5'-GAA TGT CTG CCT TCT CCA GCT T-3', GAPDH F: 5'-AAG GTC ATC CCA GAG CTG AA-3', GAPDH R: 5'-ATG TAG GCC ATG AGG TCC AC-3'. PCR amplifications were performed in a total volume of 50  $\mu$ l, containing 8  $\mu$ l of the cDNA solution, 1 U of Ex-Taq polymerase (Takara Shuzo Co., Shiga, Japan), each primer at 0.5 mM and each deoxynucleotide triphosphate at 0.25 mM in diluted 1X PCR buffer. PCR products were separated by electrophoresis on an ethidium bromide-stained 2% agarose gel.

**Statistical analysis.** Results are represented as the mean  $\pm$  SD. Data were analyzed by the Mann-Whitney U test and a P-value  $<0.05$  was considered significant.

## Results

**Evaluation of pre-neoplastic foci.** The area and size of pre-neoplastic foci were examined in the 4 weeks after initiation. Pre-neoplastic foci were clearly stained by GST-P, a marker for neoplastic and pre-neoplastic cells (Fig. 2) (15). Quantitative data for GST-P-positive foci are summarized in

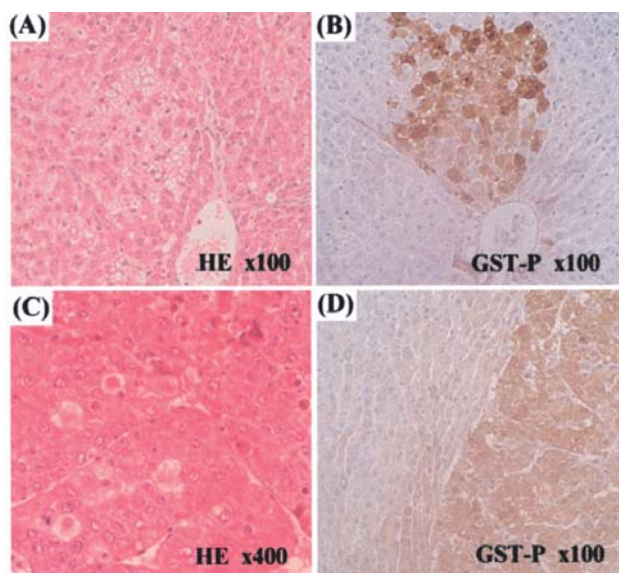


Figure 2. Histological findings of preneoplastic foci and neoplastic HCC at 4 and 40 weeks after DEN treatment. HE (A) and GST-P (B) staining of the foci, and HE (C) and GST-P (D) staining in HCC are shown. Original magnification: A, B and D, x100; and C, x400.

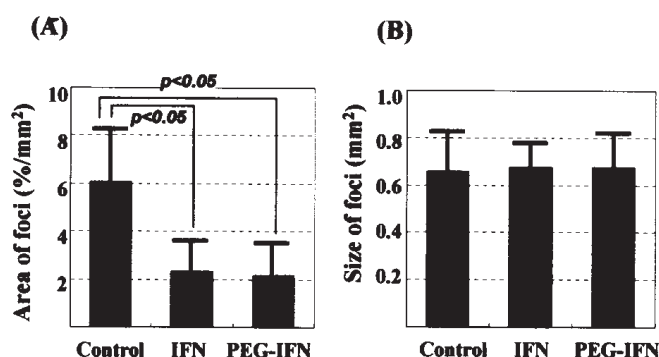


Figure 3. Effect of IFN and PEG-IFN on the development of pre-neoplastic foci. (A) The number of pre-neoplastic foci. (B) The average size of pre-neoplastic foci. The number of pre-neoplastic foci was significantly decreased in the IFN- and PEG-IFN-treated groups compared with the control group, whereas the average size of foci was almost the same among the three groups.

Fig. 3. Although no difference was shown in the average size of foci among the three groups, the area of foci in the liver was significantly reduced in the IFN- and PEG-IFN-treated groups compared with the control group.

**Liver weight and laboratory tests at 40 weeks.** At 40 weeks after initiation, the average ratio of the liver weight to the total body weight was  $3.35 \pm 0.29\%$  (control group),  $3.49 \pm 0.18\%$  (IFN-treated group) and  $3.32 \pm 0.22\%$  (PEG-IFN-treated group). In this experiment, the relative liver weight was not affected by IFN and PEG-IFN treatments (Fig. 4A). The serum alanine transaminase (ALT) level in the IFN-treated group ( $48.2 \pm 23.0$  IU/l) and PEG-IFN-treated group ( $50.0 \pm 10.6$  IU/l) was significantly lower than that of the control group ( $119.5 \pm 89.8$  IU/l) (Fig. 4B).

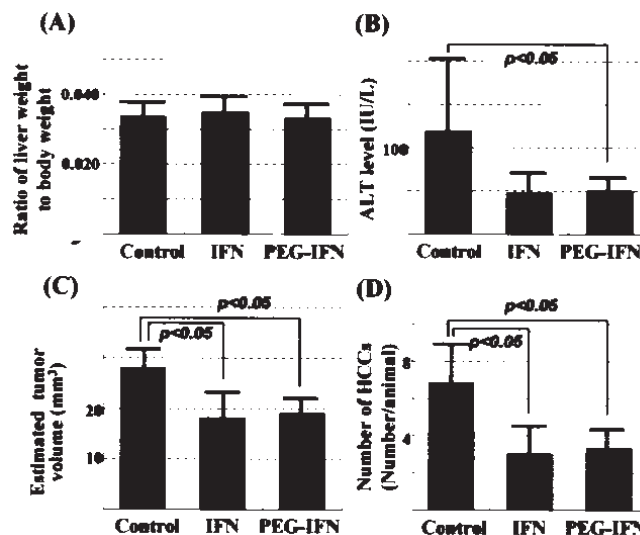


Figure 4. Effect of IFN and PEG-IFN on HCC development. (A) The ratio of the liver weight to the total body weight. (B) Serum alanine-amino-transferase (ALT) level. (C) The average size of HCCs. (D) The number of HCCs. The number and average size of HCCs was significantly decreased in the IFN- and PEG-IFN-treated groups compared with the control group.

**Generation of hepatocellular carcinoma.** The number and estimated volume of HCCs were examined. While the number was  $6.8 \pm 2.2$  (control group),  $3.0 \pm 1.6$  (IFN-treated group), and  $3.3 \pm 1.5$  (PEG-IFN-treated group), the estimated tumor volume of HCCs was  $28.0 \pm 4.0$  mm<sup>3</sup> (control group),  $18.0 \pm 5.0$  mm<sup>3</sup> (IFN-treated group), and  $19.0 \pm 2.3$  mm<sup>3</sup> (PEG-IFN-treated group), respectively. Both the number and estimated volume of HCCs were significantly suppressed in IFN- and PEG-IFN-treated groups compared with the control group (Fig. 4C and D).

**Immunohistochemical analysis and RT-PCR.** At 4 weeks after initiation, immunohistochemical analysis showed that the expression of PCNA, Notch-1, p53, 8-OHdG and TRX in the foci was higher than in surrounding tissues (Fig. 5A). On the other hand, the expression of Fas-L was lower in the foci. On RT-PCR analysis, no marked difference was shown in the expression of each molecule among the three groups (Fig. 6A).

In addition, the expression of PCNA, Notch-1, Fas-L and p53 was also examined immunohistochemically and by RT-PCR at 40 weeks after initiation. PCNA and Notch-1 expression was significantly higher in the HCC tissue and especially, PCNA was strongly stained in the nucleus of HCC tissue. On the other hand, Fas-L expression was lower in the HCC tissue compared with surrounding hepatocytes. P53 was focally expressed in the nucleus of HCC tissues (Fig. 5B). To examine the effect of IFN and PEG-IFN, RT-PCR using non-tumorous tissue was carried out. mRNA expression of Notch-1 and Hes-1, the ligand of Notch-1, was higher in the DEN-treated group (control group) compared with normal liver tissue and was lower in IFN- and PEG-IFN-treated groups compared with the control group. On the contrary, Fas-L expression was higher in the IFN- and PEG-IFN-treated groups compared with the control group (Fig. 6B).



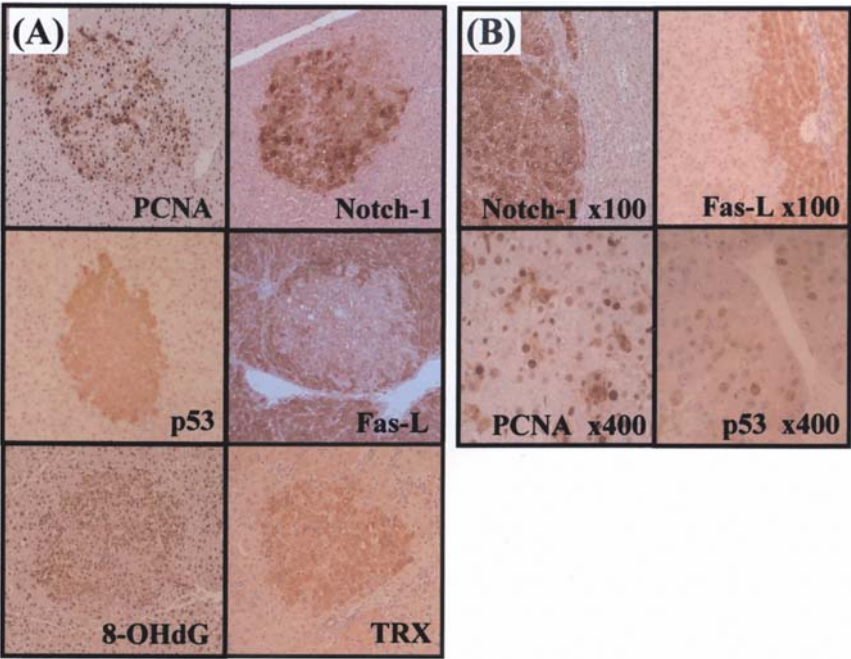


Figure 5. (A) Immunohistochemical analysis of pre-neoplastic foci. Expressions of PCNA (left upper panel), Notch-1 (right upper panel), p53 (left middle panel), Fas-L (right middle panel), 8-OHdG (left lower panel) and TRX (right lower panel) are shown. (B) Immunohistochemical analysis of HCC tissue. Expression of Notch-1 (left upper panel), Fas-L (right upper panel), PCNA (left lower panel) and p53 (right lower panel) are shown.

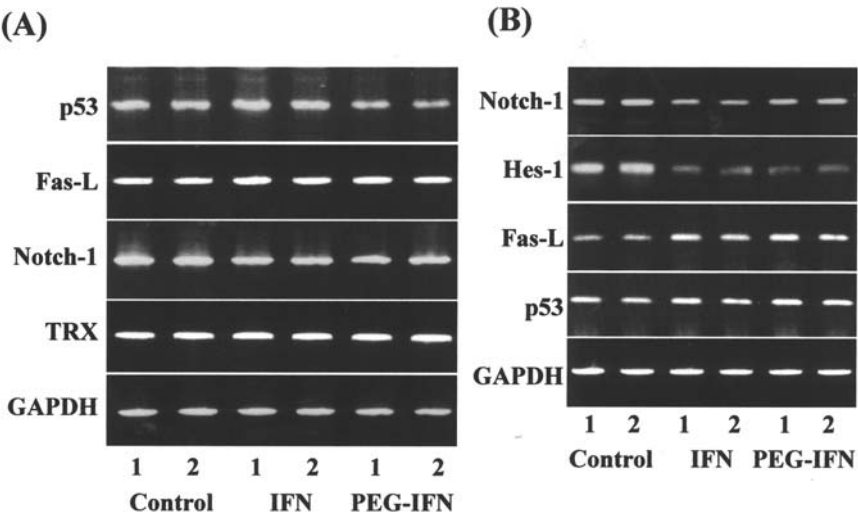


Figure 6. (A) RT-PCR of pre-neoplastic foci. mRNA expression of p53, Fas-L, Notch-1 and TRX are shown. No difference in mRNA expression was noted among the three groups. (B) RT-PCR 40 weeks after initiation. Notch-1 and Hes-1 expressions were down-regulated. On the other hand, the expression of Fas-L was up-regulated in the IFN- and PEG-IFN-treated groups.

Discussion

IFN- $\alpha$  is a multifunctional cytokine with immunomodulatory, anti-viral and anti-proliferative properties. Clinically, IFN- $\alpha$  is useful and effective for the treatment of chronic viral hepatitis (16). As for the treatment of chronic hepatitis C, IFN works as a viral eradicator to modulate immune system. In the oncogenic process, IFN- $\alpha$  possibly suppresses the generation of liver cancer in chronic viral hepatitis patients (6). Recently, many reports have shown that IFN- $\alpha$  is clinically effective for the suppression of various kinds of malignancies including renal cell cancer, malignant

melanoma and hepatocellular carcinoma (4,5,16-18). Previously, we showed that IFN- $\alpha$  prevented the development of HCC through the induction of p21 in DEN-induced rats (12). PEG-IFN has been produced and made available for the treatment of solid tumors and chronic viral hepatitis (19). Pegasys has an attached 40-kDa branched polyethylene glycol (PEG) molecule and facilitates a constant serum concentration and biologic activity. PEG-IFN appears to improve patient compliance and reduce toxicity (7). This is why PEG-IFN therapy is more universal for the treatment of chronic hepatitis C (20). Several reports showed that PEG-

IFN exhibited a strong anti-tumor effect, and that the combination of PEG-IFN and chemoreagents was effective for the treatment of several cancer types (10,21,22). It was also reported that PEG-IFN had an anti-tumor effect against HCC (11), and our results supported this. In our study, the anti-tumor effect of PEG-IFN was almost the same as that of conventional IFN.

Our study showed that the number of pre-neoplastic foci was significantly reduced in the IFN- and PEG-IFN-treated groups compared with the control group. It was suggested that IFN and PEG-IFN had an inhibitory effect against pre-neoplastic foci. IFN has an inhibitory effect on cell proliferation including cell cycle arrest and the induction of apoptosis (23). IFN-mediated apoptosis is usually initiated through the ligation of cell surface death receptors such as Fas/CD95 or TRAIL/Apo2L (24). Although p53 is an important oncogene related to apoptosis, it is still unclear how p53 is involved in IFN-mediated apoptosis (25). So, the expression of p53 and Fas-L was examined in this study. Interestingly, the expression of Fas-L in the IFN- and PEG-IFN-treated groups was higher than that in the control group at 40 weeks after initiation, although the expression of p53 showed no difference among the three groups. Immunohistochemically, Fas-L was expressed in the surrounding hepatocytes of foci at 4 weeks and non-cancerous tissues at 40 weeks after initiation. It was suggested that the accumulation of IFN would be related to activation of the p53-independent apoptotic pathway.

Oxidative stress contributes to the pathogenesis of hepatic injury induced by alcohol, viral infection, ischemia/perfusion injury and exposure to toxins (26-28). Oxidative stress and chronic inflammation have been linked to an increased risk of liver cancer. 8-OHdG, a modified base generated by reactive oxygen species, and TRX, a stress-inducible thiol-containing protein, are major oxidative stress markers. In general, oxidative stress is related to inflammation (29). DEN-induced hepatocellular carcinoma occurs in the absence of inflammation. In this study, TRX and 8-OHdG were highly expressed in pre-neoplastic foci. It was suggested that the induction of oxidative stress in the pre-neoplastic foci would be associated with hepatocarcinogenesis. In addition, it was thought that HCC was not necessarily related to inflammation, supporting the previous report (30).

Notch signaling is strongly implicated in organ regeneration and tumorigenesis (31,32). The oncogenic functions of Notch signaling involve the inhibition of apoptosis and promotion of cell proliferation. In hematological malignancies, Notch was able to inhibit the activity of pro-apoptotic molecules, such as p53 and Nur77, or increase the expression of anti-apoptotic molecules, such as FLIP and Bcl-2 (33). In breast malignancies, it was reported that Notch-1 was expressed only in tumor tissues (34). In liver tissue, Notch-1 is expressed in bile ductules, sinusoidal endothelial and small-vessel endothelial cells and hepatocyte plasma membranes (35). Using the rat regenerative model, strong expression of Notch-1 was detected in the regenerative nodule and oval cells (36). It was also reported that Notch-1 signaling results in significant growth inhibition of HCC cells both *in vitro* and *in vivo*, which is related to growth arrest and apoptosis induction (37). Based on our

results, Notch-1 was strongly expressed in the pre-neoplastic foci and HCC, and it was suggested that the Notch-1 signaling pathway is associated with the stage of liver regeneration and carcinogenesis. However, it is possible that Notch signaling works as a growth suppressor after liver cancer is generated and matured.

In this study, the expression of PCNA and Notch-1 was prominent in pre-neoplastic foci. It is suggested that pre-neoplastic foci formed a clone in which Notch signaling is activated, and cellular proliferation progressed. This supports the idea of foci as a pre-cancerous clone.

In conclusion, the activation of Notch-1 signaling and accumulation of oxidative stress in the pre-neoplastic foci might be associated with the progression of HCC in the DEN-induced hepatocarcinogenesis model. The inhibitory effect of IFN and PEG-IFN on the generation of HCC was almost the same in this model and was suggested that IFN might induce Fas-related apoptosis in the surrounding tissues.

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