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Inhibitory Function of Interferon on Hepatocarcinogenesis

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Key Words
Apoptosis · 5-Fluorouracil · Hepatocellular carcinoma · Interferon · Interferon receptor

Abstract
Objective: We examined the growth-inhibitory effect and the mechanism of action of type I interferon (IFN) in human liver cancer cell lines in vitro and in vivo. ***Methods:*** We examined the growth-inhibitory effect of 5 types of type I IFN preparations (e.g., pegylated, nonpegylated IFN-α, and IFN-β) used for the treatment of chronic hepatitis C in 13 liver cancer cell lines in vitro. After liver cancer cells were transplanted into nude mice, various doses of IFN preparations were subcutaneously administered, and the antitumor effect was examined. ***Results:*** The growth-inhibitory effect of each preparation was different, but IFN-β expressed the strongest effects in vitro. Induction of an inhibition of cell cycle progression at the G_1, S or G_2/M phase with or without apoptosis was the mechanism of action of IFN. IFN preparations induced a dose-dependent decrease in tumor volume and weight by inducing tumor cell apoptosis in vivo, and tumor growth was effectively suppressed even at the clinical dose for chronic hepatitis C treatment. The antitumor effect of pegylated IFN was significantly stronger than that of nonpegylated IFN. ***Conclusion:*** The data suggest potential clinical application of pegylated IFN for the prevention and treatment of hepatocellular carcinoma.

Introduction

Interferons (IFNs) are a family of cytokines that possess various biologic activities such as antiviral, antiproliferative, antiangiogenetic, immunomodulatory, and antitelomerase activities. IFNs are classified into two major groups, i.e., type I IFN, which includes IFN-α, IFN-β and IFN-ω, and type II IFN such as IFN-γ [1]. Human IFN-α comprises a family of structurally and functionally related genes of at least 14 subtypes [2]. Several types of IFN-α preparations are used in clinical practice, and each preparation consists of a different subtype. Both type I and type II IFNs bind to distinct cellular receptors, type I IFN receptor (IFNAR) and type II IFN receptor (IFNGR), respectively, and activate distinct and overlapping pathways [3]. Type I IFNs have been used in the treatment of virus-related chronic hepatitis and malignant diseases, such as melanoma, renal cell carcinoma and chronic myelogenous leukemia [4].

Hepatocellular carcinoma (HCC) is one of the most frequently found primary cancers, and many HCC patients have chronic hepatitis or cirrhosis caused by chronic infection of hepatitis C virus as their background disease [5]. Recently, type I IFNs have been shown to have highly suppressive effects on hepatocellular carcinogenesis [6–8] and on the recurrence of HCC after curative treatment in patients with virus-related chronic hepatitis [9, 10]. The precise mechanisms of these suppressive actions have not yet been clarified, but direct antiproliferative effects of type I IFN may be involved. In clinical practice, IFN-α alone or in combination with other antican-

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cer drugs such as 5-fluorouracil (5-FU) has been used in the treatment of malignant diseases including renal cancer [4] and advanced HCC [11, 12].

To clarify the growth-inhibitory effect of type I IFN on liver cancer cells, we conducted research on (1) type I IFN receptor expression [13], (2) in vitro and in vivo antitumor effects and the mechanism of action of 5 types of type I IFN preparations [14–19], and (3) in vitro growth-inhibitory effect and mechanism of action of combined IFN-α and 5-FU treatment in human liver cancer cells [20–22], i.e., 11 HCC cell lines and 2 combined hepatocellular and cholangiocarcinoma (CHC) cell lines. We would like to introduce some of our accumulated data in this review article.

Expression of IFNAR-2 Subunit of Type I IFN Receptor in Human HCC Cells

The effects of type I IFN are mediated by type I IFN receptor, which consists of two subunits, i.e., IFNAR-1 and IFNAR-2, and IFNAR-2 subunit occurs in a soluble, short or long form (IFNAR-2a, IFNAR-2b, or IFNAR-2c, respectively). IFNAR-2c is necessary for normal IFN binding and activation of the signal transduction pathway, while IFNAR-1 is a necessary subunit to form high affinity receptors [1, 23, 24]. We examined the mRNA expression of IFNAR-1 and IFNAR-2c in 11 human HCC cell lines and 2 CHC cell lines, which were originally established and characterized in our laboratory, by using reverse-transcription polymerase chain reaction and found that all 13 cell lines expressed both subunits. We also examined cell surface IFNAR-2 protein expression in the 13 cell lines, and the expression was identified in 12 cell lines at various levels [16]. Furthermore, we examined IFNAR-2 expression in HCC tissues and their corresponding non-HCC tissues. Immunohistochemically, IFNAR-2 expression was positive in 61 (88%) of 69 non-HCC tissues. There was no significant difference in the expression between chronic hepatitis and liver cirrhosis [13]. In 12 normal liver tissues, IFNAR-2 expression was not observed. This suggests a close relationship between chronic inflammation induced by viral infection and IFNAR-2 expression in the liver. IFNAR-2 expression was positive in 53 (77%) of 69 HCC tissues [13]. Kondo et al. [25] reported that IFNAR-2 expression was positive in 59 (61%) of 91 HCC tissues and that the expression level was related with the differentiation level of HCC. In contrast, there was no apparent relationship between IFNAR-2 expression and the histopathological characteristics of HCC, such as histological grade, fibrous capsule formation, capsular invasion, portal vein invasion, and intrahepatic metastasis in our study [13]. Ota et al. [26] reported that IFNAR-2 expression rates of esophagus, stomach, colorectal, bile duct, and pancreas cancer tissues are between 20 and 45%, and this suggests that the IFNAR-2 expression rate of HCC tissues is much higher as compared with carcinomas arising in other organs.

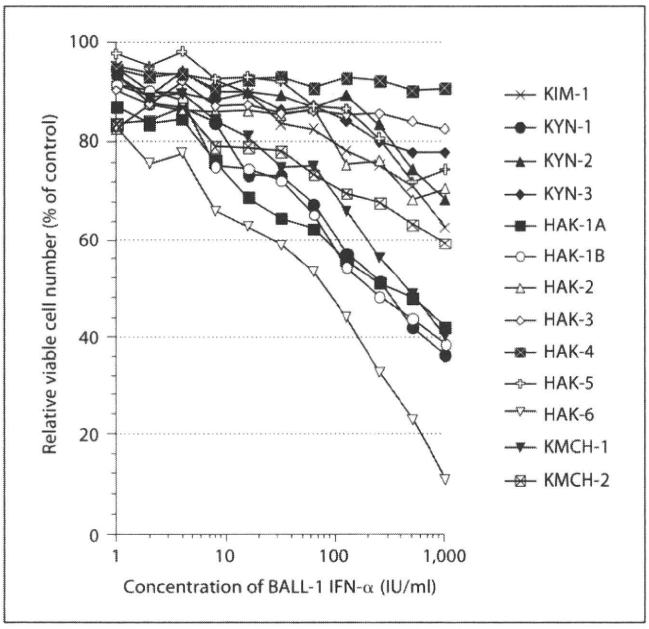

Fig. 1. Antiproliferative effects of natural human IFN-α (BALL-1 IFN-α, OIF®). Thirteen liver cancer cell lines were cultured with or without culture medium containing 1–1,024 IU/ml of BALL-1 IFN-α for 96 h, and the relative viable cell numbers were calculated and plotted on the graph.

Growth-Inhibitory Effects of Natural Human IFN-α on Liver Cancer Cell Lines in vitro

In 8 of the 13 cell lines, a time-dependent antiproliferative effect was observed at various degrees in the 96-hour cultures with 1,024 IU/ml of human lymphoblastoid IFN-α derived from Sendai virus-induced BALL-1 cells (BALL-1 IFN-α, OIF®). The relative viable cell number at 96 h after adding IFN-α (1–1,024 IU/ml) decreased in all cell lines in a dose-dependent manner (fig. 1). Sensitivity to the growth suppression effect of IFN-α was not related to the histological grade of the original tumors of each cell line. The suppressive effect was very low in cell

Table 1. Five types of type I IFN drugs used in the experiments

	Type	Source/component	Specific activity IU/mg protein
IFN-α			
OIF®	Natural	Derived from lymphoblastoid cells (BALL-1 IFN-α): IFN-α2 (75%) + IFN-α8 (25%)	20.0×10^7
Advaferon®	Recombinant	Synthesized from consensus sequence (consensus IFN)	100.0×10^7
PegIntron®	Recombinant	PEG-IFN-α2b	6.4×10^7
Intron®A	Recombinant	IFN-α2b	26.0×10^7
IFN-β			
FERON®	Natural	Derived from human fibroblasts	20.0×10^7

lines with little or no IFNAR-2 expression, but in the other cell lines, the expression level of IFNAR-2 on the cell surface was not clearly related to the growth suppression effect of IFN-α [16]. In clinical practice, Ota et al. [27] reported that in advanced HCC (Vp3 or 4) patients who received IFN-α and 5-FU combination therapy, there was a significant difference in the time-to-progression survival and the overall survival between IFNAR-2-positive and IFNAR-2-negative cases.

Growth-Inhibitory Effects of IFN-α Subtypes on Liver Cancer Cells in vitro

Human IFN-α comprises a large family of structurally related genes expressing at least 14 subtypes. The coding regions of the IFN-α genes are quite similar, and the least related are about 77% homologous. Natural IFN-α preparations, such as human lymphoblastoid IFN-α, consist of a mixture of a number of distinct IFN-α subtypes, and BALL-1 IFN-α used in the above-described experiment consists of the α2 subtype (approximately 75%) and the α8 (25%) subtype. Several studies suggest that IFN-α subtypes display significant differences in specific activities such as antiviral activity and antiproliferative activity, as well as in binding affinities to type I IFN receptor [28, 29]. The activity levels also varied greatly depending on the target cells. We examined the antiproliferative effects of five representative IFN-α subtypes (α1, α2, α5, α8 and α10) in vitro against 13 human liver cancer cell lines. We found that the antiproliferative effect of each IFN-α subtype varies according to the cell line, but that the cells are relatively or absolutely responsible for α5 and α8 subtypes [18]. On average, the antiproliferative effects were strong in descending order from α5, α8, α10, α2 and α1. The relative viable cell number started to decrease from the early culture period after the addition of IFN-α5. These results suggest that the administration of IFN-α preparations containing high proportions of IFN-α5 or IFN-α8 would be more efficient in terms of the prevention and treatment of HCC.

Growth-Inhibitory Effects and the Mechanism of Action of Type I IFN Preparations on Liver Cancer Cell Lines in vitro

We compared the growth-inhibitory effects in vitro on 13 human liver cancer cell lines among 5 types of type I IFN preparations, including BALL-1 IFN-α shown in table 1. The 5 types of type I IFN preparations can be classified into IFN-α and IFN-β, natural and recombinant, or pegylated and nonpegylated. Natural IFNs include BALL-1 IFN-α (OIF®) and fibroblast-derived IFN-β (FERON®), and the recombinant IFNs include IFN-α2, and consensus IFN (rIFN-αCon1, Advaferon®) synthesized through the scanning of several IFN-α nonallelic subtypes and assigning the most frequently observed amino acid in each position [30]. Pegylated IFN-α2b (PEG-IFN-α2b, PegIntron®) is a covalent conjugate of recombinant IFN-α2b (Intron®A) with a monomethoxy-polyethylene glycol (PEG) in a 1:1 molar ratio. Thirteen liver cancer cell lines were cultured with culture medium alone or medium containing 10–1,024 IU/ml of 1 of the 5 type I IFN preparations, and the average of relative viable cell numbers was calculated from the 13 cell lines for each type I preparation and plotted (fig. 2). The antiproliferative effect of each type I IFN preparation was different and was strongest in IFN-β, followed by consensus IFN, BALL-1 IFN-α, and IFN-α2b or PEG-IFN-α2b. A time-dependent growth inhibition was observed in most of the cell lines in the 96-hour culture with IFN-β, and at 96 h after adding IFN-β,

growth suppression occurred even at a low dose in 6 of the 13 cell lines [14]. It has been reported that the stronger growth-inhibitory effects of IFN-β may be mediated by the formation of a uniquely stable type I IFN receptor complex, greater affinity for the type I receptor complex, involvement of other receptor components, and the activation of additional signaling pathways [24, 31, 32]. In contrast, IFN-α2b with or without pegylation showed the lowest growth-inhibitory activity. This is consistent with the above-described finding, i.e., that the antiproliferative activity of the IFN-α2 subtype in vitro is relatively weak compared with other IFN-α subtypes [18].

Forty-eight to 72 h after adding each type I IFN preparation, at least 10 cell lines presented characteristics of various degrees of apoptosis, e.g., cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation (fig. 3). Sensitivity to IFN-mediated apoptosis appears to be dependent on the type and concentration of IFN, and the cell line [14–17]. We examined the mechanism of IFN-mediated apoptosis in liver cancer cells and found a release of cytochrome c and Smac/DIABLO from mitochondria to cytosole and an activation of various caspases, such as caspase-9, caspase-8, and caspase-3, in the IFN-α-mediated apoptosis-sensitive cells, suggesting the involvement of the mitochondrial apoptotic pathway in the IFN-α-mediated apoptosis [19]. Besides the mitochondrial apoptotic pathway, the involvement of the death receptor/ligand (e.g., TRAIL-R1, TRAIL-R2 and TRAIL) in the death receptor apoptotic pathway has been reported [33], and this point needs to be further studied. In addition to the induction of apoptosis, we identified the inhibition of cell cycle progression in all cell lines, i.e., blockage of the cell cycle at the S phase (11 cell lines), G_2/M phase (1 cell line), and G_1 phase (1 cell line) [16, 17].

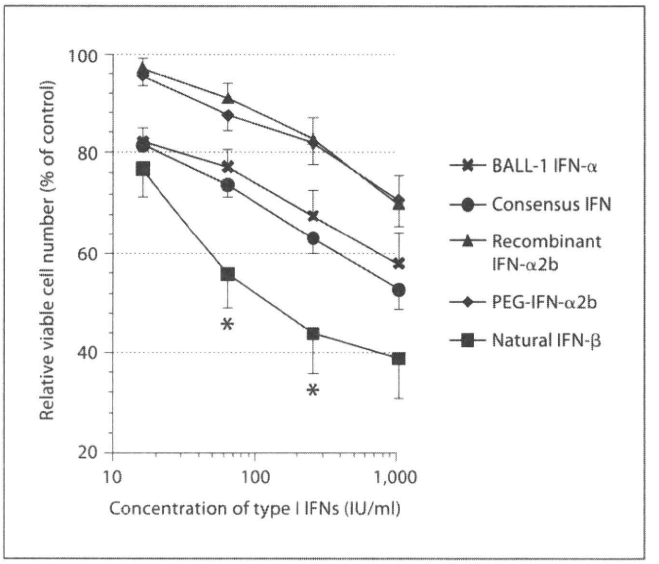

Fig. 2. Antiproliferative effects of type I IFN preparations, including BALL-1 IFN-α, consensus IFN, recombinant IFN-α2b, PEG-IFN-α2b, and natural IFN-β. Thirteen liver cancer cell lines were cultured with or without culture medium containing 10–1,024 IU/ml of one of the 5 type I IFN preparations for 96 h, and the relative viable cell numbers were calculated. The average of the relative viable cell numbers was assessed for each type I preparation and plotted. The antiproliferative effect was strongest in IFN-β, followed by consensus IFN, BALL-1 IFN-α, and IFN-α2b or PEG-IFN-α2b. * $p < 0.05$ vs. the other type I IFNs.

Growth-Inhibitory Effects and the Mechanism of Action of Type I IFN Preparations on Liver Cancer Cell Lines in vivo

Cultured HAK-1B [34] (10^7 cells/mouse) was subcutaneously (s.c.) injected into the backs of 5-week-old female BALB/c athymic nude mice (Clea Japan, Osaka, Japan). Five to 7 days later when the largest diameter of the tumor reached approximately 5–10 mm, each mouse received s.c. injection of medium containing BALL-1 IFN-α (0, 4,000, 40,000 or 400,000 IU) or consensus IFN (0, 0.01, 0.1 or 1 μg), or intraperitoneal injection of medium containing IFN-β (0, 1,000, 10,000 or 100,000 IU) once a day for 2 consecutive weeks and the growth-inhibitory effects

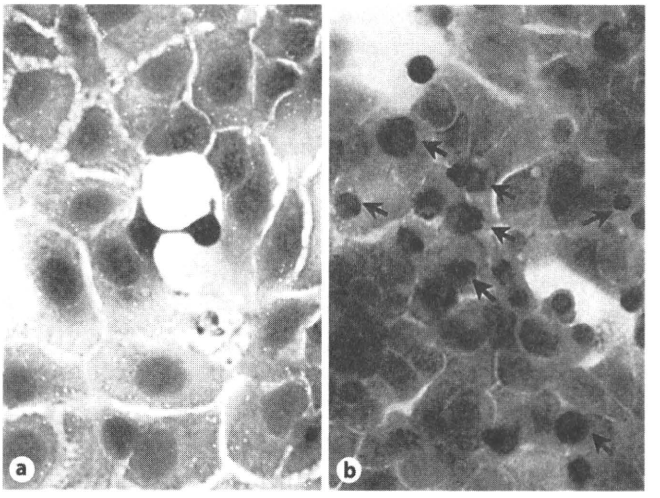

Fig. 3. KIM-1 cells cultured for 72 h on a Lab-Tek Chamber slide. **a** Without PEG-IFN-α2b in culture medium. **b** With 4,096 IU/ml of PEG-IFN-α2b in culture medium. Apoptotic cells (arrows) characterized by cytoplasmic shrinkage, chromatic condensation and nuclear fragmentation were noted. HE staining. ×200.

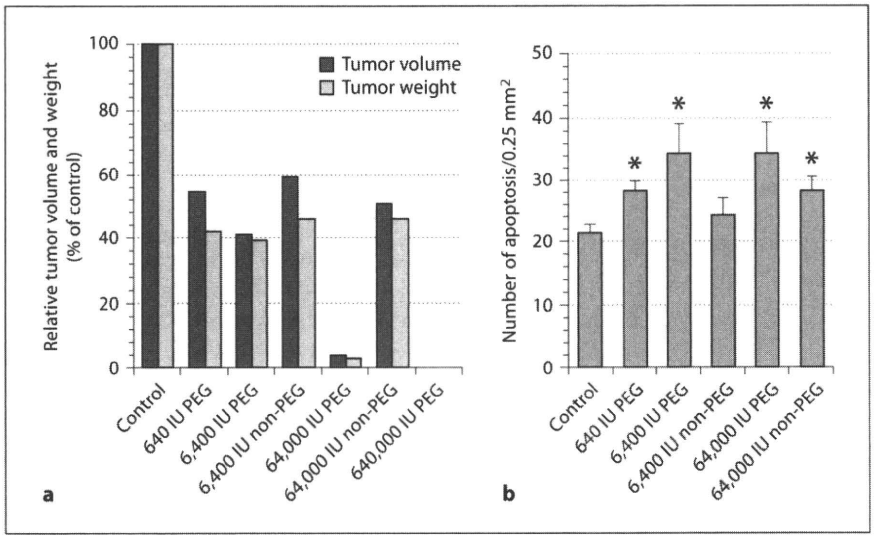

Fig. 4. a Estimated tumor volume and weight of s.c. transplanted human HCC tumors in nude mice at the end of the experiments. The mice received s.c. injection of 640, 6,400, 64,000 or 640,000 IU of PEG-IFN-α2b, or 6,400 or 64,000 IU of IFN-α2b, or medium alone (control) twice a week for 2 consecutive weeks. **b** Numbers of apoptotic tumor cells in subcutaneous human HCC tumors in nude mice that received 640, 6,400, or 64,000 IU of PEG-IFN-α2b, or 6,400 IU or 64,000 IU of IFN-α2b, or medium alone (control). * $p < 0.05$ vs. control.

and histological features of the tumor were estimated about 2 weeks after the initial injection. The lowest dose of BALL-1 IFN-α (4,000 IU/mouse) is as much as the clinical dose (2.0×10^5 IU/kg) for chronic hepatitis C treatment, and the lowest doses of consensus IFN (0.01 μg/mouse, 0.5 μg/kg) and IFN-β (1,000 IU/mouse, 5.0×10^4 IU/kg) are 1.4 times and two fifths, respectively, the clinical doses (0.36 μg/kg and 1.2×10^5 IU/kg, respectively) for chronic hepatitis C treatment. As a result, at the end of the experiment, estimated tumor volumes of mice that received the lowest doses of BALL-1 IFN-α, consensus IFN and IFN-β were about 70% (unpubl. data), 60% [15] and 85% [14], respectively, of the control. These results suggest that the clinical doses of IFNs are effective in inhibiting the proliferation of liver cancer cells in vivo. In the mice that received the highest dose of consensus IFN, the tumor completely disappeared at about 10 days after the initial injection [15]. Histological examination on s.c. tumor revealed that the numbers of apoptotic cells in the mice that received consensus IFN were significantly higher than those of the control and increased dose-dependently. The number of blood vessels in and around the tumor decreased in mice that received consensus IFN as compared with the control [15].

PEG-IFN-α2b increases patient exposure to IFN-α2b and requires less frequent administration because the absorption of the pegylated molecule is slower, its clearance rate from the plasma is lower, and its serum half-life is longer than in unmodified molecules. The in vivo PEG-IFN-α2b experiments were conducted in the same manner, but each mouse received s.c. injection of medium containing PEG-IFN-α2b (0, 640, 6,400, 64,000 or 640,000 IU) or IFN-α2b (6,400 or 64,000 IU) twice a week for 2 consecutive weeks. The lowest dose of PEG-IFN-α2b (640 IU/mouse, 3.2×10^4 IU/kg) is one third of the clinical dose (9.6×10^4 IU/kg) for chronic hepatitis C treatment. As a result, at the end of the experiments, the estimated tumor volume and tumor weight in the mice that received the lowest dose (640 IU/mouse) was 42 and 54%, respectively, of the control (fig. 4a). Induction of apoptosis was found to be the main mechanism of growth inhibition of the tumor by PEG-IFN-α2b (fig. 4b), but inhibition of angiogenesis could not be identified. The antiproliferative effect of PEG-IFN-α2b in vitro is lower than that of IFN-αCon1. Therefore, our in vivo findings would be understood as the serum half-time of IFN-α2b getting longer due to pegylation. Then PEG-IFN-α2b at high concentrations remained in the serum for a long time to affect tumor cells, resulting in much stronger antitumor effects. This consideration is also supported by our results, i.e., PEG-IFN-α2b and IFN-α2b in vitro presenting the same antiproliferative effects. However, in vivo, IFN-α2b presented significantly weaker antitumor effects and a smaller number of apoptotic tumor cells than PEG-IFN-α2b (fig. 4b) [17].

Effects of PEG-IFN-α2b on the Proliferation and Expression of IFNAR-2 Subunit

When IFN-α binds to its receptors, the IFN receptor complexes are internalized and degradated intracellularly [35], resulting in the downregulation of type I IFN re-

ceptors. Nakajima et al. [36] reported that the number of IFN receptors on peripheral blood mononuclear cells in patients with chronic hepatitis B decreased to about 50% of the baseline with a 5-fold increase in 2′,5′-oligoadenylate synthetase activity when the patients were treated with IFN for 2 or 4 weeks. These results suggest that the downregulation of the IFN receptor is not always associated with a decrease in the action of IFN. We chronologically examined the relationship between the antiproliferative effect and the expression of the IFNAR-2 subunit in HAK-1B cells up to 240 h after the addition of PEG-IFN-α2b. The expression of the IFNAR-2 subunit was significantly downregulated at 3 h compared with the control and then significantly upregulated at 48 h. Expression decreased in a time-dependent manner after 72 h, and the viable cell number continuously decreased over time [17]. Similarly, IFNAR-2 expression in the tumor was lower in mice that received PEG-IFN-α2b than in mice that received IFN-α2b or in control mice as a result of the long-term continuous action of PEG-IFN-α2b, but, in fact, the tumor size was smaller in mice that received PEG-IFN-α2b than in mice that received IFN-α2b or in control mice. The results suggest that, at least for the HCC cell line, HAK-1B, the IFNAR-2 subunit is downregulated, but an efficient antiproliferative effect is induced with continuous contact with PEG-IFN-α2b in vitro and in vivo.

Growth-Inhibitory Effects and Mechanism of Action of Combined IFN-α and 5-FU Treatment in Human Liver Cancer Cells in vitro

Alterations in cell cycle progression via upregulation of $p27^{kip1}$ [37] or cyclin A [22], induction of apoptosis by downregulation of Bcl-xl [38], modulation of the immune response via the TRAIL/TRAIL-R pathway [39] and Fas/Fas ligand pathway [40], and alteration of 5-FU metabolism (e.g., increase of 5-fluoro-2′-deoxyuridine-5′-monophosphate and decrease of thymidylate synthase) have been reported as the mechanism of synergistic antitumor action of the combined IFN-α and 5-FU treatment in HCC. We examined the growth-inhibitory effects of the combined IFN-α and 5-FU treatment in 6 HCC cell lines in vitro by using isobologram analysis and found that the cell lines could be divided into two groups: the S group (3 cell lines) showing synergistic effects and the A group (3 cell lines) showing additive effects. In addition, mRNA and protein expressions of type I IFN receptor subunits, IFNAR-1 and IFNAR-2, were specifically upregulated by 5-FU in all cell lines of the S group except IFNAR-2 in one cell line, but not in those of the A group. IFN-α modulated the protein expression levels of six enzymes (thymidylate synthase, dihydropyrimidine dehydrogenase, orotate phosphoribosyl transferase, thymidine phosphorylase, uridine phosphorylase, and thymidine kinase) regulating sensitivity to 5-FU, but none of them were altered in the same way in cells in the S or A group. We concluded that the 5-FU-induced modulation of type I IFN receptor expression, at least in part, contributes to the induction of synergistic effects of combined IFN-α and 5-FU therapy.

Conclusions

We show that (1) almost all liver cancer cell lines express type I IFN receptor, (2) each IFN-α preparation or subtype presents very different antiproliferative activities in different human liver cancer cell lines, (3) a common mechanism of in vitro growth suppression by type I IFN is cell cycle arrest with or without caspase-dependent apoptosis induction, and (4) the mechanism of in vivo growth inhibition by type I IFN is the induction of apoptosis with or without the inhibition of angiogenesis. These lines of evidence suggest that the direct antiproliferative action of type I IFN may be involved in the suppressive mechanisms of type I IFN on hepatocellular carcinogenesis. In addition, we would expect pegylated IFN-α preparations to produce more potent effects in the prevention and treatment of HCC than do nonpegylated IFN preparations.

Disclosure Statement

The author declares that he has no financial conflict of interest.

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Review

肝癌の発生・進展とインターフェロンによる制御

矢野　博久

Development and progression of hepatocellular carcinoma and chemoprevention of HCC by interferon

Hirohisa Yano

Abstract

Most early hepatocellular carcinomas (HCCs) are well differentiated, with an ill-defined nodular appearance. When a well-differentiated HCC reaches a size of about 1.5 cm in diameter, less-differentiated cancerous tissues with greater proliferative activity evolve within it. Clonally-related HCC cell lines (HAK-1A and HAK-1B) established from a single HCC nodule with a variety in the histological grade suggest that less-differentiated cancerous tissues develop from clonal dedifferentiation of well-differentiated HCC tissues. Subsequently, moderately to poorly differentiated HCC tissues gradually replace the initial surrounding HCC.

HCC frequently occurs multicentrically whether synchronously or metachronously, defying complete cure by conventional therapies; therefore, chemoprevention of HCC is very important. Interferon (IFN)-α has been used for the treatment of chronic viral liver diseases to eradicate virus. Recently, IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis. We found that type I IFN preparations inhibit the growth of 13 liver cancer cell lines at various degrees *in vitro* and *in vivo*, and the clinical dose of IFN preparations was effective *in vivo*. The data suggest that IFNs may inhibit the growth of clinically undetectable HCC cells and prevent or delay the development of HCC in patients with chronic viral liver diseases.

Key words: hepatocellular carcinoma, dedifferentiation, interferon, chemoprevention

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緒　言

本稿では，肝細胞癌（肝癌）の発生と進展に関する病理形態学的研究，肝癌の脱分化機構やインターフェロン（IFN）の肝癌細胞に対する増殖抑制作用に関する培養肝癌細胞を用いた分子・実験病理学的研究について我々の施設で集積してきたデータの一部を紹介する．

Ⅰ．肝癌の発生：早期肝癌の特徴

肝癌の多くは，ウイルス性慢性肝炎・肝硬変を背景に発生する．腫瘍径2cm前後までの小さな肝癌は，肉眼的に単純結節型と境界不明瞭型に大別される[1]．単純結節型は明瞭な結節を形成し，その約60%に被膜形成と中分化型肝癌組織が認められ古典的な肝癌の像を呈する[2]．一方，境界不明瞭型は単純結節型よりやや腫瘍径が小さいものが多く（1cm前後），癌結節が不明瞭で被膜形成は認められず，脈管侵襲も転移も示さず，高分化型肝癌組織のみから構成される[2]．「原発性肝癌取扱い規約，第4版」では小結節境界不明瞭型を「早期肝細胞癌」と定義している[1]．この境界不明瞭型肝癌は，細胞異型・構造異型の比較的乏しいため，境界病変（前癌病変）といわれている異型腺腫様過形成（high grade dysplastic nodule）との鑑別が問題となることがあるが，その際には結節内部の門脈域への癌細胞の浸潤像の有無が鑑別の指標となる．浸潤があれば高分化癌，なければ異型腺腫様過形成と診断される[2]．

Ⅱ．肝癌の進展：組織多彩性の出現と高分化型肝癌細胞の脱分化

腫瘍径が1cm前後の境界不明瞭型肝癌が高分化型肝癌組織のみから構成されるのに対し，腫瘍径2～3cmの癌結節は約40%に分化度の異なる癌組織が混在し組織多彩性が認められる[3]．この場合，分化度の低い癌組織が結節の内側に，高分化な癌組織が外側に位置している．腫瘍径の増大とともに外側に位置している高分化型肝組織は面積を減じ，3cmを超えるようになると中分化あるいは低分化型癌組織で置換される．このような形態推移の定型的なものは“nodule-in-nodule”像として肉眼的，画像的に認められる[3]．我々は，中心部が低分化型肝癌で，周囲が脂肪化を伴う，あるいは伴わない高分化型肝癌からなり“nodule-in-nodule”像を呈する肝癌結節から高分化型肝癌細胞株（HAK-1A）と低分化型肝癌細胞株（HAK-1B）の分離樹立に成功した（**Fig. 1**）[4]．HAK-1Bは，HAK-1Aに比べ形態的に低分化で，生物学的悪性度も高い．しかし，これら2つの細胞株には，p53遺伝子の242番目のコドンに共通の変異を認めることから，2つの細胞株が同一起源であり，おそらく，低分化型癌細胞が高分化型癌細胞の脱分化に由来することが示唆される．このように肝癌は，高分化型癌として発生するが，高分化型肝癌細胞の脱分化により，より低分化で悪性度の高い細胞が発生することが肝癌の進展に深く関係する可能性が示唆される[3]（**Fig. 2**）．

Ⅲ．肝癌の多中心性発生とIFNによる発生・再発抑制

筆者らの施設で実施された切除肝癌の検討では，肝硬変を背景に肝癌を発症した場合，約35%の症例で同時性多中心性発生が認められ，また，2cm以下の肝癌を発症し切除治療を受けた症例で，術後5年までに異時性多中心性発生を認めた症例は約60%に上ることが判明した[5]．多中心性発生は肝癌の1つの特徴であるが，このような患者は，背景病変にC型慢性肝炎や肝硬変を有するものが多い．このような患者にIFNを投与することにより，肝機能の改善や肝発癌率の低下が誘導されることが報告されている[6)-8)]．また，同様に肝癌の切除術後の再発防止に対するIFN投与の有用性も報告されている[9]．しかしながら，このようなIFNの発癌抑制の機序はいまだ十分に解明されていない．IFNには細胞増殖抑制作用があることから，肝癌細胞に対して直接的に作用して，発癌抑制や抗癌作用を示している可能性も考えられる．そこで，我々は，培養肝癌細胞を使用しIFNの肝癌細胞に対する直接的作用の検討を行った．

Ⅳ．IFN-α製剤及びIFN-β製剤の肝癌細胞株に対するin vitroの増殖抑制作用と増殖抑制機序

現在我が国において臨床的に使用されているI型IFN製剤には，天然型には，リンパ芽球由来の天然型IFN-α（オーアイエフ®）や線維芽細胞由来の天然型IFN-β（フエロン®）がある．遺伝子組換え型のIFNとしては，IFN-

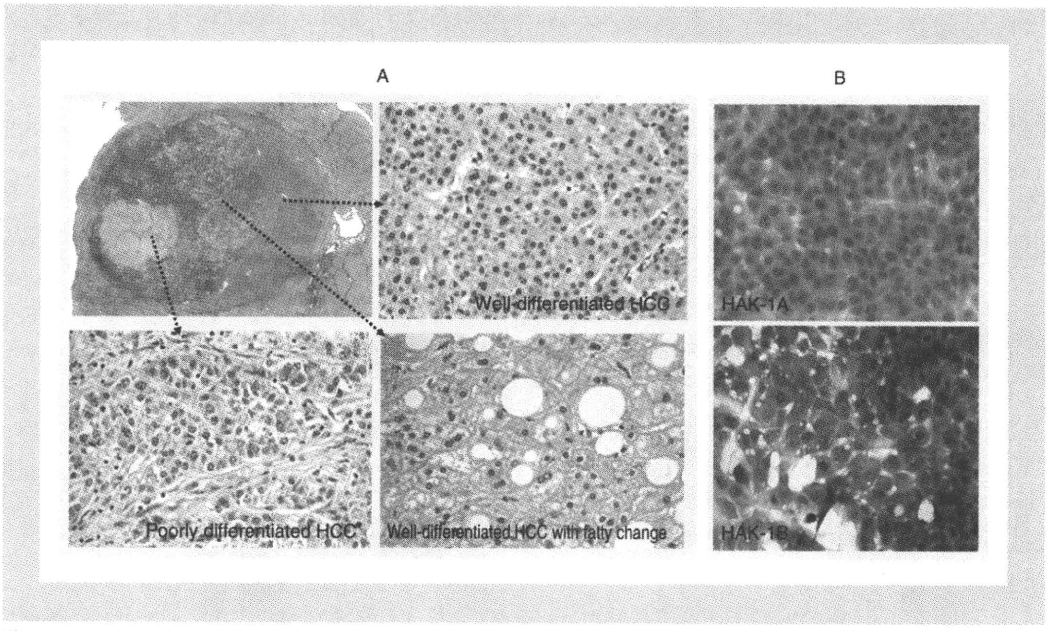

Figure 1

A: upper left, low-power photomicrograph showing the distinct three-layered pattern of the tumor and suggesting the presence of different histological features in each layer (Azan staining); upper right, cells in the outer layer show well-differentiated hepatocellular carcinoma (HCC) (H&E stain, ×100); lower left, cells in the inner layer show poorly differentiated HCC (H&E stain, ×100); lower right, cells in the intermediate layer show well-differentiated HCC with fatty change (H&E stain, ×100).

B: op, HAK-1A cells showing cobble stone-like arrangement of monomorphic cells; bottom, HAK-1B cells showing relatively large pleomorphic cells.

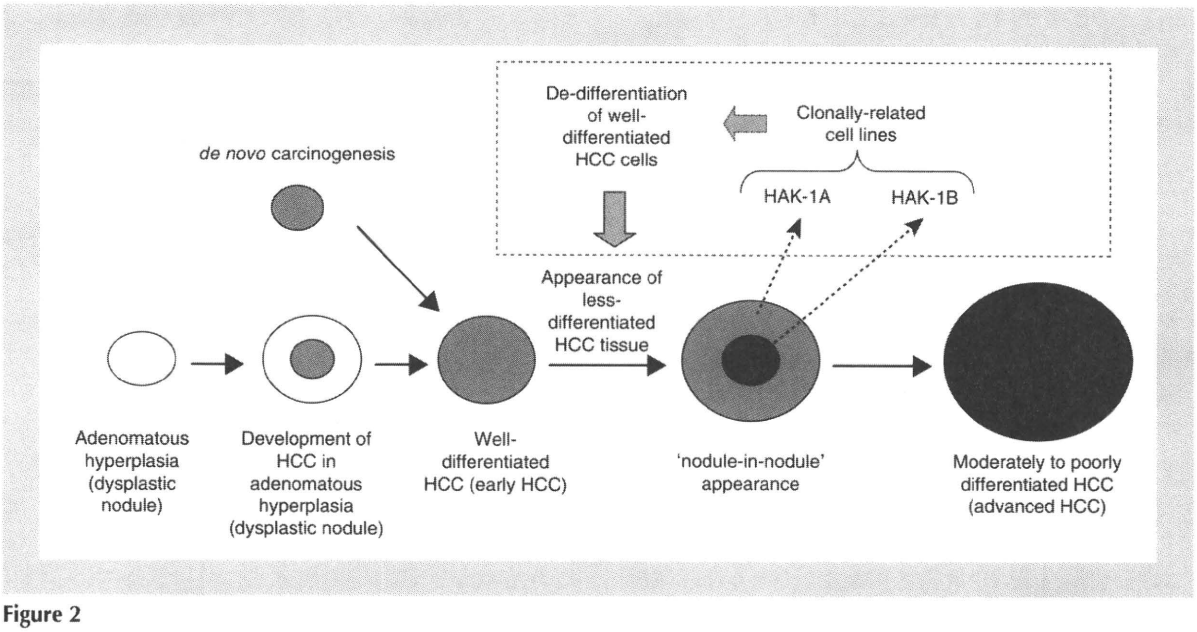

Figure 2

Development and progression of hepatocellular carcinoma (HCC). HCC is well differentiated and vaguely nodular in the early stage, and develops by dedifferentiation in a multistep fashion. Dedifferentiation of well-differentiated HCC cells produces less-differentiated and biologically aggressive HCC cells.

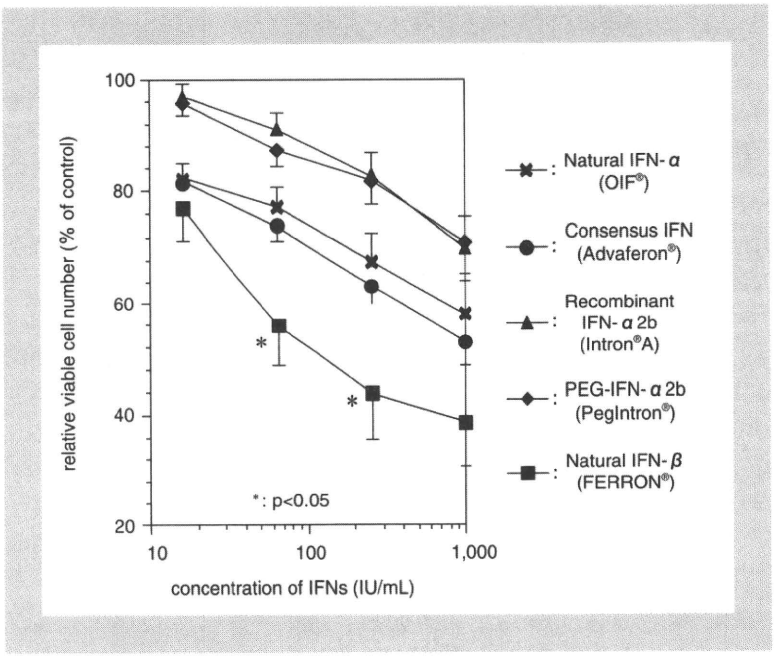

Figure 3

Antiproliferative effects of type I interferon (IFN) preparations, including natural IFN-α, consensus IFN, recombinant IFN-α2b, PEG-IFN-α2b, and natural IFN-β. Thirteen liver cancer cell lines were cultured with or without culture medium containing 10-1,024 IU/mL of one of the 5 type I IFN preparations, and relative viable cell numbers (% of control) were examined. Average of relative viable cell numbers was assessed for each type I preparation and plotted. Antiproliferative effect was strongest in IFN-β, followed by consensus IFN, natural IFN-α, and IFN-α2b or PEG-IFN-α2b.

α2b（イントロン®A）や，特殊なものとして，IFN-αの13種類のサブタイプ遺伝子のそれぞれのアミノ酸配列について，各位置で最も出現頻度が高いアミノ酸を選択することによりアミノ酸配列を決定し，人工的に作製したコンセンサスIFN（rIFN-α Con1，アドバフェロン®）がある．最近開発されたIFNとしては，遺伝子組換え型IFN-αにメトキシポリエチレングリコール（PEG）を結合させることにより生物学的半減期を延長させ，少ない投与回数で高い効果が期待可能なPEG-IFN-α2b（ペグイントロン®）やPEG-IFN-α2a（ペガシス®）がある．これらのIFNのなかからPEG-IFN-α2aを除く4種類のIFN-α製剤と，1種類のIFN-β製剤を用いて13種類の肝癌細胞株に対する増殖抑制作用について比較検討を行った．1,024 IU/mLの各種IFN添加培地で96時間培養後に，IFN非添加培養（コントロール）と比べ生細胞数の割合が50%以下まで低下した細胞株の数は，ヒト天然型IFN-αでは5株[10]，コンセンサスIFNでは7株[11]，IFN-α2bとPEG-IFN-α2bではいずれも2株[12]，天然型IFN-βでは10株であった[13]．IFN製剤別の増殖抑制作用を13株の平均値で比較すると，天然型IFN-β，コンセンサスIFN，天然型IFN-α，PEG-IFN-α2b・IFN-α2bの順に強い作用を認めた（**Fig. 3**）．特に，天然型IFN-βでは，経時的に増殖抑制作用が増大し，接触96時間後では，低濃度でも比較的強い増殖抑制効果が見られた．

各種IFNを肝癌細胞の培地に添加し，48～72時間培養し細胞形態を観察すると細胞質の縮小や核の濃縮，核の断片化など，アポトーシスに特徴的な細胞像の出現が認められた．アポトーシス誘導は，使用したIFNの種類，濃度，そして細胞株により差を認めるものの，最低でも13株中10株で認められた[10)-13)]．IFN-α誘導性アポトーシスでは，caspase-9，-8，-7，-3の活性化とともにcytochrome cやSmac/DIABLOのミトコンドリアから細胞質への放出が見られ，ミトコンドリア系のアポトーシス誘導経路の関与が示唆されるが[14]，TRAILやTRAIL-R1，-R2などの発現亢進も見られており（未発表データ），デスリガンド-デスレセプターを介した経路の関与も考えられ，今後更なる検討が必要である．アポトーシス誘導以外の増殖抑制の機序としてすべての細胞株で細胞周期の進行停止誘導が認められ，S期での停止誘導が11株，G_2/M期での停止誘導が1株，G_1期での停止誘導が1株で認められた[10) 12)]．

V. IFN-α製剤及びIFN-β製剤の肝癌細胞株に対するin vivoの増殖抑制作用と増殖抑制機序

肝細胞癌細胞株HAK-1B[4]をヌードマウスの皮下に接種し，約1週間後5～10mmの腫瘍径の腫瘍が形成された時点から，各種IFN製剤を投与し*in vivo*における増殖抑制作用の検討を行った．天然型IFN-αは，C型慢性肝炎患者の治療に使用される投与量にほぼ相当する量（臨床量）（4,000 IU/mouse，2.0×10^5 IU/kg），その10倍量あるいは100倍量を14日間連日マウスの皮下に接種し，腫瘍の経時的な推定体積や，15日目に摘出された腫瘍の組織像を比較検討した．コンセンサスIFN-αは，臨床量の約1.4倍量（0.01 μg/mouse，0.5 μg/kg），その10倍量，100倍量を同様に投与し，天然型IFN-βは，臨床量の約半量（1,000 IU/mouse，5.0×10^4 IU/kg），その10倍量，100倍量を腹腔内に投与し同様に検討した．その結果，14日目の腫瘍体積は，最小量のIFN投与によりIFNを投与しなかったマウス（コントロール）に比べ，天然型IFNで30%前後（未発表データ），コンセンサスIFN-αで40%前後[11]，天然型IFN-βで15%前後[13]減少した．このように，臨床量前後のIFN製剤の投与は，*in vivo*において肝癌細胞の増殖を抑制した．IFNを投与されたマウスの腫瘍組織では，IFNの濃度依存性に肝癌細胞のアポトーシス数の増加を認め，コンセンサスIFN-αを投与されたマウスの腫瘍では，腫瘍内血管の減少も認められた．

PEG-IFN-α2bは，前述のごとくPEG化により吸収・排泄速度が低下し，通常のIFNに比べ生物学的半減期が数倍延長する結果，長時間IFN-α2bの血液濃度が維持されるという特徴を有する．臨床量の1/3量（640 IU/mouse，3.2×10^4 IU/kg），その10倍量，100倍量，1,000倍量を1週間に2回，合計4回皮下に投与し腫瘍の経時的な推定体積や，15日目に摘出された腫瘍の重量や組織像を比較した．その結果，臨床量の1/3量の投与でコントロールに比べ約50%前後の腫瘍の体積及び重量の減少が認められた[12]（**Fig. 4A**）．増殖抑制機序としては，アポトー

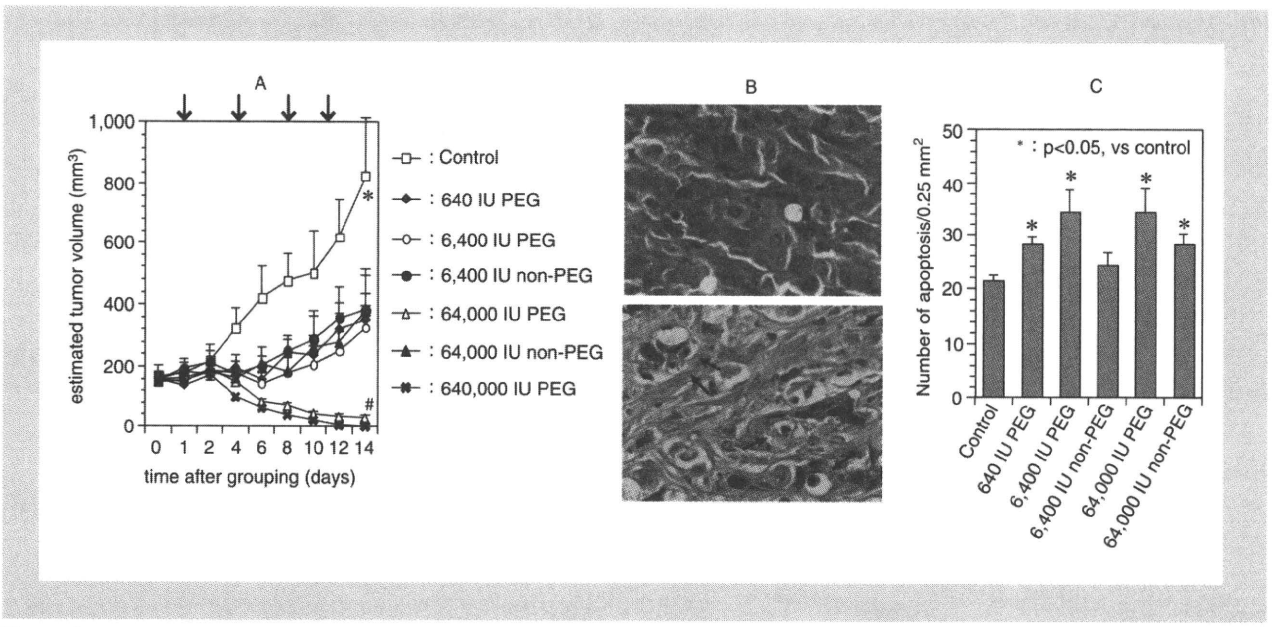

Figure 4

A: Time-course change in estimated tumor volumes of subcutaneously transplanted human hepatocellular carcinoma (HCC) tumors in nude mice. The mice received a subcutaneous injection of 640, 6,400, 64,000, or 640,000 IU of PEG-IFN-α2b, or 6400 or 64,000 IU of IFN-α2b, or medium alone (control). The arrows show the days of injection. * : p<0.001, vs the other groups, # : p<0.0001, vs IFN-α2b (64,000 IU).

B: Photomicrograph of subcutaneous human HCC tumor in nude mice. Top, a control mouse that received culture medium alone; bottom, a mouse that received a subcutaneous injection of 6,400 IU PEG-IFN-α2b, showing some apoptotic tumor cells (arrows).

C: The numbers of apoptotic tumor cells in subcutaneous human HCC tumors in nude mice that received 6,400 IU or 64,000 IU of IFN-α2b, or 640, 6,400, or 64,000 IU of PEG-IFN-α2b, or medium alone (control).

シスの誘導を認めたが，血管新生抑制は確認できなかった（**Fig. 4B**）．PEG-IFN-α2bと同じ活性（IU）のIFN-α2bを同様の方法で投与し，抗腫瘍作用をPEG-IFN-α2bを投与した場合と比較すると，PEG-IFN-α2bを投与した方が，腫瘍のアポトーシス誘導は高度であり，有意により強い抗腫瘍作用を認めた[12]（**Fig. 4C**）．*In vitro* では，PEG-IFN-α2bはIFN-α2bと同程度に増殖抑制効果が最も低いIFN-α製剤であったが，PEG化により長時間血中IFN-α2bの濃度が維持されたことにより，肝癌細胞に持続的に作用し，非PEG化IFN-α2bや他のIFN-α製剤より強い増殖抑制作用を発揮したと推察される．

結　語

肝癌の発生・進展に関して，肝癌の多くは，ウイルス性慢性肝炎・肝硬変を背景に高分化型肝癌として発生するが，腫瘍径の増大とともに増殖活性の高いより低分化な癌組織が高分化型癌細胞の脱分化により発生・増殖し，その結果，進行肝癌は，中・低分化型癌組織のみで占められるようになる．肝癌は，再発や多中心性発生を示す頻度も高いが，IFN製剤の投与はこれらを有意に抑制する．今回紹介したIFN製剤が肝癌細胞に対しアポトーシスなどを誘導し直接的に抗腫瘍作用を発揮するという実験結果は，IFN製剤による肝発癌抑制機序の１つとして，ごく初期のまだ不顕性な段階の肝癌細胞に対する直接的な増殖抑制作用が発癌抑制に寄与している可能性を支持している．

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