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Temporal treatment with interferon-β prevents hepatocellular carcinoma in hepatitis B virus X gene transgenic mice☆

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***Background/Aims*: The preventive effect of interferon (IFN) against hepatocellular carcinoma (HCC) has been confirmed clinically. We sought to determine whether the temporal administration of IFN-β prevents hepatocarcinogenesis in a mouse model where HCC develops without necroinflammation.**

***Methods*: Hepatocarcinogenic mice that are transgenic for the hepatitis B virus X gene (HBx-Tg) were treated with IFN-β or saline (control) for three months, from 3 to 6 months of age, and the incidence of HCC was determined at 18 months of age. The effects of IFN-β on DNA synthesis and apoptosis were tested.**

***Results*: The incidence of HCC was significantly lower in the IFN-β-treated mice than the controls (0 vs. 50%, $P < 0.01$). Inhibition of DNA synthesis in hepatocytes by IFN-β was observed in the livers of HBx-Tg, without any significant induction of apoptosis. Although the treatment of IFN-β was temporal, the number of hepatocytes with DNA synthesis remained lower 3 and 12 months later in life.**

***Conclusions*: Temporal administration of IFN-β has a significant preventive effect on the occurrence of HCC in a mouse model where HCC develops without inflammation. The mechanisms are the inhibition of DNA synthesis and cell cycle progression of hepatocytes.**

***Keywords*: Interferon-β; Hepatocarcinogenesis; Temporal treatment; Hepatitis B virus X gene; Transgenic mouse; DNA synthesis; Cell cycle; Primary hepatocyte culture; WRL-68**

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. Necroinflammation caused by hepatitis viruses or other toxic agents induces a broad variety of genetic alterations which ultimately lead to the clinical manifestation of HCC [2–4].

A number of studies have shown that interferon (IFN) treatment may prevent hepatocarcinogenesis in patients with chronic hepatitis C [5–8] and hepatitis B [9]. Inhibition of HCC development has been shown clearly in patients with chronic hepatitis C who achieved

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viral eradication or normalization of alanine aminotransferase (ALT) [5–8] as well as those who could not achieve these outcomes [10].

The mechanisms of the inhibitory effects of IFN on HCC involve multiple factors, which include: (i) suppression of viral replication, (ii) immunomodulative effects, and (iii) direct antiproliferative activity such as the induction of apoptosis or inhibition of the cell cycle [11,12]. Although the antiproliferative activities of IFN in the context of anti-tumor effects have been investigated extensively using HCC-derived cell lines [13–16], only a few studies have examined this effect on primary hepatocytes [17]. Unfortunately, there have been no studies extrapolating this approach to the preventive action of IFN on HCC.

IFN-β belongs to the type-I IFNs [11,12] and has been reported to inhibit the recurrence of HCC in patients with chronic hepatitis [18]. Although the biological functions of IFN-β are similar to those of IFN-α, more pronounced growth-inhibitory effects have been reported [19–23].

The question we asked was whether the antiproliferative effect of IFN-β has a preventive role in the development of HCC, even with temporal administration. To investigate this hypothesis, we used a hepatocarcinogenic transgenic mouse model which contained the hepatitis B virus X (HBx) gene (HBx-Tg) [24–26] and in which there was no necroinflammatory activity in the pathology of liver. Enhanced DNA synthesis and apoptosis may eventually result in the development of HCC from 12 months of age [25].

So far, a few reports have addressed the preventive effect of IFN *in vivo* [27–29] but none has successfully shown the inhibition of hepatocarcinogenesis by temporal treatment with IFN. Merle et al. showed a preventive effect of IFN-α for HCC by continuous treatment of a highly-hepatocarcinogenic transgenic mouse [27,28]. However, because they gave the mice lifelong IFN treatment, both a tumor-killing effect and an antiproliferative effect on normal pre-cancerous hepatocytes might have contributed to the anti-hepatocarcinogenic effect.

The aim of this study was to clarify whether short term-treatment with IFN-β carried out in our mouse model at a young age when the livers of the animals were free from cancers and precancerous lesion, had a preventive effect against HCC.

2. Materials and methods

2.1. In vivo experimental protocol

Human and mouse IFN-β were kindly supplied by Toray Co., Ltd (Tokyo, Japan). A highly-hepatocarcinogenic mouse model (HBx-Tg) was used [24]. The experimental protocol is shown in Fig. 1A: 5000 U/gram body weight of mouse-IFN-β (23 mice, Male:Female = 12:11) or saline (17 mice, M:F 6:11) was administered intraperitoneally [30] to three-month-old HBx mice three times per week for three months. Four (M:F = 2:2) and three (M:F = 1:2) mice from the respective groups were sacrificed at the end of treatment (6 months old). Four (M:F = 2:2) mice from each group were sacrificed 3 months after the end of the treatment (9 months old), otherwise mice (M:F = 8:7 for IFN-β and M:F = 3:7 for controls) were sacrificed at 18 months of age.

All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Niigata University and performed in accordance with the National Institutes of Health Guidelines.

2.2. Hepatocyte proliferation assay in vivo and in vitro

HBx-Tg were treated at 12 weeks of age with 5000 U/gram body weight IFN-β or saline intraperitoneally for 10 days, and water with 1 mg/ml bromodeoxyuridine (BrdU) was given to the mice for the last 5 days prior to sacrifice [31]. Paraffin-embedded liver sections were subjected to staining with anti-BrdU (Dako, Japan). Cell proliferation also was assessed in the livers of the mice sacrificed at 18 months of age by the incubation of the slides with anti-PCNA antibodies (Dako) at 4 °C overnight.

Primary cultured hepatocytes were prepared from 6-week-old mouse livers using a conventional collagenase perfusion method [32].

BrdU incorporation assays in primary hepatocyte culture were carried out as described previously [33,34].

2.3. Quantification of apoptosis

Quantitative analysis of apoptosis was performed by a terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) method (In Situ apoptosis Detection kit, Chemicon International, Temecula, CA) for mouse hepatocytes *in vivo* and *in vitro,* and by caspase-Glo™ 3/7 assay (Promega, Madison, WI) for WRL-68 cells. Liver tissues from the HBx-Tg, obtained using the protocol for the cell proliferation analysis, also were subjected to TUNEL analysis. WRL-68 cells were incubated with or without IFN-β for 3 or 6 h and assayed by a luminometer (Luminescencer-JNR, AB 2100, Atto, Japan).

2.4. Effect of IFN-β on cell proliferation and cell cycle in a normal hepatocyte cell line

A normal human hepatocyte cell line (WRL-68: American Type Culture Collection, Manassas, VA) [35] was obtained commercially.

To analyze the effect of IFN-β on WRL-68 cell proliferation, the number of viable cells was determined by a modified MTT assay (Cell counting kit-8, Dojindo, Japan) according to the manufacturer's instructions.

For cell cycle analysis, a time chase experiment was carried out on cells that had incorporated BrdU to determine the effect of IFN-β on the cell cycle [36–38].

2.5. Quantification of HBx mRNA using TaqMan-PCR

Primary hepatocytes (1×10^5 cells/ml) obtained from an HBx-Tg were cultured with or without 100,000 U/ml IFN-β. Cells were harvested at 0, 1, and 3 h, and RNA was extracted. Similarly, RNA was extracted from IFN-β or PBS treated-mice (described in Section 2.2). After digestion with DNase, one microgram of RNA was converted to cDNA (Transcriptor first strand cDNA synthesis kit, Roche, Germany). Aliquots of cDNA samples were subjected to *Taq*Man-PCR using a light cycler (Roche). Primers for detecting HBx mRNA were 5′-CCCGTCTGTGCCTTCTCA-3′ (sense) and 5′-AGCAATGTCAA CGACCGACC-3′ (antisense), and the (*Taq*Man) probe: 6FAMS 5′-C CGTGTGCACTTCGCT-3′ TAMRA to detect amplicons of 148 bps. Mouse β-actin mRNA was quantified as an endogenous control. PCR protocols were as follows: denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 15 s. HBx mRNA levels in each sample were standardized by levels of β-actin mRNA at each time point. For the RNA obtained from primary hepatocytes, values from six samples were obtained at each time point (two experiments in triplicate), and the relative values of HBx mRNA at 1 and 3 h to the baseline levels (means ± SD) were calculated. Statistical comparisons were made between the baseline level of HBx mRNA and >that obtained at each time point. For the RNA obtained from the HBx-Tg liver, six (IFN-treated) and five (saline) samples were analyzed.