의학 박사학위 논문

Roles of Interferon Lambda on Hepatitis B Virus Replication

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의 학 과

홍 승 호

Roles of Interferon Lambda on Hepatitis B Virus replication

by

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홍승호의 의학 박사학위 논문을 인준함.

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-ABSTRACT-

Roles of Interferon Lambda on Hepatitis B Virus Replication

Background/Aims: Chronic hepatitis B virus (HBV) infection is a major cause of liver disease. Only interferon-α (IFN-α) and the nucleosidic inhibitors of the viral polymerase, lamivudine and adefovir, are approved for therapy. However, these therapies are limited by the side effects of interferon and by the substantial resistance of the virus to nucleosidic inhibitors. New antiviral molecules suitable for monotherapy or combination therapy are highly desired. Recently described IFN-λ family utilizing its distinct cellular receptor has been reported to exert inhibitory effect on replication of several viruses. In this study, I wanted to know whether IFN-λ1 inhibits HBV replication in human hepatoma cell lines and whether HBV modulates expression of CRF2-12, a subunit of IFN-λ1 receptor. In addition, I wanted to establish ELISA for IFN-λ1 to examine HBV mediated modulation of IFN-λ1 production.

Methods: I produced IFN-λ1 using *E.coli* expression system. For antiviral activity of IFN-λ1, I used a functional form of MBP-IFN-λ1 and for generation of antibody against IFN-λ1, I used His-IFN-λ1. I analyzed HBV replication in WT10 and PEB8 human hepatoma cell lines supporting HBV replication, after treatment of these cells with IFN-λ1 by real-time PCR and Southern blotting. After treatment of PEB8 with IFN-λ1, I examined the transcription level of HBV by Northern blotting

and the amount of secretory viral Ag by ELISA. I performed the RT-PCR to investigate the induction of antiviral proteins, MxA and 2’5’-OAS, by IFN-λ1. I investigated the expression of CRF2-12 by RT-PCR and Western blotting. I generated monoclonal Abs against IFN-λ1 using splenocytes from immunized mice and determined the detection limit of ELISA using these Abs.

Results: HBV replication in PEB8 but not in WT10 was suppressed by IFN-λ1 treatment. In both cell lines, similar amount of CRF2-12 to that in their parental cell line was expressed, and similar amount of transcripts of MxA as well as 2’5’-OAS were induced by IFN-λ1. In PEB8, neither HBV transcripts nor secretory Ag was affected by IFN-λ1 treatment. IFN-λ1 was detected by ELISA using monoclonal Abs and polyclonal Ab generated in this study with the detection limit of 40 ng/ml.

Conclusions: Antiviral activity of IFN-λ1 on HBV was demonstrated in one of two human hepatoma cell lines suggesting that the effect of IFN-λ1 may be dependent on the cellular factors, and/or viral factors. My results showed that CRF2-12 expression was not regulated by HBV replication. Finally, an assay for IFN-λ1 production was established.

Key words : Hepatitis B Virus, IFN-lambda-1 protein, Virus replications

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ABBREVIATION

- 2’5’-OAS: 2’5’-Oligoadenylate Synthetase
- Ab: Antibody
- BSA: Bovine Serum Albumin
- CRF2: Type II Cytokine Receptor Family
- ECL: Enhanced Chemical Luminescence
- FPLC: Fast Protein Liquid Chromatography
- HBV: Hepatitis B Virus
- HCMV: Human Cytomegaly Virus
- HCV: Hepatitis C Virus
- HRP: Horseradish Peroxidase
- IFN-α: Interferon alpha
- IFN-λ: Interferon lambda
- Ig: Immunoglobulin
- MBP: Maltose Binding Protein
- MxA: Myxovirus resistance A
- p-NPP: p-Nitrophenyl Phosphate
- PBS: Phosphate Buffered Saline
- RT-PCR: Reverse Transcription-Polymerase Chain Reaction
- STAT: Signal Transducer and Activator of Transcription

I. INTRODUCTION

A. Background

1. Hepatitis B Virus (HBV)

HBV belongs to the family of hepadnaviruses, a group of small-enveloped viruses with major liver tropism (Seeger and Mason, 2000). HBV infection may lead to acute liver disease, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC).

HBV consists of an envelope, a nucleocapsid core and a viral genome. The outer envelope encloses the nucleocapsid core of the virus, within which lies the viral genome. The viral genome is a relaxed circular, partially double-stranded DNA molecule of 3.2 kb in length, and contains four partially overlapping open reading frames (ORFs) (Karayiannis, 2003). The Pre-S/S ORF encodes the three envelope glycoproteins that are known as the large (L), middle (M) and small (S) HBsAgs. The precore/core ORF yields two translation products, the precore polypeptide being the precursor of the soluble hepatitis B e antigen (HBeAg), and the nucleocapsid or core protein. One of the other two ORFs encodes for the X protein and the other one the polymerase, which acts as a reverse transcriptase and DNA polymerase. The core and polymerase genes are essential for viral DNA replication, and the envelope proteins are essential for envelopment of nucleocapsids. Hepatitis X protein (HBx) activates stimulation of signal transduction and the transcription of host gene (Seeger

and Mason, 2000).

The life cycle of HBV is characterized by the synthesis of a 3.2 kb partially double-strand, relaxed-circular DNA (rcDNA) genome by reverse transcription of a 3.5 kb pregenomic RNA. HBV initiates infection with attachment on the receptor of hepatocytes which is the primary site of viral DNA replication. During initiation of infection the nucleocapsids are imported into nucleus and the viral genome in the nucleocapsid is released (Rabe et al., 2003). The viral relaxed circular DNA genome, with the reverse transcriptase attached to the 5' end of the minus DNA strand and a short RNA attached to the 5' end of the plus DNA strand, is converted into a covalently closed circular DNA (cccDNA). The cccDNA serves as the template for transcription of viral mRNAs (Fig. 1). The pregenome serves as the mRNA for the synthesis of core protein and the viral reverse transcriptase. The reverse transcriptase binds to the 5' end of its own mRNA template, and the complex is then packaged into nucleocapsids, where viral DNA synthesis occurs. Once partially double-stranded DNA has been produced, nucleocapsids can undergo a maturation event that facilitates their acquisition of an outer envelope via budding into the ER. These nucleocapsids can also migrate to the nucleus to increase the copy number of cccDNA (Seeger and Hu, 1997). Enveloped particles containing all three envelope proteins are thought to be transported through the ER into the Golgi complex (Huovila, 1992). Glycosylation at an asparagine residue located in the S domain of the envelope proteins occurs during the phase of the assembly process, which is completed with the secretion of mature virions into the bloodstream.

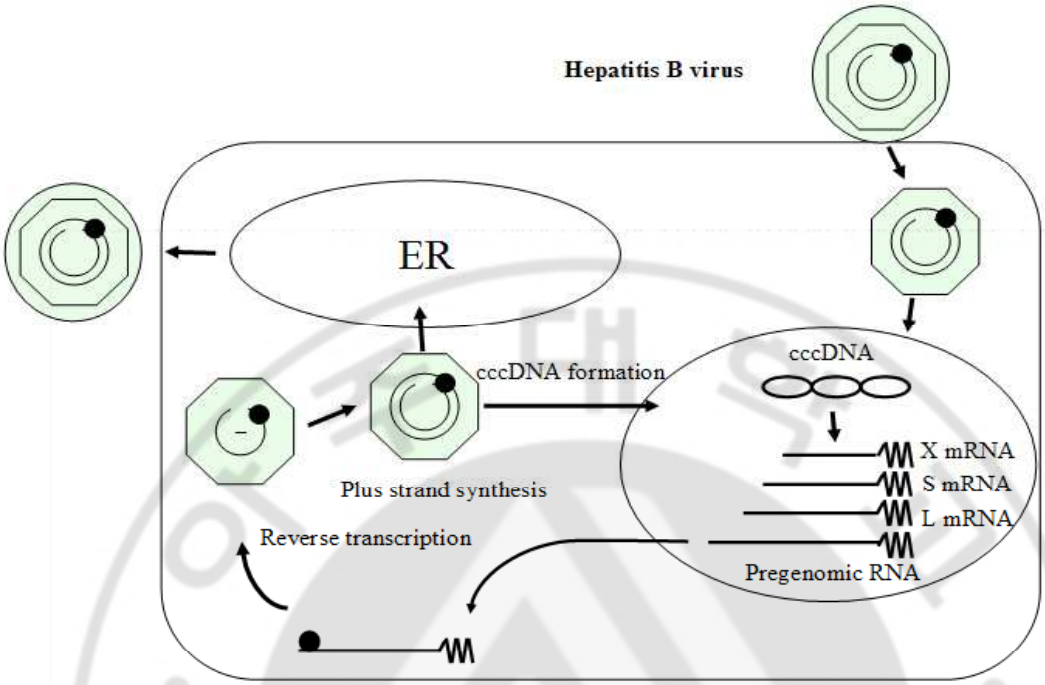


Fig. 1. HBV life cycle. The virion is internalized and uncoated in the cytosol, whence the genome translocates to the nucleus, where it is converted into a double-stranded covalently closed circular DNA (cccDNA) molecule, by completion of the synthesis of the shorter plus DNA strand and repair of the nick in the minus DNA strand. cccDNA serves as a template for synthesis of viral transcripts. The reverse transcriptase binds to the 5' end of its own mRNA, pregenomic RNA, and the resulting complex is then packaged into nucleocapsids, where viral DNA synthesis occurs. Once partially double-stranded DNA has been produced, nucleocapsids can undergo a maturation event that facilitates their acquisition of an outer envelope via budding into the ER. These nucleocapsids can also migrate to the nucleus to increase the copy number of cccDNA.

HBV infects more than 350 million people worldwide. As these patients are at increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma, therapeutic intervention offers the only means of interrupting this progression. The ultimate goals of treatment are to achieve sustained suppression of HBV replication and remission of liver disease. The agents currently available for the treatment of chronic HBV infection are divided into two main groups: Interferon-α (IFN-α), and the nucleoside/nucleotide analogues, among which lamivudine, adefovir dipivoxil, and famciclovir are the most well-known. At present however, only IFN-α, lamivudine and adefovir dipivoxil are approved for chronic HBV treatment. Nucleoside analogues are chemically synthesized drugs that are able to mimic natural nucleosides (Karayiannis, 2003). As such, they are incorporated into newly synthesized HBV-DNA causing chain termination, and thus inhibiting viral replication. The drug contains a sulphur atom instead of carbon at the 3′ position of the sugar ring, which does not allow chain elongation by phosphodiester bond formation, in the absence of the normal 3′ hydroxyl group. Since lamivudine acts by terminating viral DNA synthesis and competitively inhibiting the viral polymerase/reverse transcriptase, it is equally effective in patients of any race. Adefovir dipivoxil is a nucleotide analogue of adenosine monophosphate that inhibits both HBV reverse transcriptase and DNA polymerase activity. Adefovir has been shown to be effective in suppressing not only wild-type HBV but also lamivudine-resistant HBV mutants (Mark and John, 2006)

2. Interferon alpha (IFN-α)

The IFNs are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation and immune activation. By gene homology and binding receptor as well as cellular source, the IFNs are classified into two major groups: type I IFNs and type II IFNs. Type I IFNs are produced in direct response to virus infection and consist of the products of the IFN-α multigene family and the IFN-β. While IFN-α is predominantly synthesized by leukocyte, IFN-β is synthesized by most cell types but particularly by fibroblasts. On the other hand, type II IFN consists of the product of the IFN-γ gene and, rather than being induced directly by virus infection, is synthesized in response to the recognition of infected cells by activated T lymphocytes and natural killer cells(Goodbourn et al., 2000).

The biological activities of IFN-α are initiated by the binding of IFN-α/β to their cognate receptors on the surface of cells. The IFN-α/β receptor is composed of two major subunits, IFNAR1 and IFNAR2. Each subunit consists of the extracellular domain for binding of IFN, the transmembrane domain and the intracellular domain for binding for Janus kinase (JAK) family, which transduces the intracellular signal. Prior to stimulation, the cytoplasmic domains of IFNAR1 and IFNAR2 are respectively associated with the Janus tyrosine kinase Tyk2 and Jak1, which also associate with signal transducer and activator of transcription 2 (STAT2). On IFN-α/β binding, IFNAR1 and IFNAR2 associate, facilitating the tyrosine-phosphorylation and activation of Tyk2 and Jak1 (Novick et al., 1994). Activated Tyk2 phosphorylates the tyrosine at position 466 (Tyr466) on IFNAR1, creating a new

docking site for STAT2 (Colamonici et al., 1994). STAT2 is then phosphorylated by Tyk2 at Tyr690 and serves as a platform for the recruitment of STAT1 (Qureshi et al., 1996), which is subsequently phosphorylated on Tyr701 (Shuai et al., 1993). The phosphorylated STAT1/STAT2 heterodimers formed dissociate from the receptor and are translocated to the nucleus, where they associate with the DNA-binding protein p48 to form a heterotrimeric complex called IFN-α/β-stimulated gene factor 3 (ISGF3), which binds interferon-stimulated response element (ISRE) and induces transcription of genes under ISRE (Pestka S et al., 2004).

Of genes induced by IFN-α, only a few genes are reported to be involved in viral replication. The well-characterized proteins involved in anti-viral response by IFN-α are 2’5’ oligoadenylate synthetase (2’5’-OAS) and Myxovirus resistance A (MxA).

2’5’-OAS is activated by binding to dsRNA and synthesizes oligoadenylates with 2’5’-phosphodiester linkages and with varying chain length from two to more than twenty of adenosine, which are collectively called 2-5A. It activates RNase L by binding to the enzyme. The activated RNase L catalyzes the cleavage of single-stranded RNA including mRNA, leading to inhibition of protein synthesis. RNase L also cleaves 28S ribosomal RNA in a site-specific manner, leading to ribosomal inactivation and thus translational inhibition of viral genes (Iordanov et al., 2000).

Human MxA is a 76 kDa GTPase protein belonging to the dynamin superfamily of large GTPases. MxA is selectively up-regulated by type I IFNs but not other cytokines such as IFN-γ, TNF-α/β, IL-1, IL-2, IL-6 or GM-CSF and is

accepted as a sensitive marker of IFN activity (Roers et al., 1994; von Wussow et al., 1990). It accumulates in the cytoplasm of the cells at 4 h after type I IFN induction. Its half-life (about 2 days) is considerably longer than that of the IFNs. Experimental evidences have established that MxA is able to inhibit a broad spectrum of negative and positive strand RNA viruses (Haller and Kochs, 2002), including influenza, parainfluenza, vesicular stomatitis virus (Schwemmle et al., 1995), thogoto, and measles virus. The antiviral activity of MxA is not restricted to RNA viruses, but also includes a DNA virus (HBV) (Gordien et al., 2001). In vitro, MxA is able to inhibit HBV replication a post-transcriptional level and acts, at least in part, by inhibiting the nucleocytoplasmic export of viral mRNAs. Recent reports have shown that antiviral mechanism of MxA may involve its direct interaction with HBV nucleocapsid (Fernandez et al., 2003; Schwemmle et al., 1995). In the liver of female HBV/MxA double transgenic mice, MxA expression moderately downregulates the expression of viral proteins and HBV replication by reducing the synthesis of HBV DNA without affecting the steady-state levels of HBV RNAs (Peltekian et al., 2005). It therefore suggests a post-transcriptional inhibitory effect of MxA on HBV. However, it was also published that IFN-α suppressed HBV replication in MxA-deficient HEp2 cells (Rang A et al., 2002). It indicates that MxA is not essential for these activities. The role of MxA in the host defense against HBV is still controversial.

3. Interferon lambda (IFN-λ)

In 2003, Kotenko group and Sheppard group have reported new cytokine family and its receptor (Kotenko et al., 2003; Sheppard et al., 2003). While Kotenko group designated these cytokines as IFN-λ1, IFN-λ2 and IFN-λ3, Sheppard group designated them as IL-28A, IL-28B and IL-29. Their distinct receptor complex is composed of two subunits, CRF2-12 (IL-28R) and IL-10R2. At the same time, CRF2-12 was identified and termed likely interleukin or cytokine receptor-2 (LICR2) by Dumoutier group through screening genomic DNA databases for similarity with IL-22R (Dumoutier et al., 2003).

The subtypes of IFN-λ family is IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). The open reading frame (ORF) size of IFN-λ1, IFN-λ2 and IFN-λ3 is 856 bp, 734 bp, 595 bp, respectively. IFN-λ1, IFN-λ2 and IFN-λ3 are composed of all 200 amino acids. The predicted molecular weight of IFN-λ1, IFN-λ2 and IFN-λ3 is 21.90 kDa, 22.29 kDa, 22.19 kDa, respectively. While IFN-λ2 and IFN-λ3 are identical with 96% amino acid identity, IFN-λ1 and IFN-λ2/IFN-λ3 are identical with 81% amino acid identity. Both genes are located on chromosome 19. Also IFN-λ1 and IFN-λ2/IFN-λ3 are composed of 5 exons and 6 exons, respectively, but type I IFN is composed of one exon (Kotenko et al., 2003; Sheppard et al., 2003).

Although the biological activities of IFN-λ have not fully investigated, its antiviral activity has been demonstrated. For antiviral activity, some researchers have demonstrated that IFN-λ reduced cytopathogenic effect (CPE) by vesicular stomatitis virus (VSV) infection in HT29, A549 and HaCaT cells. Also, IFN-λ inhibited the

replication of several viruses, including Hepatitis C virus (HCV) (Robek et al., 2005) and human cytomegaly virus (HCMV) (Vlotides et al., 2004). Like IFN-α, IFN-λ was capable of inducing transcription 2'5'-OAS and MxA protein. It was suggested that the antiviral potency of IFN-λ is comparable to that of IFN-α.

IFN-λ receptor is a heterodimer of IL-10R2 and CRF2-12. At RNA level, CRF2-12 expression was observed in various normal tissues such as pancreas, thyroid, skeletal muscle, heart, prostate and testis and in several cell lines such as Raji, HL-60, SW480, HepG2 and HuH7 (Sheppard et al., 2003)Brand et al., 2005). It showed that CRF2-12 appears to be constitutively expressed across a broad range of cell lines and tissues. CRF2-12 forms three different transcripts by alternative splicing: IL-28Rαv1, IL-28Rαv2 and IL-28Rαv3. The ORF of IL-28Rαv1, IL-28Rαv2 and IL-28Rαv3 is 1,563 bp, 1476 bp and 674 bp, respectively. IL-28Rαv1, IL-28Rαv2 and IL-28Rαv3 are composed of 520 amino acids (aa), 491 aa, and 211 aa, respectively. The predicted molecular weight of IL-28Rαv1, IL-28Rαv2 and IL-28Rαv3 are 57.65 kDa, 54.41 kDa and 23.69 kDa, respectively. While IL-28Rαv1 is consisted of cytokine-binding domain, transmembrane and intracellular region, IL-28Rαv2 differs only in the intracellular domain of 29 amino acids deletion from IL-28Rav1. IL-28Rαv3 is a soluble form of IL-28Ra that transmembrane and intracellular region is deleted (Sheppard et al., 2003). The gene is located together with *IL-22* on chromosome 1. The gene encoding CRF2-12 is composed of 7 exons. The first exon contains the 5'-UTR and the signal peptide. Exons 2, 3, 4 and 5 and a part of exon 6 encode the extracellular domain. Exon 6 also encodes the

transmembrane domain and the beginning of the intracellular domain. Exon 7 covers the rest of the intracellular domain the 3'-UTR. Both CRF2-12 and IL-10R2 required for IFN-λ binding. Although intracellular signal transduction of IFN-λ receptor remains to be elusive, some signaling pathways are known. IFN-λ receptor mediates the tyrosine phosphorylation of STAT1, STAT2, STAT3, STAT4 and STAT5. STAT2 tyrosine phosphorylation is dependant on tyrosines 343 and 517 of CRF2-12 (Dumoutier et al., 2004). STAT activation induced by CRF2-12 was mediated by JAK1. Therefore, CRF2-12 associates with JAK1, which activates STAT, and IL-10R2 associates with Tyk2 (Dumoutier et al., 2003) (Fig. 2).

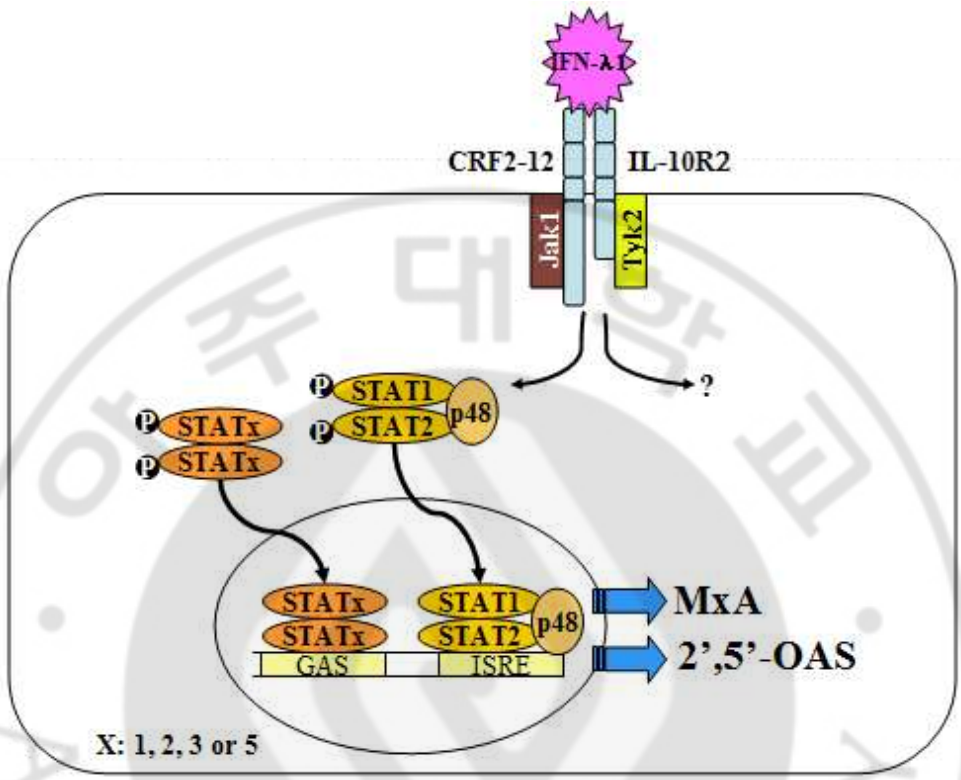


Fig. 2. Transcriptional induction of MxA and 2'5'-OAS by IFN-λ1. The IFN-λ receptor complex consists of CRF2-12 and IL-10R2 chains. Upon binding of IFN-λ receptor with IFN-λ, STAT1 and STAT2 are phosphorylated, then associated with p48, translocated to the nucleus and activated genes, including MxA and 2'5'-OAS that contain the IFN-stimulated response element (ISRE).

B. Aims

In spite of the several reports regarding the antiviral activity of IFN-λ against several RNA viruses, there was no report about the antiviral activity of IFN-λ against HBV when started this study. Although this year Robeck et al. showed that HBV replication in a murine hepatocyte cell line was inhibited by IFN-λ treatment (Robek et al., 2005), it has not been investigated that HBV replication in a human cell line is affected by IFN-λ1.

It is conceivable that HBV or its protein may modulate antiviral activity of IFN-λ. HBx inhibits proteasome function, which is required for the inhibitory action of IFN-α on HBV replication (Zhang et al., 2004). By interfering with the NF-kB pathway, recombinant HBs inhibited LPS-induced IL-18 production, which has been shown to inhibit HBV infection in vivo (Cheng et al., 2005). However, except for HCV, the antagonistic effect of virus to IFN-λ1 activity has not been studied.

I wanted to know whether IFN-λ1 suppresses HBV replication in two HBV replicating human hepatoma cell lines, and which stage of viral replication is affected by IFN-λ1.

2. I wanted to know whether HBV regulates expression of the receptor for IFN-λ1, and planned to establish ELISA system for analyzing the production of IFN-λ1.

II. MATERIALS AND METHODS

A. Expression vectors for IFN-λ1 and its receptor subunit CRF2-12

For the production of functional form of IFN-λ1, prokaryotic expression vector pMAL-IFN-λ1 (kindly provided by Dr, Kotenko, New Jersey Medical School, USA) containing DNA sequences encoding human IFN-λ1 gene adjacent to the 3' end of a gene for maltose binding protein (MBP) was used because it was reported that MBP-IFN-λ1 produced by *E.coli* transformed with this vector retains functional activity (Fig. 1A) (Kotenko et al., 2003).

In order to produce a large amount of IFN-λ1 with a tag of low antigenicity, pPROEX-IFN-λ1 was constructed. A DNA segment of IFN-λ1 produced by digestion of pMAL-IFN-λ1 with *Xmn*I and *Eco*RI was subcloned into pPROEX1 (Life Technologies Inc., Gaithersburg, MD, USA) using *Ehe*I and *Eco*RI recogintion sites (Fig. 1B). Because the expressed IFN-λ1 is fused with histidine tag, it can be purified using Ni-NTA column.

In order to produce a large amount of CRF2-12 with a tag of low antigenicity, pPROEX-CRF2-12 EC was constructed A DNA segment of extracellular domain of CRF2-12 produced by digestion of pEF2-CRF2-12 (kindly provided by Dr, Kotenko) with *Xho*I and *Eco*RI was subcloned into pPROEX1 (Life Technologies Inc., Gaithersburg, MD, USA) (Fig. 1C).

In order to produce CRF2-12 protein expressed from eukaryotic cell, pIgG2A-CRF2-12 EC was constructed. The extracellular domain gene of CRF2-12 was amplified by PCR with sense primer CRF2-12 EX5(BamHI) (5'-CGGGATCCCAGGGAGGCCCCGTCTG-3') and antisense primer CRF2-12EX3(EcoRI) (5'-CGGAATTCGTTGGCTTCTGGGACCTCCA-3'). A DNA segment of extracellular domain of CRF2-12 produced by digestion of PCR product with *Bam*HI and *Eco*RI was subcloned into pIgG2A (kindly provided by Dr. Chwae, Pochon Cha University College of Medicine, South Korea), which has secretory signal sequence and was derived from pSecTag2C (Invitrogen, Carsbade, CA, USA) (Fig. 1D). Because the expressed CRF2-12 is fused with mouse immunoglobulin (mIg), it can be purified using Protein A column.

B. Purification of the recombinant proteins

1. Expression and purification of MBP-IFN-λ1 in *E. coli*. pMAL-IFN-λ1 was transformed into *E. coli* TB1. A single colony was incubated in 1 L LB containing 100 ug/ml ampicillin at 37℃ with 250 rpm until A_{600} reaches 0.8~1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 0.3 mM was added to the culture to induce the expression of IFN-λ1. The culture was incubated at 37℃ for 4 h and was centrifuged at 5,500 rpm for 20 min. The cell pellet was resuspended in 100 ml of 30 mM Tris-HCl, 20 % sucrose (pH 8.0) and 200 ul of 0.5 M EDTA (pH 8.0). It was centrifuged at 7,500 rpm at 4℃ for 20 min. The cell pellet

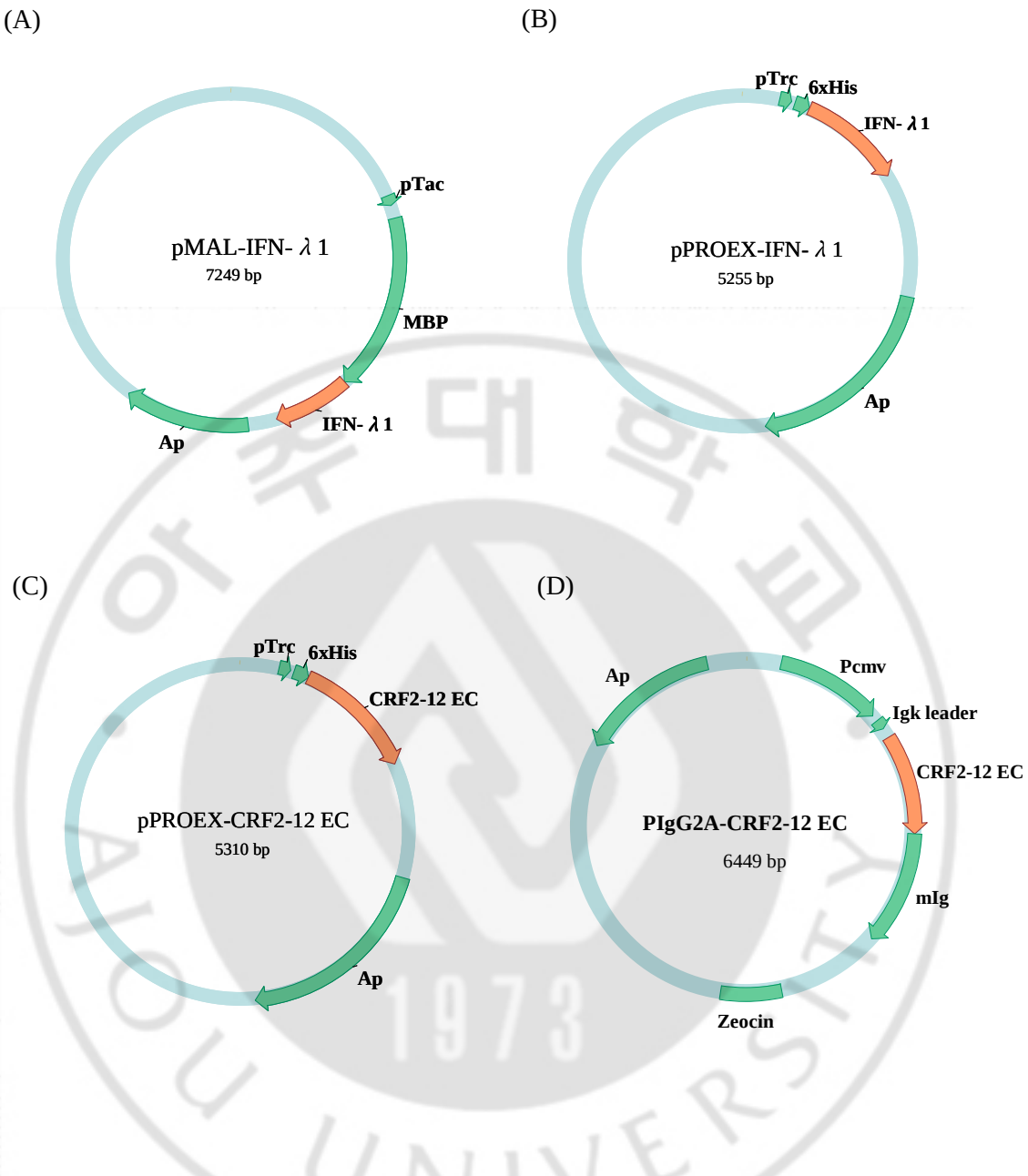


Fig. 3. Expression vectors for IFN-λ1 and CRF2-12. (A) Expression vector for MBP-IFN-λ1. (B) Expression vector for His-IFN-λ1. (C) Expression vector for His-CRF2-12. (D) Expression vector for CRF2-12-mIg.

was resuspended in ice-cold 100 ml of 5 mM $MgSO_4$. After centrifugation, 8 ml of 1 M Tris-HCl (pH 7.4) was added into the supernatant, which was loaded into amylose column. The column was washed with column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA). The protein was eluted with 10 mM maltose. The eluted MBP-IFN-λ1 was applied to Fast Protein Liquid Chromatography (FPLC) (Amersham Biosciences Co., Piscataway, NJ, USA) in order to remove MBP protein.

2. Expression and purification of His-IFN-λ1 and His-CRF2-12 in *E. coli.*

pPROEX-IFN-λ1 or pPROEX-CRF2-12 EC was transformed into *E. coli* PRT. A single colony was incubated in 1 L LB containing 100 ug/ml ampicillin at 37℃ with 250 rpm until A_{600} reaches 0.8~1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 0.3 mM was added to the culture to induce the expression of IFN-λ1. The culture was incubated at 37℃ for 4h and was centrifuged at 5,500 rpm for 20 min. The cell pellet was stored at -20℃ overnight and thawed for 15 min on ice. It was resuspended in 20 ml lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 8.0). The lysate was centrifuged at 9,000 rpm for 30 min at room temperature. The supernatant was loaded into Ni-NTA column (Qiagen, Valencia, CA, USA). The column was washed with 30 ml of washing buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 6.3). The protein was eluted with 20 ml of elution buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 4.5).

3. Expression and purification of CRF2-12-mIg in COSM6. Sixteen micrograms of pIgG2A-CRF2-12 EC were transfected into COSM6 (2x 10^6) using lipfectamin 2000 (Invitrogen, Carsbade, CA, USA). Next day the media were exchanged with IgG-depleted FBS-DMEM. At fourth day posttransfection, the culture supernatant was collected and centrifuged at 1,000 rpm for 15 min. The supernatant was mixed with nProtein A Sepharose (Amersham Biosciences Co., Piscataway, NJ, USA) at 4℃ for 1 hr. The mixture was centrifuged at 5,000 rpm for 2 min. The pellet was washed 3 times with phosphate buffered saline (PBS) by centrifugation of 5,000 rpm for 5 min. CRF2-12-mIg protein was eluted with 200 ul of 0.1 M citrate (pH 3.0), followed by neutralization of 40 ul of 0.1M Tris-HCl (pH 9.0).

C. SDS-PAGE

The concentration of the purified recombinant proteins was measured and the proteins were separated by 12% SDS-PAGE. The acrylamide gels were incubated with Coomassie Blue staining solution (Amersham Biosciences Co., Piscataway, NJ, USA) for 4 h. It was incubated with destaining solution (30% Methanol, 10% glacial acetic acid, 60% distilled water) for 12 h.

D. Western blotting

To examine the purity of recombinant proteins, the purified protein was separated by 12% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore co. Bedford, MA, USA). The membrane was kept for 1 h in 3% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS) at room temperature, and reacted for 1 h with primary Ab such as rabbit anti-MBP antiserum (New England Biolabs Inc., Beverly, MA, USA) (1:10,000) or mouse anti-His Ab (Qiagen Inc., Valencia, CA, USA) (1:1,000). Then the membrane was washed with PBS containing 0.1% Tween 20 for 1 h. The membrane was incubated for 1 h in 3 % BSA containing horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) conjugate or HRP-goat anti-mouse IgG (H+L) conjugate (Zymed, SanFrancisco, CA, USA) (1:20,000). Then the membrane was washed with PBS containing 0.1% Tween 20 for 1 h. After washing, the membrane was incubated in enhanced chemical luminescence (ECL) solution (Amersham Biosciences Co., Piscataway, NJ, USA) and developed with X-ray.

To examine the reactivity of monoclonal Abs against IFN-λ1, Western blottig was performed as above except followings. Purified His-IFN-λ1 was separated by SDS-PAGE. The membrane was kept for 1 h in 10% skim milk in phosphate buffered saline (PBS), and reacted for 1 h with culture supernatants of anti-IFN-λ1 Ab producing hybridoma. The membrane was incubated for 1 h in 10 % skim milk containing horseradish peroxidase (HRP)-goat anti-mouse IgG (H+L) conjugate (1:2,000).

E. RNA preparation

Hepatoma cell lines (HuH7 and HepG2) and HBV replicating cell lines (WT10 and PEB8) were lysed by the addition of 200 ul of RNAzol Bee (Tel-Test, Inc, Friendswood, TX, USA) followed by the addition of 20 ul of chloroform. After vigorous shaking, the cell lysate was incubated on ice for 5 min and centrifuged at 12,000 rpm for 15 min at 4℃. The aqueous phase was transferred to the fresh tube. The RNA was precipitated by adding the equal volume of isoporpanol and incubated for 1 h at 4℃. After centrifugation, the pellet was washed with 75% ethanol. The pellet was dried and then dissolved in diethyl pyrocarbonate (DEPC)-treated distilled water.

F. Reverse transcription-polymerase chain reaction (RT-PCR)

The cDNA was synthesized from 500 ng of total RNA in a reaction mixture containing 0.5 ul of RNase H^- reverse transcriptase (Invitrogen, Carsbade, CA, USA), 0.5 ul of oligo $(dT)_{12\text{-}18}$ (500 ug/ml), 2 ul of 5x first-strand buffer and 1 ul of 0.1 M DTT, 0.25 ul 10mM dNTP mixture. The cDNA was amplified using specific primers (MxA(5) and MxA(3) for MxA, OAS(5) and OAS(3) for 2'5'-OAS, IL-28RA(R) and IL-28RA(F) for CRF2-12, GAPDH(5) and GAPDH(3) for GAPDH) (Table 1). PCR reaction mixture contained 2 ul of cDNA, 2.5ul of 10x buffer, 2 ul of dNTP (2.5mM), 1 ul of 2.5mM sense primer, 1 ul of 2.5mM antisense primer, 0.15 ul of rTaq (5

units/ul) and 17.25 ul of distilled water. PCR reaction condition was 94℃ for 5 min, 30 cycles at 94℃ for 30s, 55℃ for 30s, 72℃ for 1 min; final extension was 72℃ for 7min. Amplified cDNA products were separated by electrophoresis in 1% agarose gel.

Table 1. Primers used for amplificating of MxA, 2’5’-OAS and CRF2-12 cDNAs

Primer Name	Sequence
MxA(5)	GCTACACACCGTGACGGATATGG
MxA(3)	CGAGCTGGATTGGAAAGCCC
2,5-OAS(5)	ATTGACAGTGCTGTTAACATCATC
2,5-OAS(3)	AGATCAATGAGCCCTGCATAAACC
IL-28RA(R)	TCTACACGTTCAGTGTCCCGAAATAC
IL-28RA(F)	CCAGAGGCGGCTCCGGTGGTAAG
GAPDH(5)	GTCAAGGCTGAGAACGGGAAG
GAPDH(3)	CACCACCTTCTTGATGTCATC

G. Isolation of HBV core DNA

WT10 or PEB8 cells (5x 10^6) were seeded in 10 cm dishes. After 1 day or 2 days, the cells were treated with 0.5 ug/ml of poly(I:C) (Amersham Biosciences Co., Piscataway, NJ, USA), which was transfected by lipofectamin 2000 (Invitrogen, Gaithersburh, MD, USA), or IFN-α (10,000 U/ml) (PBL Biomedical Laboratories, Piscataway, NJ, USA), MBP-IFN-λ1 (3,000 ng/ml; equivalent of 1,000 ng/ml of IFN-λ1) and MBP (1,000 ng/ml). At 1 day or 2 days after treatment, WT10 or PEB8 cells were harvested and lysed in 1% NP-40 containing 10mM Tris-HCl (pH 8.0), 50 mM NaCl and 1 mM EDTA at 4°C for 5 min followed by centrifuging for 5 min at 12,500 rpm. The cleared cell lysate was treated by 20 U DNase I (SIGMA, St. Louis, MO, USA), 60 U of micrococcal nuclease (Calbiochem, San diego, CA, USA) with 10 mM $MgCl_2$ and 8 mM $CaCl_2$ at 37°C for 4 h. The virus in the cleared culture medium was precipitated by polyethylene glycol (PEG) 8000 (40%) in 50 mM NaCl and 50 mM EDTA at 4°C overnight. After centrifugation for 15 min at 12,000 rpm, the virus was resuspended in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl and 2 % sodium dodecyl sulfate (SDS), and digested with 4 ug of proteinase K for 4 h at 37°C. After phenol extraction, the intracellular virion DNAs were recovered by ethanol precipitation (Imanaka et al., 2005).

H. Real-time PCR

Primers and probes were designed against a highly conserved region among 25 published HBV genome sequences in GenBank and EMBL, representing genotypes A-F (Li et al., 2005). As the primers, HBV(sens) (5'-AGTGTGGATTCGCAC TCCT-3') and HBV(anti) (5'-GAGTTCTTCTTCTAGGGGACCTG-3') was used. The Taqman HBV probe (5'-CCAAATGCCCCTATCTTATCAACACTTCC-3') was labeled with a reporter dye FAM (6-Carboxy-uorescein) at its 5' end and with a quencher dye TAMRA (6-Carboxy-tetramethyl-rhodamine) at its 3' end. The amplification was performed in a 25 ul reaction mixture containing 12.5 ul TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 0.7 mM of HBV(sens) and HBV(anti) primers, and 0.15 mM of TaqMan HBV probe (Li et al., 2005). Five microliters of DNA template were added to each reaction. The product was first digested with uracil-N-glycosylase at 50℃ for 2 min to destroy the potential carry-over contamination. An incubation of 10 min at 95℃ allowed the activation of the AmpliTaq Gold DNA polymerase and the denaturation of the nucleic acids. Forty-five cycles of denaturation at 95℃ for 15 sec and annealing-extension at 53℃ for 1 min were carried out allowing the amplification detection of HBV genomes. The detection of amplified product (120 bp fragment) was carried out in an ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA).

I. Southern blotting

Isolated HBV core DNA was separated by 1% agarose gel electrophoresis. The gel was washed with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 1 h, and then with neutralization solution (1.5 M NaCl, 0.5 M Tris-Cl (pH 7.0)). HBV core DNA in the gel was transferred through 20x SSC (3 M NaCl, 0.3 M $Na_3Citrate_2H_2O$, pH 7.0) to nylon membrane (Schleichr & Schuell, Keene, NH, USA). Alpha-[^{32}P]-labeled probe specific for the HBV sequences was random-primed for 12 h in 50 ul of reaction buffer, including 10 ul of 5x labeling buffer (Promega co., Madison, WI, USA), 5 mM dNTP without dATP, 1x BSA, 150 ng of HBV DNA and 2 ul of DNA polymerase I large (Klenow) fragment (Promega co., Madison, WI, USA). The membrane-associated HBV DNA was hybridized to α-[^{32}P]-labeled probe specific for the HBV sequences at 68℃ for 12 h. The membrane was washed with washing solution (0.1% SDS, 2x SSC) for 1 h. The autoradiogram was scanned using the imaging analyzer FLA-3000 (Fuji Photo Film co., Tokyo, Japan).

J. Northern blotting

Total RNA (10 ug) was denatured and electrophoressed on a 1% agarose gel containing 40% formaldehyde, 1x MOP and 0.1% diethyl pyrocarbonate (DEPC)-distilled water. RNA in the gel was transferred to a nylon membrane (Schleichr & Schuell, Keene, NH, USA). Hybridization of probe into RNA on the membrane was

performed as above.

K. Enzyme-linked immunosorbent assay (ELISA)

1. Secretion of HBsAg and HBeAg. ELISA was performed using ELISA kit specific for HBsAg (GENDIA, GreenCross, Kyunggi, Korea) and for HBeAg (DiaSorin S.p.A., Saluggia, Italy) according to the manufacturer's protocol, respectively.

2. Screening of monoclonal anti-IFN-λ1 Ab. Microtiter plate was coated with His-IFN-λ1, MBP-IFN-λ1 and MBP at concentration of 10 ug/ml, followed by being blocked with 3% BSA. Then the hybridoma culture supernatant was added to each well and incubated at room temperature. The microtiter plate was incubated with alkaline phosphatase-goat anti-mouse IgG (H+L) conjugate (PIERCE, Rockford, IL, USA) in 3% BSA (1:5,000). After washing, p-nitrophenyl phosphate (p-NPP) (SIGMA, St. Louis, MO, USA) was used as a substrate and absorbance at 415nm was determined using Microplate reader model 680 (Bio-Rad, Melville, NY, USA).

3. Sensitivity of anti-IFN-λ1 Abs against IFN-λ1. Microtiter plate was coated with IFN-λ1 (R&D systems Inc., Minneapolis, MN, USA) at the concentration of 1 ng/well to 10 ng/well, followed by being blocked with 3% BSA. After washing, the purified monoclonal anti-IFN-λ1 Ab in 3% BSA was added at the concentration of 10

ug/ml to each well and incubated overnight at 4℃. After washing microtiter plate was incubated with alkaline phosphatase-goat anti-mouse IgG (H+L) conjugate (PIERCE, Rockford, IL, USA) in 3% BSA (1:1,000). After washing, p-nitrophenyl phosphate (p-NPP) (SIGMA, St. Louis, MO, USA) was used as a substrate and absorbance at 415nm was determined using Microplate reader model 680 (Bio-Rad, Melville, NY, USA).

L. Cell fusion

For a primary immunization, 50 ug of the purified His-IFN-λ1 protein was mixed with an equal volume of complete Freund's adjuvant (SIGMA, St. Louis, MO, USA). The emulsified solution was injected into three Balb/c mice (Daehan Biolink co., LTD, Chungbuk, Korea) intravenous. For secondary and third immunization, 25 ug of the purified His-IFN-λ1 protein was mixed with an equal volume of incomplete Freund's adjuvant (SIGMA, St. Louis, MO, USA) after 2 and 4 weeks. The emulsified solution was injected into the Balb/c mice intravenous. After 2 weeks, 25 ug of the purified MBP-IFN-λ1 protein was injected into the Balb/c mice intraperitoneal. After 3 days, final boosted Balb/c mouse was sacrificed and its spleen was removed. The spleen was teased apart using a couple of slide glasses. The cells were resuspended in incomplete RPMI1650 and centrifuged at 1,500 rpm for 10 min. The cell pellet was resuspended in 5 ml of red blood cell (RBC) lysis buffer for 5 min. The splenocytes and the cultured F0 myeloma cells were washed three times

by centrifugation at 1,500 rpm for 10 min. The splenocytes and the F0 myeloma cells were mixed in 5 ml of incomplete RPMI1650 in the ratio of 3 to 1. Cell mixture was centrifuged at 1,500 rpm for 15 min. One milliliter of 50% polyethylene glycol (PEG) (SIGMA, St. Louis, MO, USA) was slowly added to the cell pellet for 1 min while resuspending the cells with complete RPMI1650 by stirring the tube. The cell mixture was centrifuged at 1,500 rpm for 10 min and then cell pellet was resuspended in 30 ml complete RPMI1650. One hundred microliter of cells was dispensed into the wells of 96-well plate, which was placed at 37℃ in a CO_2 incubator. Next day, the culture media were exchanged with HAT media (SIGMA, St. Louis, MO, USA).

M. Limiting dilution

The anti-IFN-λ1 Ab-producing hybridoma cell was transferred from 96-well plate to 24-well plate. The cell was incubated for 3 days in 24-well plate. The number of the cell was counted, and the cell was diluted to be one cell per 3 wells. One hundred microliter of the diluted cell was added into 96-well plate. It was incubated at 37℃ for 10 days. When colony was appeared, the supernatant was applied to ELISA. For single-cell cloning, the limiting dilution was repeated.

N. Isotyping of monoclonal Abs

The supernatant of anti-IFN-λ1 Ab was collected from the selected hybridomas. The isotyping of the monoclonal Abs was performed according to the protocol of immunotype mouse monclonal antibody isotyping kit (SIGMA, St. Louis, MO, USA).

O. Large production of monoclonal anti-IFN-λ1 Ab

Three Balb/c mice (Daehan Biolink co., LTD, Chungbuk, Korea) were primed by injecting 500 ul of pristane (2,6,19,14-tetraamethyldecanoic acid) into the peritoneum. After 7 days, the mice were injected with HL2 hybridoma cell (1x 10^6) intraperitorial. Within 2 weeks following the injection of the cells, the mice were noticeably large. Ascite was withdrawn with 18 gauge needle attached to a 5 ml syringe. The fluid was incubated at 37℃ for 1 h and then transferred to 4℃ overnight. It was centrifuged at 3,000g for 10 min. The supernatant was removed from the cell pellet. Anti-IFN-λ1 Ab was eluted through nProtein A Sepharose column (Amersham Biosciences Co., Piscataway, NJ, USA).

P. The production of polyclonal anti-IFN-λ1 Ab and anti-CRF2-12 Ab

For a primary immunization, 250 ug of the purified His-IFN-λ1 or His-CRF2-12 protein was mixed with an equal volume of complete Freund's adjuvant (SIGMA, St. Louis, MO, USA). The emulsified solution was injected into each rabbit (Daehan Biolink co., LTD, Chungbuk, Korea) subcutaneous. For secondary and third immunization, 125 ug of the purified His-IFN-λ1 or His-CRF2-12 protein solution was mixed with an equal volume of incomplete Freund's adjuvant (SIGMA, St. Louis, MO, USA) after 2 and 4 weeks. The emulsified solution was injected into each rabbit subcutaneous. After 2 weeks, 125 ug of the purified MBP-IFN-λ1 or His-CRF2-12 protein was mixed with an equal volume of incomplete Freund's adjuvant. The emulsified solution was final boosted into each rabbit subcutaneous. After 3 days of the injection, each rabbit was sacrificed. The serums were collected and then stored at -20℃.

III. RESULTS

A. Expression and purification of IFN-λ1

For the experiment of antiviral activity of IFN-λ1, MBP-IFN-λ1 was purified based on the report that this form of IFN-λ1 retains functional activity (Kotenko et al., 2003). The purified MBP-IFN-λ1 using amylose column affinity chromatography and Fast Protein Liquid Chromatography (FPLC) was subjected to SDS-PAGE and Western blotting (Fig. 4A). In SDS-PAGE, the major band of around 65 kDa, which corresponds to MBP-IFN-λ1, and the minor band of 43 kDa, which corresponds to MBP, were observed. In Western blotting, all of two bands were detected with anti-MBP Ab. Thus the major band was confirmed as MBP-IFN-λ1 and the minor one as MBP.

As antigen for the generation of monoclonal Ab specific to IFN-λ1, IFN-λ1 with histidine tag (His-IFN-λ1) was expressed in *E. coli* and purified due to the lower antigenicity of histidine tag than MBP. Another advantage of His-IFN-λ1 was the improved expression level. The yield of purification of His-IFN-λ1 was two times greater than that of MBP-IFN-λ1. In SDS-PAGE of His-IFN-λ1 purified using Ni-NTA column, the expected size (22 kDa) of single band was detected, and in Western blotting this protein reacted with antibody specific to histidine tag (Fig. 4B).

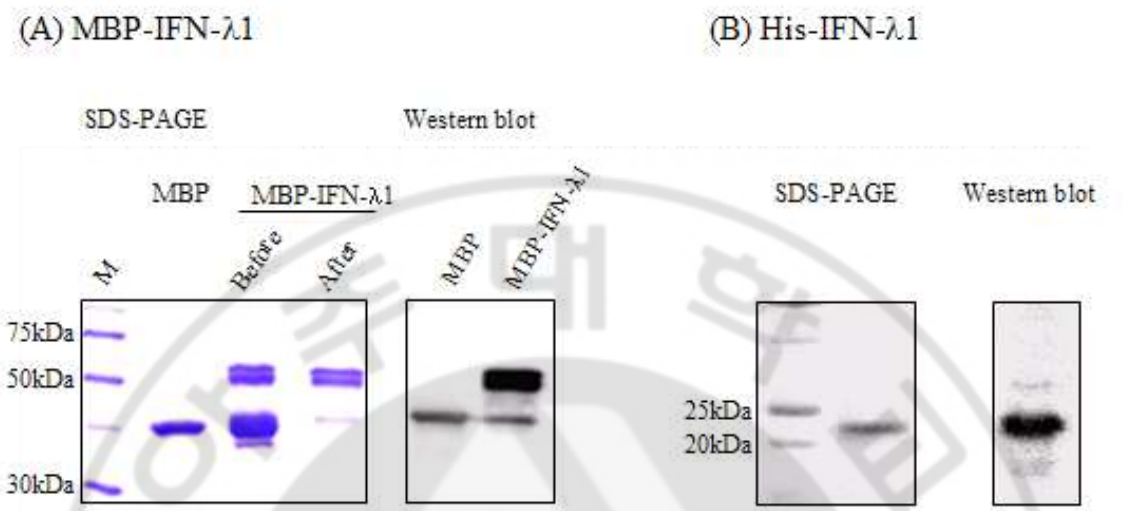


Fig. 4. The purification of IFN-λ1 in *E. coli*. (A) IFN-λ1 fused with MBP was purified using FPLC after amylose column chromatography and analyzed by SDS-PAGE (left) and Western blotting (right). Western blotting was performed with rabbit anti-MBP antibody and HRP conjugated anti-rabbit Ig antibody. Before: the protein before being purified using FPLC, After: the protein after being purified using FPLC(B) IFN-λ1 with histidine was purified using Ni-NTA column and analyzed by SDS-PAGE (left) and Western blotting (right). Western blotting was performed with mouse anti-His antibody and HRP conjugated anti-mouse Ig antibody.

B. Functional activity of IFN-λ1

Next the functional activity of purified MBP-IFN-λ1 was examined. Human hepatoma cell lines, HepG2 and HuH7, were treated with MBP-IFN-λ1, and the transcriptional activation of MxA was analyzed by RT-PCR (Fig. 5). As a positive control IFN-α was used, and as a negative control MBP was used. In MBP-treated HuH7 cells as well as untreated cells, the transcription of MxA was not induced. In HepG2 cells without any treatment or with MBP treatment, low level of transcription of MxA was induced. As expected, IFN-λ1, like IFN-α, induced transcription of MxA both in HuH7 and HepG2 cells. This indicates that the purified MBP-IFN-λ1 has functional activity and that induction of MxA mRNA by MBP-IFN-λ1 is not due to the contaminant of bacterial product.

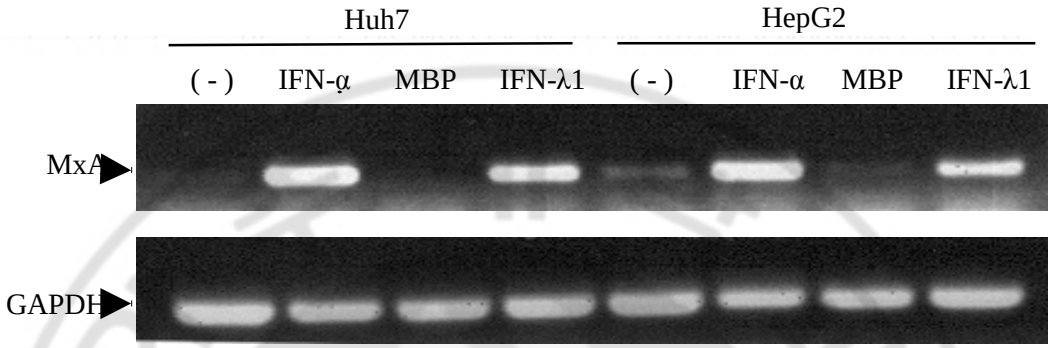


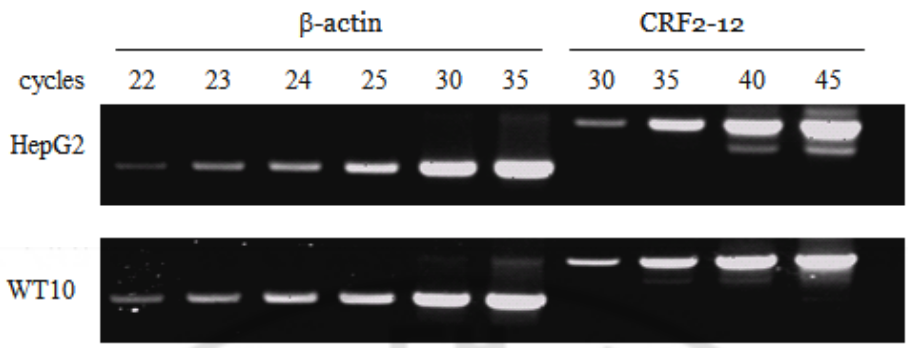
Fig. 5. The functional activity of purified MBP-IFN-λ1. HepG2 (1x 10^5) and HuH7 (5x 10^4) were seeded in 24 well plates. At 16 hr after treatment of cells with IFN-α (1,000 U/ml), MBP (1,000 ng/ml) or MBP-IFN-λ1 (3,000 ng/ml), total RNAs were isolated and RT-PCR was performed. The transcription of MxA was induced by purified MBP-IFN-λ1 in HuH7 and HepG2.

C. Expression of CRF2-12 in HBV replicating human hepatoma cell lines

IFN-λ receptor is a heterodimer of IL-10R2, ubiquitously expressed, and CRF-2-12. Expression of CRF2-12 mRNA was demonstrated in normal liver and human hepatic cancer derived cell lines (HepG2, HuH7 and HEP3B) (Sheppard et al., 2003; Vlotides et al., 2004), but not in cells supporting HBV replication.

Semiquantitative RT-PCR was performed using HBV replicating human hepatoma cell lines, WT10 (Imanaka et al., 2005) (Fig. 6). Both in HepG2 and WT10 cells, the predicted length (799 bp) of PCR product was amplified. With increased cycle numbers, smaller PCR product was appeared, which seems to be derived from alternative splicing form of CRF2-12 since the predicted length of alternative form was small by 87 bp than major band. Normalized band intensity of CRF2-12 in WT10 cells was similar to that in HepG2 cells. This result demonstrates that the mRNA level of CRF2-12 is not affected by HBV replication.

(A)



(B)

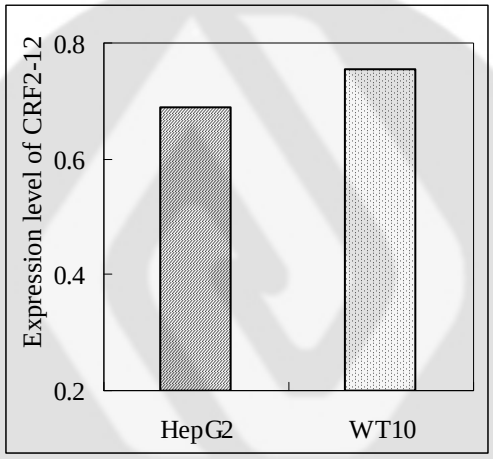


Fig. 6. The transcription level of CRF2-12 in HBV replicating cell. The transcripts of CRF2-12 were amplified by semiquantitative RT-PCR using RNAs isolated from HepG2 and WT10. (A) Amplified PCR products were analyzed by agarose gel electorphoresis. (B) The expression levels of CRF2-12 normalized to the expression levels of β-actin were comprared. It was calculated as intensity of CRF2-12 (35 cycles)/intensity of β-actin (23 cycles). Two independent experiments were done.

To examine the expression of CRF2-12 at protein level, rabbit polyclonal anti-CRF2-12 Ab was generated by immunization of extracellular domain of CRF2-12, which was expressed in *E. coli* and purified using Ni-NTA column. The purity of His-CRF2-12 used as immunogen was shown in SDS-PAGE (Fig. 7A). The reactivity of rabbit polyclonal anti-CRF2-12 Ab was determined by Western blotting using the immunogen. Like anti-histidine tag Ab, anti-CRF2-12 Ab reacted with extracellular domain of CRF2-12 with histidine tag (Fig. 7B). To know whether anti-CRF2-12 Ab may recognize CRF2-12 expressed in eukaryotic cells, fusion protein of CRF2-12 and mouse Ig was expressed in COSM6 cells and purified using nProtein A Sepharose column. The binding activity of rabbit polyclonal anti-CRF2-12 Ab to this protein was examined by Western blotting (Fig. 7C). The expected band was detected at size of 56 kDa. Anti-CRF2-12 Ab but not normal rabbit Ig reacted with CRF2-12-mIg. With this anti-CRF2-12 Ab, the CRF2-12 expression in hepatoma cell lines was explored. In all these cells CRF2-12 expression was detected at similar levels as 58 kDa size (Fig. 7D). Consistent with finding of RT-PCR, this finding indicates that HBV replication does not affect the expression of CRF2-12.

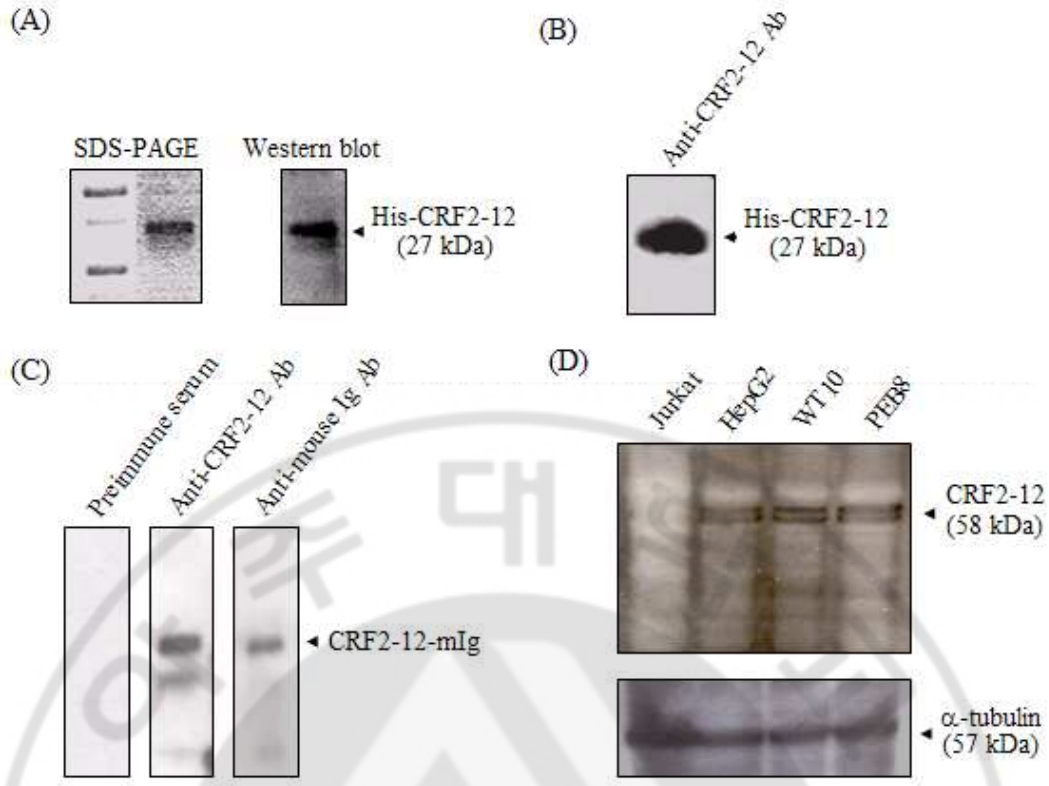
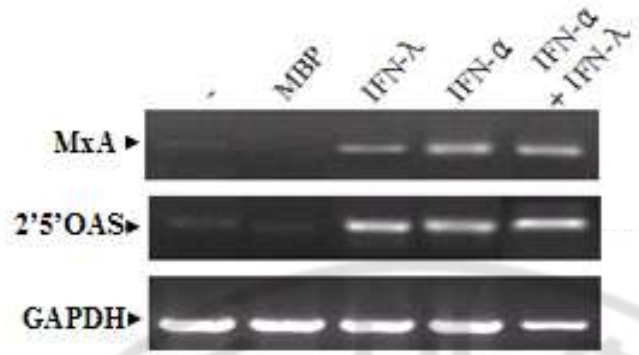


Fig. 7. The expression of CRF2-12 in WT10 and PEB8 at protein level. (A) The purified His-CRF2-12 in *E. coli* was visualized by SDS-PAGE (left) and Western blotting (right). Western blotting was performed with mouse anti-His Ab and HRP conjugated anti-mouse Ig Ab. (B) The reactivity of rabbit polyclonal anti-CRF2-12 Ab with His-CRF2-12 was examined by Western blotting using HRP conjugated anti-rabbit Ig Ab. (C) The reactivity of rabbit polyclonal anti-CRF2-12 Ab with CRF2-12 expressed in eukaryotic cells was examined by Western blotting using recombinant CRF2-12 containing mouse Ig as a fusion partner secreted from COSM6 cells. As a positive control, CRF2-12-mIg was detected by HRP-conjugated goat anti-mouse Ig Ab, and as the negative control it was reacted with preimmune rabbit serum and HRP-conjugated goat anti-rabbit Ig Ab. (D) The expression of CRF2-12 in HepG2, WT10 and PEB8 was analyzed by Western blotting using the rabbit polyclonal anti-CRF2-12 Ab. As a negative control, Jurkat T cell line was used.

D. Induction of antiviral proteins in HBV replicating human hepatoma cell lines by IFN-λ1

To know whether IFN-λ1 induces transcription of MxA and 2’5’-OAS in HBV replicating human hepatoma cell lines, RT-PCR was performed. Compared to controls (untreated and MBP-treated cells), transcriptional levels of MxA and 2’5’-OAS were increased both in WT10 and PEB8 treated with IFN-λ1 (Fig. 8). These findings indicate that HBV replication may not affect the transcriptional activation of MxA and 2’5’-OAS by IFN-λ1.

(A) WT10



(B) PEB8

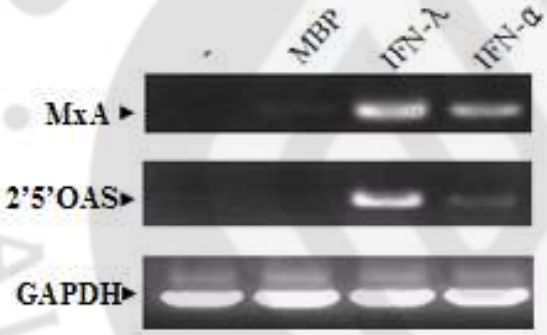
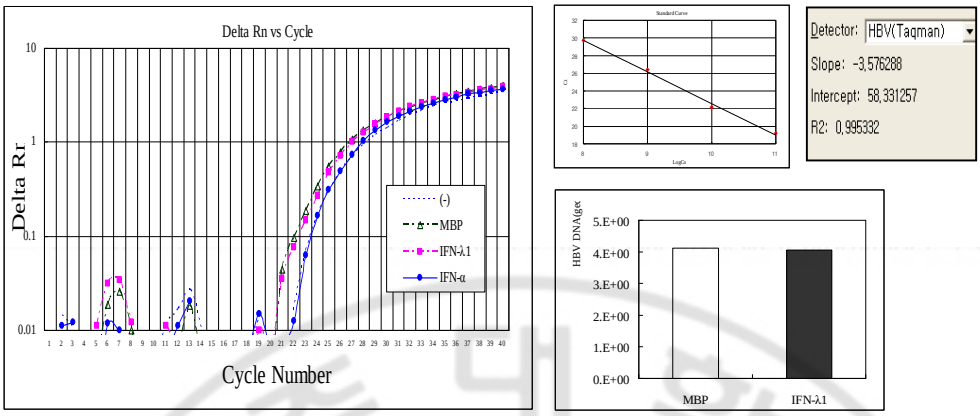


Fig. 8. The transcription of antiviral proteins by IFN-λ1 in WT10 and PEB8. WT10 (A) and PEB8 (B), respectively, were treated with MBP (1,000 ng/ml), MBP-IFN-λ1 (3,000 ng/ml), IFN-α (1,000 U/ml) or both IFN-α (1,000 U/ml) and IFN-λ1 (3,000 ng/ml) for 18 h. The induction of transcription of MxA and 2'5'-OAS was analyzed by RT-PCR. MxA: 289 bp, 2'5'-OAS: 400 bp, GAPDH: 600 bp.

E. The effect of IFN-λ1 on HBV replication

To determine the effect of IFN-λ1 on HBV replication in human hepatoma cells, real-time PCR was performed (Fig. 9). In HBV replication, HBV DNA synthesis occurred in nucleocapsid, where the HBV pregenomic RNA and reverse transcriptase complex is packaged. HBV DNA in the nucleocapsid was isolated from cells treated with IFN-λ1 for 24 h or 48 h. For the comparison, poly(I:C) or IFN-α was used. Poly(I:C) for induction of natural IFN-α was transfected into PEB8, because HepG2 does not respond to extracellular poly(I:C) (Li et al., 2005). Amplification plot showed that the level of HBV DNA in intracellular nucleocapsids isolated from WT10 treated with IFN-λ1 was almost same as that seen in WT10 treated with MBP. Even 3 times higher concentration of IFN-λ1 did not influence the level of HBV DNA in WT10 (data not shown). However, the level of HBV DNA in intracellular nucleocapsids isolated from PEB8 treated with IFN-λ1 was reduced to 73% of that seen in PEB8 treated with MBP. The reduction in HBV DNA level by IFN-λ1 was comparable to that by poly(I:C). These findings indicate that IFN-λ1 is able to inhibit HBV replication in certain human cell lines albeit its inhibitory effect is not so great. To support these findings, Southern blotting was done (Fig. 10). IFN-λ1 reduced the level of HBV DNA in intracellular nucleocapsids isolated from PEB8 at 48 hr after treatment of IFN-λ1 to about 70% of the level of MBP-control. But the level of HBV DNA isolated at 24 hr after treatment of IFN-λ1 was similar to that of MBP-control. These findings clearly show the antiviral activity of IFN-λ1 against HBV.

(A) WT10



(B) PEB8

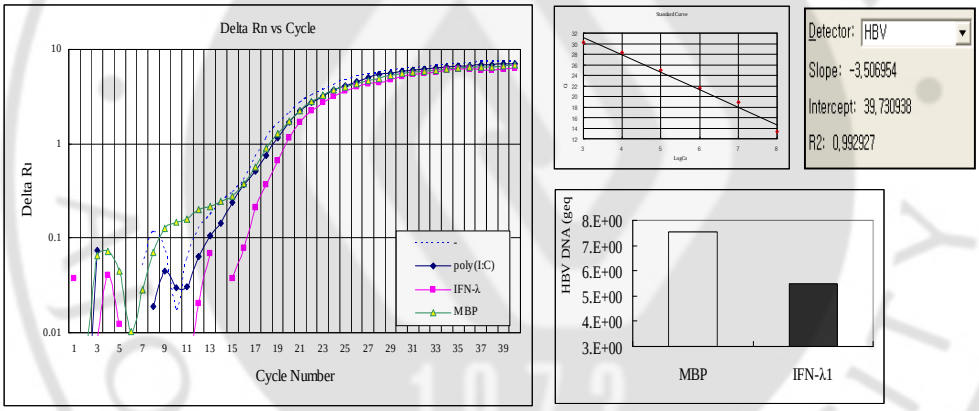


Fig. 9. Real-time PCR analysis of antiviral activity against HBV. WT10 (A) and PEB8 (B) were treated with MBP (1,000 ng/ml), MBP-IFN-λ1 (3,000 ng/ml), IFN-α (1,000 U/ml) or poly(I:C) (0.5 ug/ml) for 48 h. HBV core DNAs were prepared from cell lysates and subjected to real-time PCR with HBV Taqman probe.

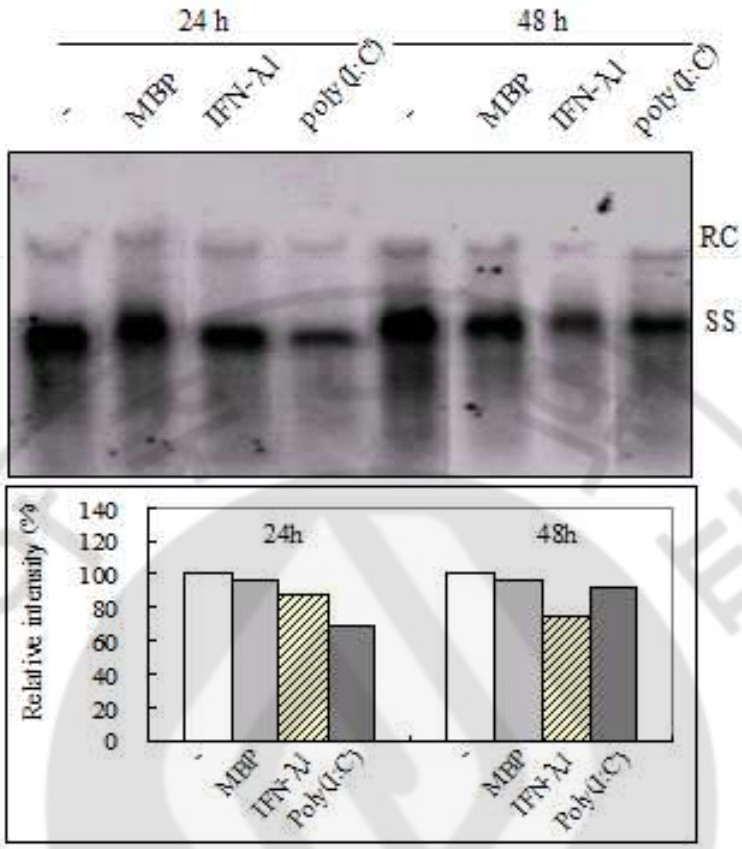


Fig. 10. Southern blot analysis of antiviral activity against HBV. PEB8 was treated with MBP (1,000 ng/ml), MBP-IFN-λ1 (3,000 ng/ml) and poly(I:C) (0.5 ug/ml). After final 24 h or 48 h, HBV core DNAs were prepared from cell lysates and subjected to Southern blotting. RC: relaxed circular DNA, SS: single stranded DNA. The figure is representative of three independent experiments.

F. The effect of IFN-λ1 on HBV transcription

To determine whether the inhibitory effect of IFN-λ1 on HBV replication occurs in RNA level, Northern blotting was performed (Fig. 11). The amount of 3.5 kb HBV transcript isolated from cells treated with IFN-λ1 was similar to controls (untreated cells or MBP-treated cells). It indicates that the inhibition of HBV replication by IFN-λ1 may not occur at HBV transcriptional level.

G. The effect of IFN-λ1 on the secretion of HBsAg and HBeAg

The effect of IFN-λ1 on the production of HBsAg and HBeAg by PEB8 was examined by ELISA (Fig. 12). Compared to control, IFN-λ1 did not have significant effect on the secretion of HBsAg and HBeAg both at 24 h and 48 h, but poly(I:C) significantly reduced the secretion of HBeAg at both times and HBsAg at 24 h, suggesting that antiviral mechanism by IFN-λ1 may be different from that by poly(I:C).

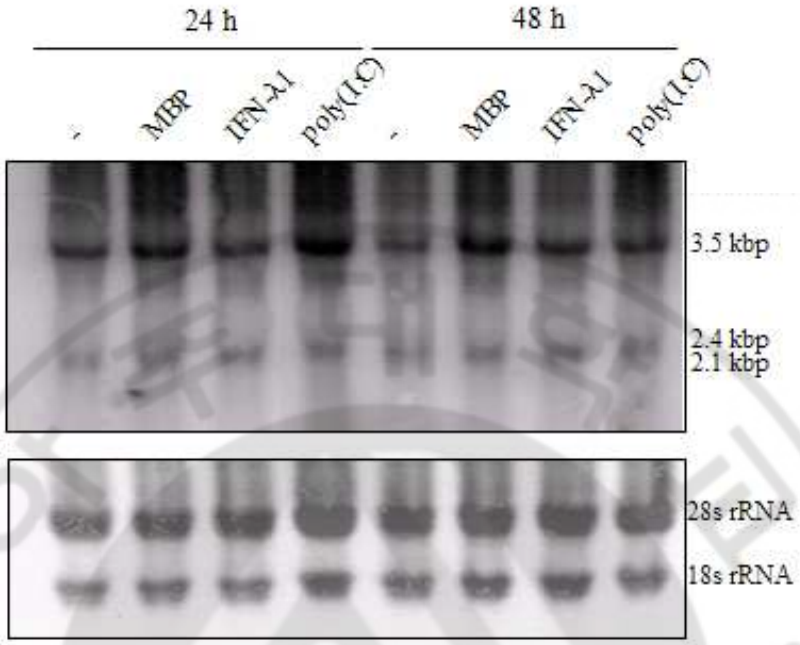
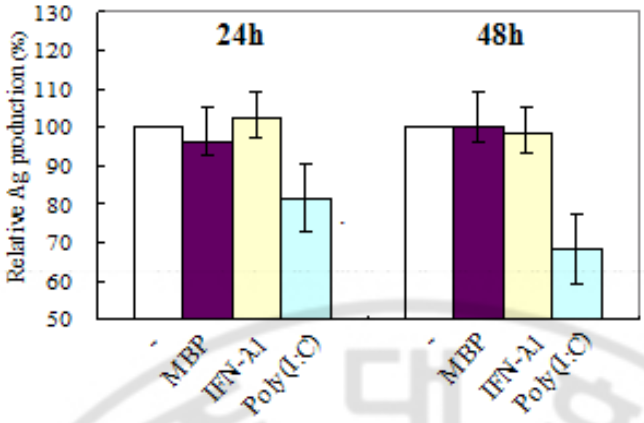


Fig. 11. The effect of IFN-λ1 on HBV transcription in PEB8. PEB8 was treated with MBP (1,000 ng/ml), MBP-IFN-λ1 (3,000 ng/ml) and poly(I:C) (0.5 ug/ml). After 24 h or 48 h, total HBV RNAs were isolated from the cells and subjected to Northern blotting. Upper panel: three transcripts of HBV; lower panel: ribosomal RNA (rRNA). The figure is representative of two independent experiments.

(A) HBsAg



(B) HBeAg

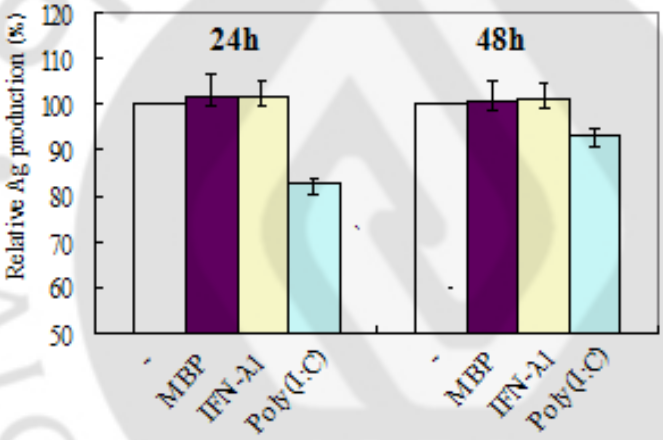


Fig. 12. The effect of IFN-λ1 on the secretion of HBsAg and HBeAg by PEB8. PEB8 was treated with MBP (1,000 ng/ml), MBP-IFN-λ1 (3,000 ng/ml) or poly(I:C) (0.5 ug/ml). After 24 h or 48 h, the production of HBsAg (A) and HBeAg (B) was analyzed by ELISA. Relative Ag production was calculated as (OD of each condition/OD of negative control) x100.

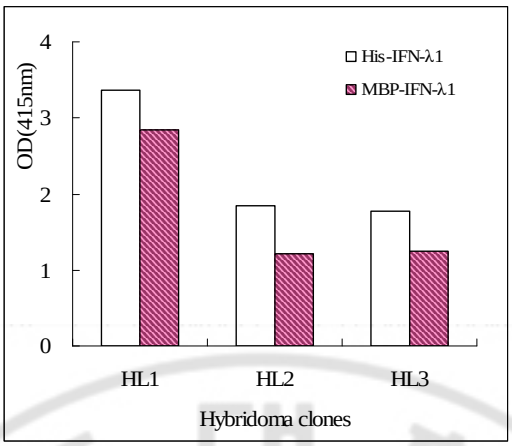
H. Generation of the polyclonal and the monoclonal Ab against IFN-λ1

Although it was reported that IFN-λ1 mRNA expression was induced or enhanced by IFN-α and other stimulators, the amount of IFN-λ1 was not quantitated at protein level (Siren et al., 2005; Osterlund et al., 2005). The reason may be because polyclonal or monoclonal anti-IFN-λ1 Ab was not commercially available at that time. In order to establish a quantitation system to measure the production of IFN-λ1, the polyclonal and the monoclonal anti-IFN-λ1 Abs were generated.

First, from three immunized mice 25 hybridoma cells producing Ab against IFN-λ1 were selected and three clones of these, designated as HL1, HL2 and HL3, were established through limiting dilution. The binding activity of these monoclonal Abs to IFN-λ1 was revealed by ELISA using MBP-IFN-λ1 as well as His-IFN-λ1 (Fig. 13A) and by Western blotting using His-IFN-λ1 (Fig. 13B). The isotype of each monoclonal Ab was determined; for HL1, G1 and for HL2 and HL3, G2b.

Next, the reactivity of polyclonal rabbit anti-IFN-λ1 Abs to native and denatured form of IFN-λ1 was shown in Westren blotting and ELISA (Fig. 13B and Fig. 14A).

(A)



(B)

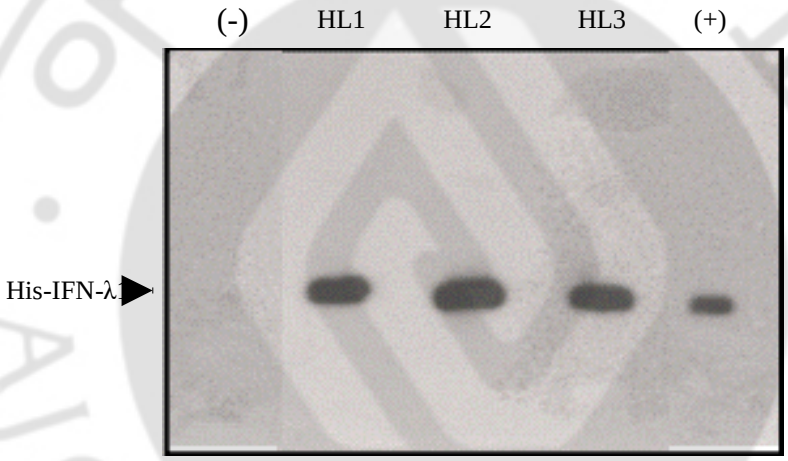
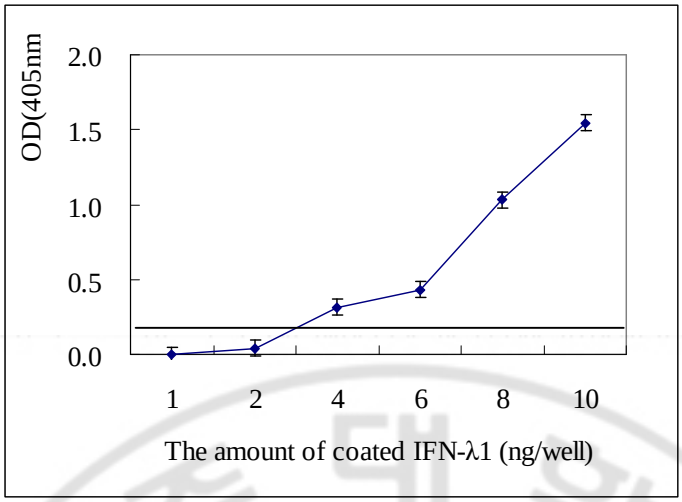


Fig. 13. The reactivity of monoclonal Abs against IFN-λ1. (A) The reactivity of supernatants of monoclonal Abs to IFN-λ1 was examined by ELISA using recombinant MBP-IFN-λ1 (1 ug/well) and His-IFN-λ1 (1 ug/well). (B) The reactivity of supernatants of monoclonal Abs to denatured IFN-λ1 was examined by Western blotting using His-IFN-λ1 (500 ng/lane) and HRP conjugated anti-mouse Ig Ab. (-): myeloma cell culture supernatant. (+): rabbit polyclonal anti-IFN-λ1 Ab.

I. Establishment of ELISA for IFN-λ1

Indirect ELISA for IFN-λ1 was established using polyclonal anti-IFN-λ1 Ab (Fig. 14A) and HL2 monoclonal anti-IFN-λ1 Ab (Fig. 14B). The detection limits were 40 ng/ml for both polyclonal and monoclonal anti-IFN-λ1 Abs. Sandwich ELISA using both polyclonal and monoclonal Abs was performed but the detection limit was not better than indirect ELISA (data not shown).

(A) Polyclonal anti-IFN-λ1 Ab



(B) HL2 monoclonal anti-IFN-λ1 Ab

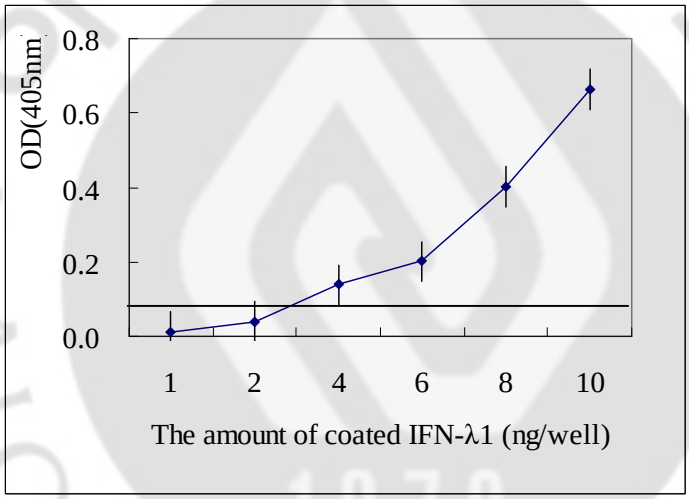


Fig. 14. The sensitivity of ELISA for IFN-λ1 using polyclonal and monoclonal anti-IFN-λ1 Abs. Serially diluted IFN-λ1 was detected by ELISA (A) using polyclonal rabbit anti-IFN-λ1 Ab (1:1,000) or (B) using HL1 monoclonal anti-IFN-λ1 Ab (10 ug/ml). IFN-λ1 expressed in eukaryotic cells was purchased from R & D systems Inc. Values represent the mean ± S.D. of quadriplicates.

IV. DISCUSSION

In this study, it was demonstrated that IFN-λ1 can inhibit HBV replication in PEB8 but not in WT10. Additionally, it was shown that HBV does not modulate the expression of CRF2-12, an IFN-λ1 receptor subunit. Also, ELISA for analyzing of production of IFN-λ1 was established.

Although antiviral activity of IFN-λ1 against several RNA viruses and cytomegalovirus has been reported (Robek et al., 2005), the effect of IFN-λ1 on HBV replication in human cells has not. A recent report showed that HBV replication in a murine hepatocyte cell line was inhibited by IFN-λ treatment (Robek et al., 2005). They treated murine hepatocyte cells with 1 to 10 ng/ml of murine IFN-λ2 expressed in *E.coli* (PEPROTCH, Rocky Hill, NJ, USA), analyzed HBV replication by Southern blotting at 24 h after the addition of IFN-λ2 and showed about 90% reduction of HBV DNA replication intermediates. My results using human hepatoma cell lines revealed that 1,000 ng/ml of IFN-λ1 (equivalent of 3,000 ng/ml of MBP-IFN-λ1) reduced the level of viral DNA by 30% (corresponding to 100 fold reduction of viral progeny) in PEB8 cells but not in WT10 cells. Similar results were obtained in cells treated with 3,000 ng/ml of IFN-λ1 (data not shown). In this study recombinant MBP-IFN-λ1 was used because when I started these experiments IFN-λ1 was commercially unavailable. Then why did IFN-λ1 suppress HBV replication in PEB8, but not in WT10? Both cell lines originated from HepG2 cells. In both cell lines, comparable level of expression of IFN-λRs and IFN-λ-mediated antiviral gene

induction were observed. The known difference is that PEB8 was established by transfection of plasmid pcDNA containing a 1.05 HBV genome-length DNA of *adw*R9 subtype, while WT10 was made using retrovirus containing a 1.1 HBV genome length DNA of *adr* subtype (Fu L and Cheng YC, 2000). HBV *adw*R9 subtype belongs to genotype A, B and G, and HBV *adr* subtype belongs to genotype C. HBV genotype was reported to be an important predictor of response to treatment of IFN-α; better responses in A and B genotypes than those in C genotype was observed (Wai et al., 2002; Janssen et al., 2005; Thuy et al., 2005). Thus my results raise a question whether the antiviral activity of IFN-λ may vary in HBV genotypes.

Neither HBV transcription nor secretion of viral Ags was suppressed by the treatment of PEB8 with IFN-λ1. Similar to IFN-λl, type I IFN does not affect either viral transcription or production of secretory Ag. IFN-α-mediated suppression of HBV replication in HBV transgenic mouse occurs by reducing the intracellular content of HBV RNA-containing capsids without altering either HBV gene expression, translation, capsid maturation or virus secretion (Wieland et al., 2000). IFN-β treatment of immortalized HBV transgenic hepatocytes results in rapid clearance of HBV RNA-containing capsids (Pasquetto et al., 2002). In an inducible HBV replication system, type I IFN suppresses HBV replication by preventing the formation of replication-competent core particles not by destabilization of pgRNA-containing capsids (Robek et al., 2005). To dissect the stage of HBV replication process affected by IFN-λl, further study is needed

IFN-λ l up-regulated the transcripts of MxA and 2’5’-OAS in HBV replicating

cell lines. The involvement of MxA in inhibitory activity of IFN-α on HBV replication has been reported (Gordien et al., 2001). In MxA transgenic mouse model, HBV replication was downregulated. By contraries in MxA deficient cells HBV replication was still inhibited by IFN-α (Rang A et al., 2002). Since MxA was induced by IFN-λl in WT10 which was nonresponsive to IFN-λl, it may be reasonable that MxA is not a major molecule for the suppression of HBV replication or at least MxA is not working in WT10 cellular context. Identification of antiviral proteins responsible for the activity of IFN-λl against HBV remains to be solved.

The expression level of an IFN-λ1 receptor subunit, CRF2-12 was not different in hepatoma cell lines regardless of HBV replication. It indicates that HBV does not modulate the expression of IFN-λR in order to resist to IFN-λ. As an escaping mechanism from the antiviral activity of IFN-α, downregulation of MxA promoter by HBV through direct interaction of it with precore/core proteins was reported (Fernandez et al., 2003). Also SOCS-1 expression in livers of patients with chronic HCV infection seems to be involved in resistance to IFN-α or IFN-λ (Brand et al., 2005; Imanaka et al., 2005). Based on the failure of IFN-λ in suppressing HBV replication in WT10 cells, it is conceivable that there may be an escaping mechanism against IFN-λ adopted by HBV.

Murine monoclonal Abs and rabbit polyclonal Ab able to react with native form as well as denatured form of IFN-λ1 was produced. Indirect ELISA using these Abs has the detection limit of 40 ng/ml. Although the sensitivity of ELISA need to be improved, analysis of IFN-λ1 production at protein level is now possible.

For many years, administration of IFN-α has been the mainstay of therapy for chronic HBV. In about 30 percent of patients who tolerated this regimen, successful responses have been reported. However, the side effects of therapy with IFN-α (fever, myalgias, thrombocytopenia, and depression) make it untolerable for many patients. Moreover, in some patients autoimmunity occurs during administration of IFN-α. This phenomenon may reflect the immunomodulatory activity of IFN-α, such as upregulation of MHC class I antigens, inhibition of apoptosis of cytotoxic T lymphocytes and direction of helper T cell differentiation to T_H1 (Ganem and Prince, 2004). Although IFN-λ receptor is broadly expressed on non-hematopoietic tissues, it does not seem to be present on leukocytes. This may provide a possible therapeutic advantage for IFN-λs as a therapeutic agent with less adverse effects (Brand et al., 2005).

This study suggests that IFN-λl may be useful to repress HBV replication in selected chronic hepatitis B patients. I raised the possibility that HBV genotype may be a determinant for the responsiveness of IFN-λl treatment. Further studies are required to test this possibility and to identify other determinants leading to suppression of HBV replication by IFN-λl.

V. CONCLUSION

IFN-λ family has antiviral activity against some viruses, but its effect against HBV in human hepatocytes, natural host cells of HBV, has not been known. I examined whether IFN-λ1 has the suppressive effect on HBV replication in human hepatoma cells. It was demonstrated that HBV replication was inhibited by IFN-λ1 in PEB8, one of two HBV-replicating human hepatoma cell lines examined. Although it is not explainable why HBV replication was not inhibited in the other cell line, in both cell lines the transcription of the antiviral proteins, MxA and 2'5'-OAS was induced by IFN-λ1. In PEB8, treatment of IFN-λ1 did not suppress transcription of HBV nor did it reduce the secretion of HBsAg and HBeAg, indicating that the posttranscriptional stage of HBV replication may be affected by IFN-λ1.

The expression of the unique receptor subunit for IFN-λ1 was analyzed in HBV-replicating human hepatoma cell lines. In both cell lines, the amount of transcripts and protein of CRF2-12 was similar to that of their parent cell line, suggesting that HBV may not modulate the expression of IFN-λ1 receptor.

Finally, I produced the polyclonal and the monoclonal Abs reactive to native form and denatured form of IFN-λ1. Using these Abs the ELISA system with sensitivity of 40 ng/ml was established, which enables further study to analyze the production of IFN-λ1 by HBV infection.

My results indicated that the suppressive effect of IFN-λ1 on HBV replication may be affected by factors of cellular origin and/or viral origin. IFN-λ1 can be useful

in the treatment of HBV infection.

REFERENCES

1. Brand S, Zitzmann K, Dambacher J, Beigel F, Olszak T, Vlotides G, Eichhorst ST, Goke B, Diepolder H and Auernhammer CJ: SOCS-1 inhibits expression of the antiviral proteins 2',5'-OAS and MxA induced by the novel interferon-lambdas IL-28A and IL-29. *Biochem Biophys Res Commun* 331, 543-548, 2005

2. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diebold J, Diepolder H, Adler B, Auernhammer CJ, Goke B, Dambacher J: IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *Am J Physiol Gastrointest Liver Physiol* 289, G960-G968, 2005

3. Cheng J, Imanishi H, Morisaki H, Liu W, Nakamura H, Morisaki T, Hada T: Recombinant HBsAg inhibits LPS-induced COX-2 expression and IL-18 production by interfering with the NFkB pathway in a human monocytic cell line, THP-1.

4. Chen RW, Piiparinen H, Seppanen M, Koskela P, Sarna S and Lappalainen M: Real-time PCR for detection and quantitation of hepatitis B virus DNA. *J Med Virol* 65, 250-256, 2001

5. Colamonici O, Yan H, Domanski P, Handa R, Smalley D, Mullersman J, Witte M, Krishnan K and Krolewski J: Direct binding to and tyrosine

phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase. *Mol Cell Biol* 14, 8133-8142, 1994

6. Dumoutier L, Lejeune D, Hor S, Fickenscher H and Renauld JC: Cloning of a new type II cytokine receptor activating signal transducer and activator of transcription (STAT)1, STAT2 and STAT3. *Biochem J* 370, 391-396, 2003

7. Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV and Renauld JC: Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. *J Biol Chem* 279, 32269-32274, 2004

8. Fernandez M, Quiroga JA and Carreno V: Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins. *J Gen Virol* 84, 2073-2082, 2003

9. Fu L and Cheng YC: Characterization of novel human hepatoma cell lines with stable hepatitis B virus secretion for evaluating new compounds against lamivudine- and penciclovir-resistant virus. *Antimicrob Agents Chemother* 44, 3402-3407, 2000

10. Ganem D and Prince AM: Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 350, 1118-1129, 2004

11. Goodbourn S, Didcock L and Randall RE: Interferons: cell signalling, immune

modulation, antiviral response and virus countermeasures. *J Gen Virol* 81, 2341-2364, 2000

12. Gordien E, Rosmorduc O, Peltekian C, Garreau F, Brechot C and Kremsdorf D: Inhibition of hepatitis B virus replication by the interferon-inducible MxA protein. *J Virol* 75, 2684-2691, 2001

13. Haller O and Kochs G: Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. *Traffic* 3, 710-717, 2002

14. Huovila AP, A.M. Eder, and S.D.Fuller: Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. *J. Cell. Biol* 118, 1305-1320, 1992

15. Imanaka K, Tamura S, Fukui K, Ito N, Kiso S, Imai Y, Naka T, Kishimoto T, Kawata S and Shinomura Y: Enhanced expression of suppressor of cytokine signalling-1 in the liver of chronic hepatitis C: possible involvement in resistance to interferon therapy. *J Viral Hepat* 12, 130-138, 2005

16. Iordanov MS, Paranjape JM, Zhou A, Wong J, Williams BR, Meurs EF, Silverman RH and Magun BE: Activation of p38 mitogen-activated protein kinase and c-Jun NH(2)-terminal kinase by double-stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. *Mol Cell Biol* 20, 617-627, 2000

17. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y,

Simon C, So TM, Gerken G, de Man R A, Niesters HG, Zondervan P, Hansen B, Schalm SW: Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 365, 123-9, 2005

18. Karayiannis P: Hepatitis B virus: old, new and future approaches to antiviral treatment. *J Antimicrob Chemother* 51, 761-785, 2003

19. Kotenko SV: The family of IL-10-related cytokines and their receptors: related, but to what extent? *Cytokine Growth Factor Rev* 13, 223-240, 2002

20. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H and Donnelly RP: IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4, 69-77, 2003

21. Li K, Chen Z, Kato N, Gale M, Jr. and Lemon SM: Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* 280, 16739-16747, 2005

22. Mark EM and John LG: Emerging therapeutics for chronic hepatitis B. *Annu Rev Med* 57, 24.1-24.12, 2006

23. Novick D, Cohen B and Rubinstein M: The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell* 77, 391-400, 1994

24. Osterlund P, Veckman V, Siren J, Klucher KM, Hiscott J, Matikainen S and Julkunen I: Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *J Virol* 79, 9608-9617, 2005

25. Pasquetto V, Wieland SF, Uprichard SL, Tripodi M, Chisari FV: Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. *J Virol* 76, 5646-53, 2002

26. Pestka S, Krause CD, Walter MR: Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202, 8-32, 2004

27. Qureshi SA, Leung S, Kerr IM, Stark GR and Darnell JE, Jr.: Function of Stat2 protein in transcriptional activation by alpha interferon. *Mol Cell Biol* 16, 288-293, 1996

28. Rabe B, Vlachou A, Pante N, Helenius A and Kann M: Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc Natl Acad Sci U S A* 100, 9849-9854, 2003

29. Rang A, Bruns M, Heise T, Will H: Antiviral activity of interferon-alpha against hepatitis B virus can be studied in non-hepatic cells and Is independent of MxA. J Biol Chem 277, 7645-7, 2002

30. Robek MD, Boyd BS, Wieland SF, Chisari FV: Signal transduction pathways

that inhibit hepatitis B virus replication. *Proc Natl Acad Sci U S A* 101, 1743-7, 2004

31. Robek MD, Boyd BS and Chisari FV: Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 79, 3851-3854, 2005

32. Roers A, Hochkeppel HK, Horisberger MA, Hovanessian A and Haller O: MxA gene expression after live virus vaccination: a sensitive marker for endogenous type I interferon. *J Infect Dis* 169, 807-813, 1994

33. Schwemmle M, Richter MF, Herrmann C, Nassar N and Staeheli P: Unexpected structural requirements for GTPase activity of the interferon-induced MxA protein. *J Biol Chem* 270, 13518-13523, 1995

34. Schwemmle M, Weining KC, Richter MF, Schumacher B and Staeheli P: Vesicular stomatitis virus transcription inhibited by purified MxA protein. *Virology* 206, 545-554, 1995

35. Seeger C and Hu J: Why are hepadnaviruses DNA and not RNA viruses? *Trends Microbiol* 5, 447-450, 1997

36. Seeger C and Mason WS: Hepatitis B virus biology. *Microbiol Mol Biol Rev* 64, 51-68, 2000

37. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin

J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D and Klucher KM: IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 4, 63-68, 2003

38. Shuai K, Stark GR, Kerr IM and Darnell JE, Jr.: A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* 261, 1744-1746, 1993

39. Siren J, Pirhonen J, Julkunen I and Matikainen S: IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. *J Immunol* 174, 1932-1937, 2005

40. Thuy le TT, Ryo H, Van Phung L, Furitsu K, Nomura T: Distribution of genotype/subtype and mutational spectra of the surface gene of hepatitis B virus circulating in Hanoi, Vietnam. *J Med Virol* 76, 161-9, 2005

41. von Wussow P, Jakschies D, Hochkeppel HK, Fibich C, Penner L and Deicher H: The human intracellular Mx-homologous protein is specifically induced by type I interferons. *Eur J Immunol* 20, 2015-2019, 1990

42. Wai CT, Chu CJ, Hussain M, Lok AS: HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 36, 1425-30, 2002

43. Wieland SF, Guidotti LG and Chisari FV: Intrahepatic induction of alpha/beta

interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. *J Virol* 74, 4165-4173, 2000

44. Zhang Z, Protzer U, Hu Z, Jacob J and Liang TJ: Inhibition of cellular proteasome activities enhances hepadnavirus replication in an HBX-dependent manner. *J Virol* 78, 4566-4572, 2004

-국문요약-

B형 간염 바이러스의 감염과 관련된 인터페론 람다 시스템의 역할과 조절

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연구배경 및 목적: B형 간염 바이러스 (HBV)에 의해 진행되는 만성 감염은 간경변과 간암을 유발할 수 있으므로 효과적인 치료법이 요구된다. 현재 사용되고 있는 인터페론 알파 (IFN-α)와 라미부딘 (lamivudine) 등은 치료효율과 부작용 등에서 개선이 요구된다. 인터페론 람다 (IFN-λ)는 CRF2-12와 IL-10R2로 이루어진 고유의 수용체에 결합하며 몇몇 바이러스의 증식을 억제하는 것으로 최근 보고되어 HBV 치료에 이용될 가능성을 보인다. 본 연구에서는 IFN-λ가 HBV의 증식을 억제하는가를 알아보고, CRF2-12 발현이 HBV 증식에 의해 조절되는 지에 대해 알아보고자 하였다. 또한 HBV에 의해 IFN-λ의 생산이 조절되는가를 알아보기 위해 IFN-λ에 대한 ELISA를 확립하고자 하였다.

연구 방법: 두 종류의 IFN-λ1을 세균에서 발현시켰다: 항바이러스 활성 조사를 위해 MBP-IFN-λ1와 IFN-λ1에 대한 항체 생산을 위해 His-IFN-λ1. HBV가 증식하는 사람 간암 세포주로는 WT10과 PEB8을

사용하였다. HBV 증식에 미치는 IFN-λ1의 영향을 알아보기 위해 IFN-λ1을 두 세포주에 처리후 real-time PCR과 Southern blotting으로 분석하였다. PEB8 세포주에서 HBV의 전사 수준에 미치는 IFN-λ1의 영향을 알아보기 위해 Northern blotting으로 조사하였고, 분비되는 바이러스 항원의 양에 미치는 IFN-λ1의 영향을 ELISA로 조사하였다. 항바이러스 단백질인 MxA와 2’5’-OAS의 전사가 IFN-λ1에 의해 유도하는 지를 조사하기 위해서 RT-PCR을 실시하였다. CRF2-12의 발현은 RT-PCR과 Western blotting으로 조사하였다. 면역한 마우스에서 분리한 비장세포를 사용하여 단클론 항체를 만들었고, 이 항체들을 통해 ELISA의 민감도를 측정하였다.

연구 결과: IFN-λ1은 PEB8 세포에서의 HBV 증식만을 억제하고 WT10 세포의 HBV 증식은 억제하지 못하였다. 하지만, 두 세포주 모두에서 CRF2-12의 발현 양과 IFN-λ1에 의해 유도되는 MxA와 2’5’-OAS의 전사체 양은 유사하였다. IFN-λ1은 PEB8 세포에서 HBV 전사체와 분비되는 항원에는 영향을 미치지 못하였다. 본 연구에서 만들어진 IFN-λ1에 대한 단클론 항체와 다클론 항체의 ELISA 민감도는 40 ng/ml이었다.

연구 결론: IFN-λ1이 HBV에 미치는 항바이러스 활성이 두 가지 사람 간암 세포주 중 한 가지 세포주에서만 영향을 미쳤다. 이는 IFN-λ1이 HBV에 미치는 영향이 세포내 인자, 또는 바이러스 인자에 의해 이루어질 수 있음을 제시하는 것이다. 또한 CRF2-12의 발현이 HBV의 복제에 의해 조절되지 않음을 알 수 있었다. 끝으로 감염이나 자극에 의해 생산되는 IFN-λ1의 양을 측정하는 방법으로 IFN-λ1에 대한 ELISA가 확립되었다.

핵심어: B형 간염 바이러스, 인터페론 람다 단백질, 바이러스 증식