

addition, HBx affects various cellular signal transduction pathways [9–13]. Furthermore, the biologic activities of HBx may be induced by its direct binding to cellular target proteins [14–21].

Another HBV protein, preS/S protein, may also act as a transcriptional transactivator. Both pre-S1 [22] and pre-S2/S [23] proteins activate transcription of a particular gene, suggesting that these proteins, as well as HBx, substantially contribute to modifications of the host cellular function. Therefore, it is important to determine the phenotypic changes in the host cell due to expression of the full-length HBV genome.

Recent advances in DNA array technology make it possible to simultaneously examine the expression levels of hundreds to thousands of genes. In the present study, we compared the gene expression profile of cells persistently expressing full-length HBV (HB611 cells) [24] with that of negative control cells using a DNA array assay, and comprehensively investigated the alterations in the gene expression of host cells in response to the expression of complete HBV proteins.

## Materials and Methods

### Cell Culture

HB611 cells were established from a human hepatoblastoma cell line, Huh-6, by transfection with the plasmid 3HBneo carrying a 3-tandem repeat of the full length of HBV adr4 strain [24]. HB611 cells are capable of transcribing the pregenome RNA and other viral mRNAs from the integrated HBV DNA through regulation by their own promoter/enhancer, followed by constitutive production of viral proteins and the release of Dane-like particles into the culture medium [24]. Huh-6 neo cells were generated by transfection with only the neomycin-resistant gene and used as a negative control. Both HB611 and Huh-6 neo cells were kindly provided by Prof. K. Matsuura (Institute for Molecular and Cellular Biology, Osaka University Graduate School of Medicine, Osaka, Japan). These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B and 200 µg/ml G418 sulfate (Gibco BRL/Life Technologies, Inc., Tokyo, Japan) at 37 ° under an atmosphere of 5% CO<sub>2</sub>-95% air.

### DNA Array Analysis

In the present study, a commercially available DNA array system (Atlas Human Array 1.2, Clontech Laboratories, Inc., Palo Alto, Calif., USA) was used for the analysis. This is a broad-coverage DNA array system that is capable of simultaneously analyzing the 1,176 genes examined in this study. The DNA array procedures were conducted according to the manufacturer's instructions. Briefly, total RNA was extracted from both HB611 and Huh-6 neo cells in a confluent state with TRIZOL reagent (Gibco BRL/Life Technologies), and the mRNA was selected using an oligo-dT column (Roche Diagnostic Co. Ltd., Tokyo, Japan). The mRNA sample was treated with

RNase-free DNase I (Promega Co., Madison, Wisc., USA), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitation with ethanol. The resulting mRNA sample (1 µg) was used for the DNA array analysis. After cDNA synthesis, parallel hybridization with cDNA samples derived from HB611 and Huh-6 neo cells was performed using two identical membranes loaded with the gene probes. Finally, the arrays were exposed for 24 h using the bioimaging analyzer BAS-2500 (Fuji Photo Film Co. Ltd., Tokyo, Japan). Quantitation of the signal intensity of each gene was performed using Atlas Image software (Clontech Laboratories).

### Reverse Transcription-Polymerase Chain Reaction Analysis

For the reverse transcription (RT)-polymerase chain reaction (PCR) assay, total RNA was extracted from both the HB611 and Huh-6 neo cells with TRIZOL reagent (Gibco BRL/Life Technologies), as described above. After RNase-free DNase I (Promega) treatment, cDNA was synthesized using mutated Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, Toyobo, Co. Ltd., Osaka, Japan) and oligo (dT)<sub>20</sub> primer (Toyobo). Table 1 shows the primers for the PCR analysis used in this study. An aliquot of the cDNA product was subjected to PCR reaction (94 ° for 30 s, 55 ° for 1 min and 72 ° for 2 min), followed by a final extension at 72 ° for 10 min. PCR reaction for 34, 31, 28 and 25 cycles was performed in each experiment, and the appropriate cycles for the comparison of the gene expression level between HB611 and Huh-6 neo cells were determined for each gene. As an internal control, β-actin mRNA was also examined. The PCR product was separated using agarose gel electrophoresis and visualized with ethidium bromide staining under an ultraviolet lamp.

### Western Blot Analysis

For the Western blot analysis, cells in a confluent state were lysed and separated using SDS-PAGE. After transfer onto a nitrocellulose membrane (Hybond-P, Amersham Pharmacia Biotech Co. Ltd., Buckinghamshire, UK), the membrane was blocked with 5% milk. The membrane was then incubated with the primary antibody, followed by further incubation with immunoglobulin coupled with horseradish peroxidase as a secondary antibody. Finally, the proteins were detected by chemiluminescence (Supersignal, Pierce Chemical, Rockford, Ill., USA). The following antibodies were used in this study: anti-27-kD heat shock protein (HSP-27; Upstate Biotechnology, Lake Placid, N.Y., USA), anti-NM23-H1 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), anti-caspase-3 (MBL Co. Ltd., Nagoya, Japan) and anti-BAX (Santa Cruz Biotechnology).

## Results

### Results of the DNA Array Analysis

To investigate the changes in the gene expression profile caused by transfection of the full-length HBV DNA, DNA array analysis was performed using mRNA samples derived from HB611 and Huh-6 neo cells. Genes with a HB611 to Huh-6 neo signal intensity ratio of 3 or greater were regarded as upregulated, whereas genes with an HB611 to Huh-6 neo signal intensity ratio of 0.33 or less were regarded as downregulated. Figure 1 shows the com-

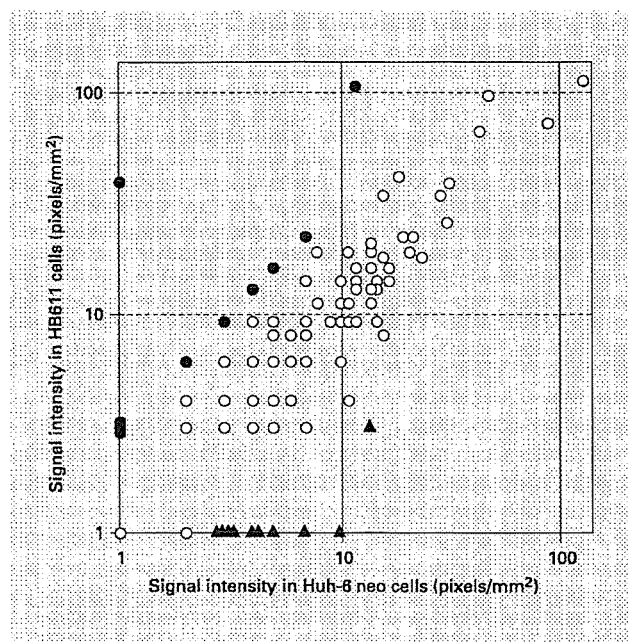
**Table 1.** Sequences of primers for PCR amplification used in this study

Genes		Primer sequence (5' to 3')
CD44	sense	CATCTACCCCAGCAACCCTA
	antisense	CTTCTGCCACACCTTCTTC
HMG-I	sense	AGTGAGTCGAGCTCGAAGTC
	antisense	GTCTCTTAGGTGTTGGCACT
TB-10	sense	GGAAATCGCCAGCTTCGATA
	antisense	AATCCCTCCAGGATCTTAGG
$\alpha_1$ -AT	sense	GGGTCAACTGGGCATCACTA
	antisense	CCATGAAGAGGGGAGACTTG
$\alpha_1$ -AG	sense	AGAGTACCAGACCCGACAGG
	antisense	CTCTCCTTCTCGTGCTGCTT
IGFBP-1	sense	GAGAGCACGGAGATAACTGAGG
	antisense	AACCACTGTACCTCTCGGAAGC
BTEB2	sense	ACTTACTTTCCCCCGTCACC
	antisense	CAGCCTTCCCAGGTACACTT
TOPO-II $\alpha$	sense	TGTCACCATTGCAGCCTGTA
	antisense	GTCGAGAAGGGTATAATAGG
HIF-1 $\alpha$	sense	TGTAATGCTCCCCTCACCCAACGAA
	antisense	GTGACCCTGATAATCCGAGTCCACT
ROR1	sense	CCTCATGACAGAGTGCTGGA
	antisense	GAGGACCTGTTGGCTGGTAG
$\beta$ -Actin	sense	ACACTGTGCCCATCTACGAGG
	antisense	AGGGGCCGGACTCGTCATACT

parison of the gene expression levels between HB611 and Huh-6 neo cells. The expression of 10 genes was significantly enhanced, whereas the expression of 10 genes was reduced due to persistent expression of HBV. Thus, 20 of the 1,176 genes (1.7%) examined in this study were initially judged to be altered by HBV expression in the DNA array analysis.

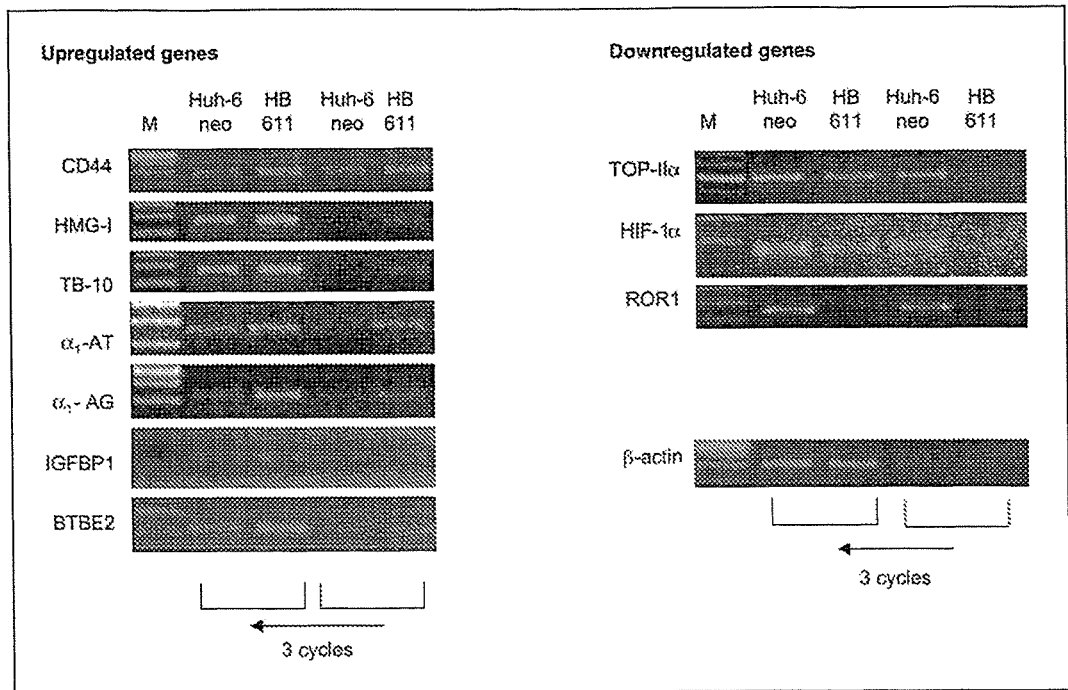
#### Results of Corroboration Assays by RT-PCR and Western Blot

For the 20 genes initially regarded to be HBV responsive in the DNA array analysis, RT-PCR analysis (for 14 genes) or Western blot analysis (for 6 genes) was further performed for corroboration. The RT-PCR results are shown in figure 2. In the RT-PCR assay, 7 genes, i.e. CD44, high mobility group protein-I (HMG-I), thymosin beta-10 (TB-10), alpha-1-antitrypsin ( $\alpha_1$ -AT), alpha-1-acid glycoprotein 1 ( $\alpha_1$ -AG), insulin-like growth factor-binding protein 1 (IGFBP-1) and basic transcription ele-

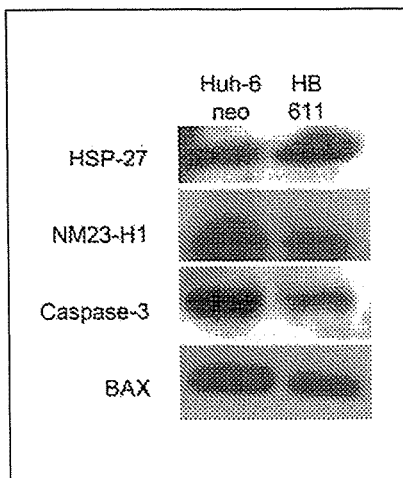


**Fig. 1.** Comparison of gene expression levels between Huh-6 neo and HBV-expressing HB611 cells in DNA array analysis. The signal intensities of each gene were quantitated with Atlas Image software (Clontech Laboratories). ● = Genes upregulated by HBV expression, with a HB611 to Huh-6 neo signal intensity ratio  $\geq 3$ ; ▲ = genes downregulated by HBV expression, with a HB611 to Huh-6 neo signal intensity ratio  $\leq 0.33$ ; ○ = genes whose expression levels were not altered by HBV expression.

ment-binding protein 2 (BTEB2), were upregulated, whereas 3 genes, i.e. DNA topoisomerase II alpha (TOPO-II $\alpha$ ), hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) and protein-tyrosine kinase transmembrane receptor ROR1, were downregulated due to expression of HBV proteins. In addition, Western blot analysis revealed that HBV expression induced 1 upregulated gene, HSP-27, and 3 downregulated genes, NM23-H1, caspase-3 and BAX (fig. 3). Expression of the remaining 6 genes was not different between HB611 and Huh-6 cells by RT-PCR assay (4 genes) or by Western blot (2 genes), and these genes were regarded as 'false positives' of the DNA array analysis. The specificity of our DNA array analysis was 70% (14 of 20 genes). The high specificity indicates that the DNA array analysis used in this study was a reliable experimental method to simultaneously examine the expression levels of many genes. The 14 HBV-responsive genes identified in this study are summarized in table 2.



**Fig. 2.** RT-PCR assay for the detection of HBV-responsive genes. Total RNA samples were extracted from Huh-6 neo and HB611 cells and used for RT-PCR assay. The left panel represents upregulated genes (8 genes), whereas the right panel represents downregulated genes (6 genes). The right bottom panel shows the  $\beta$ -actin mRNA as an internal control. M = 100-bp ladder.



**Fig. 3.** Western blot analysis for the detection of HBV-responsive genes. Total cellular lysates were extracted from Huh-6 neo and HB611 cells and subjected to Western blot analysis to examine HSP-27, NM23-H1, caspase-3 and BAX expression levels.

## Discussion

In the present study, we identified 14 genes whose expression levels were altered by expression of the full-length HBV DNA through the screening of 1,176 genes using the DNA array method. Among these HBV-responsive genes, there were 6 genes, CD44, NM23-H1, BAX, HMG-I, TB-10 and HSP-27, which have been suggested to be closely associated with the development or progression of HCC or other kinds of carcinomas. CD44 is a cell surface glycoprotein that possesses functions in cell-cell and cell-matrix adhesions. High levels of CD44 expression are related to the invasive and metastatic potential of HCC [25, 26]. HBx induces metastatic potential by modifying CD44-dependent migratory behavior, as determined using HBx-overexpressing cultured cells [27]. NM23-H1, a nucleotide diphosphate kinase, is an antimetastatic molecule [28]. In HCC, the reduced expression of NM23-H1 is closely correlated with the presence of intrahepatic metastasis [29] and higher recurrence rates after surgical resection [30]. HMG-I is involved in the regula-

tion of chromatin structure and function, and regulates transcription activity by interacting with several transcription factors. Elevated expression of HMG-I is frequently observed in various carcinomas, such as thyroid tumors [31], colorectal carcinomas [32] and pancreatic duct cell carcinomas [33]. In addition, the forced expression of the HMG-I gene in cultured epithelial cells leads to a malignant and metastatic phenotype of the cells [34]. TB-10 is an acidic polypeptide originally isolated from the calf thymus, and belongs to the  $\beta$  class of the thymosin family. Overexpression of TB-10 is frequently detected in colon, breast, ovarian and uterine carcinomas and germ cell tumors [35]. HSP-27, a member of the heat shock protein family, is thought to have an important role in the regulation of intracellular homeostasis. HSP-27 expression might be associated with a poor clinical outcome in prostate [36] and breast cancers [37], suggesting that overexpression of HSP-27 is linked to the aggressiveness of malignant tumor cells. Transient transfection of HBx into cultured cells enhances expression of HSP-27 [38].

The levels of two acute-phase response proteins,  $\alpha_1$ -AT and  $\alpha_1$ -AG, were also increased by HBV expression in the present study. Serum  $\alpha_1$ -AT and  $\alpha_1$ -AG levels tend to be higher in patients with HCC than in patients with chronic hepatitis without HCC [39]. Furthermore, a high level of serum  $\alpha_1$ -AT might also be significantly correlated with shorter survival [40].

Thus, a series of previous studies in either a clinical setting or laboratory investigations suggest that enhanced expression of CD44, HMG-I, TB-10, HSP-27,  $\alpha_1$ -AT and  $\alpha_1$ -AG, and reduced expression of NM23-H1 might be involved in the acceleration of carcinogenesis in the liver or other organs. These findings suggest that each of these alterations in gene expression levels have an important role in the course of carcinogenesis, although the precise mechanisms through which cells would gain a malignant phenotype have not been fully clarified. It is noteworthy that such carcinogenesis-related changes in gene expression levels are caused by expression of the full-length HBV genome, suggesting that persistent expression of HBV might accelerate hepatocarcinogenesis accompanied by alterations in gene expression levels of the host liver cell.

Furthermore, HBV expression suppressed the expression levels of two important apoptosis-inducing molecules, caspase-3 and BAX. Caspase-3, a member of the cysteine protease family, activates its target molecules by proteolytic cleavage and has a crucial role in cellular apoptosis. Transfection of the HBx gene into cultured cells inhibits caspase-3 activity and results in the resistance of

**Table 2.** HBV-responsive genes determined by DNA array screening and the corroboration assays

*Genes upregulated by HBV expression (n = 8)*  
(HB611 to Huh-6 neo ratio  $\geq 3$ )

CD44 antigen (M59040)  
High mobility group protein-I (M23619)  
Thymosin beta-10 (M92381)  
27-kD heat shock protein (X54079)  
Alpha-1-antitrypsin precursor (X02920)  
Alpha-1-acid glycoprotein 1 precursor (X02544)  
Insulin-like growth factor-binding protein 1 (M31145)  
Basic transcription element-binding protein 2 (D14520)

*Genes downregulated by HBV expression (n = 6)*  
(HB611 to Huh-6 neo ratio  $\leq 1/3$ )

Metastasis inhibition factor NM23 (X17620)  
Caspase-3 (U13737)  
Apoptosis regulator BAX (L22474)  
DNA topoisomerase II alpha (J04088)  
Hypoxia-inducible factor 1 alpha (U22431)  
Protein-tyrosine kinase transmembrane receptor ROR1 (M97675)

Another 6 genes were initially judged to be HBV-responsive, but were subsequently found to be false positive by RT-PCR or Western blot analysis for corroboration. Numbers in parentheses represent the GenBank accession numbers.

cellular apoptosis under various stimuli [7]. In the present study, we demonstrated that HBV expression reduced caspase-3 transcription and expression levels. BAX is a proapoptotic member of the BCL-2 family. Downregulation of BAX is observed in HCC tissues with overexpression of the tumor suppressor p53 [41]. In light of this, the HBV-mediated suppression of caspase-3 and BAX might result in prolonged survival of the host cell and contribute to carcinogenesis in the liver.

In the present study, 5 additional HBV-responsive genes were identified. IGFBP-1 takes part in the regulation of the function of insulin-like growth factor (IGF) by binding to IGF [42]. The modulatory effect of IGFBP-1 on the mitogenic activity of IGF, however, has not been fully clarified in the liver cell. TOPO-II $\alpha$  is a nuclear enzyme that changes the topology of DNA and is essential for chromosome segregation at mitosis. High expression levels of TOPO-II $\alpha$  are reported in lung cancer [43], in contrast to the suppression of TOPO-II $\alpha$  by HBV expression observed in the present study. HIF-1 $\alpha$  is involved in the transcriptional regulation of a variety of genes related to angiogenesis [44]. HBV expression, however, sup-

presses HIF-1 $\alpha$  levels, despite the fact that angiogenesis has a key role in the progression of malignant cells. As for ROR1, an orphan cell surface receptor with strong homology to the tyrosine kinase domain of growth factor receptors [45], and BTEB2, a transcription factor involved in phenotypic changes of smooth muscle cells [46], their functions in the liver have not yet been clarified. Thus, the biologic significance of HBV-mediated alterations in these 5 genes remains unclear, especially with respect to carcinogenesis.

In conclusion, our DNA array results suggest that HBV expression induces the host cell to adopt a malignant phenotype through alterations in the expression levels of a set of carcinogenesis-related genes. These findings may serve as a first step toward the comprehensive understanding of HBV-mediated development or progression of HCC. It remains unclear, however, which parts of the HBV region are responsible for the change in the expression level of each HBV-responsive gene. Also, the detailed functions of these HBV-responsive genes in the acceleration of carcinogenesis should be clarified.

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## Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas

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**Background/Aims:** MHC class I-related chain A (MICA), a human ligand of natural killer (NK) cell stimulatory receptor NKG2D, is expressed in human hepatocellular carcinomas (HCC). Earlier research demonstrated that the soluble form of MICA (sMICA) is released from some types of tumors, but its presence and role in HCC was not determined.

**Methods:** Serum sMICA was studied in 26 patients with HCC. In vitro experiments were performed to examine the impact of sMICA on NK cell expression of NKG2D and subsequent dendritic cell (DC) activation.

**Results:** The levels of sMICA were frequently elevated in patients with advanced HCC. The elevation of sMICA was associated with down-regulated NKG2D expression and impaired activation of NK cells. In vitro experiments revealed that sMICA derived from advanced HCC was responsible for down-modulation of NKG2D expression and NK cell functions. NK cells upon stimulation of human hepatoma cells induced maturation of DC and enhanced the allostimulatory capacity of DC; maturation and activation of DC were completely abolished when NK cells were pre-treated with sMICA-containing serum.

**Conclusions:** sMICA is present in sera of patients with advanced HCC and may serve as a tumor evasion mechanism by negatively modulating both innate and adaptive immunity.

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**Keywords:** Liver; Tumor; HCC; NK, MIC; MIA; DC; NKG2D

### 1. Introduction

Human hepatocellular carcinoma (HCC) has the unique characteristic of a high risk of development from chronic inflammatory liver diseases. Despite recent advances in new therapeutic modalities, a significant number of HCC show frequent recurrence and progression to an advanced stage with few curative options [1]. In this regard, the identification and manipulation of molecules that are specifically present in advanced HCC may offer new

strategies for improving and broadening therapeutic options.

Natural killer (NK) cells are a major component of innate lymphocytes that predominantly reside in the liver [2], and play a critical role in innate resistance against tumors [3,4]. In addition, recent studies have revealed that NK cells can modulate the functions of dendritic cells (DC), the major sentinel between innate and adaptive immunity [5,6]. Therefore, NK cells may also affect the magnitude and direction of adaptive immune responses against tumors. NK cell functions are regulated by a balance of negative and positive signals, which are mediated by inhibitory and activating receptors; the former includes killer cell immunoglobulin-like receptors (KIRs) and C-type lectin-like molecules, such as CD94 and NKG2A/E, and the latter includes the NKG2D activating receptor [7,8].

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A stress-inducible MHC class I-related chain A (MICA) was recently identified as a human ligand of NKG2D [9]. MICA is expressed in many carcinoma cells such as in lung, breast, ovary, prostate, colon cancer, but is usually absent from normal tissue [10,11]. This raises the possibility of MICA being an important 'on' signal for NK cell-mediated innate immune surveillance against tumor cells. A tumor-specific expression pattern of MICA has also been observed in human HCC, and NK cells recognize hepatoma cells via MICA-NKG2D interaction [12]. These findings suggest that MICA-NKG2D may serve as an efficient innate pathway of immune surveillance against HCC.

Recent studies have suggested that MICA is released as a soluble form from the cell surface of tumor cells and can be detected in gastrointestinal malignancy, prostate cancer and leukemia [11,13–16]. In addition, the soluble form of MICA (sMICA) was found to sequester NKG2D in the cytoplasm and to inhibit cell-surface NKG2D expression and NKG2D-mediated effector functions of immune cells in progressive malignant tumors [11,13,15] and rheumatoid arthritis [17]. Therefore, sMICA may represent one of the factors involved in tumor evasion of host immunity. However, definitive evidence is still lacking as to whether these mechanisms can also be applied to human HCC.

In the present study, we investigated the presence of sMICA in patients with HCC and its function in NK cell activation in human HCC. We also elucidated the role of sMICA on the NK cell-mediated functional regulation of DC.

## 2. Materials and methods

### 2.1. Subjects

Twenty six patients with HCC, 15 patients with chronic hepatitis C, 9 patients with chronic hepatitis B and 10 healthy individuals were enrolled in this study after informed consent had been obtained. The profiles of patients with HCC are summarized in Table 1. The classification of HCC by tumor node metastasis (TNM) staging [18] was based on diagnostic modalities such as computed tomography and magnetic resonance imaging. Serum levels of alpha-fetoprotein (AFP) were measured using the commercially available enzyme-linked immunosorbent assay (ELISA) kit (Eiken Chemical Co., Tokyo, Japan). HCV and HBV infections were diagnosed by the presence of serum HCV-RNA and hepatitis B surface antigen, respectively.

### 2.2. Quantification of serum sMICA

A human MICA ELISA kit (IMMATICS Biotechnologies, Turbigen, Germany) was used for the detection of sMICA from each sample of serum according to the manufacturer's protocol. The threshold limit of detection of these ELISA systems is 10 pg/mL.

### 2.3. Isolation and propagation of NK cells

NK cells were isolated from peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Bergisch-Gladbach, Germany). More than 90% of the cells were CD56<sup>+</sup>CD3<sup>-</sup> lymphocytes.

In some cases, enriched NK cells were cultured in 24-well-culture plates ( $5.0 \times 10^5$ /well) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (complete medium). For treatment of NK cells with serum from HCC patients, NK cells from healthy donors were cultured for 48 h in complete medium supplemented with 10% patient sera.

### 2.4. NKG2D expression of human NK cells

NK cells were stained with purified anti-NKG2D mAb 1D11, followed by PE-labeled goat anti-mouse immunoglobulin light chain  $\lambda$  (BD-Biosciences, San Jose, CA) as described previously [19]. 1D11 was kindly provided by Drs V. Groh and T. Spies (Fred Hutchinson Cancer Research Institute, Seattle, WA). They were analyzed using a FACScan system, and data analysis was performed using CELLQuest software (BD Biosciences).

### 2.5. Tumor cell lines and culture

The Huh7 human hepatoma cell line was used as MICA-positive cells [12]. The human chronic myeloid leukemia cell line K562 was obtained from American Type Culture Collection (Rockville, MD).

### 2.6. Cytolytic activity of NK cells

Target cells (Huh7 or K562) labeled with  $^{51}\text{Cr}$  were incubated with NK cells for 4 h at an effector/target ratio of 20/1. The supernatants were obtained after the incubation and subjected to  $\gamma$ -counting. The maximum or spontaneous release was defined as counts from samples incubated with 5% Triton-X or medium alone, respectively. Cytolysis was calculated with the following formula: % lysis = (release in experiment – spontaneous release)  $\times$  100 / (maximum release – spontaneous release). The spontaneous release in all assays was less than 20% of the maximum release.

### 2.7. Measurements of IFN $\gamma$ production of NK cells

NK cells were cultured with Huh7 or K562 at a ratio of 1:1 for 24 h. Interferon  $\gamma$  (IFN $\gamma$ ) in the culture supernatant was determined using the ELISA kit (Endogen, Woburn, MA) according to the manufacturer's instructions.

### 2.8. Generation of monocyte-derived DC

Monocytes were isolated from peripheral venous blood by their adherence to plastic wells and were supplemented with GM-CSF (50 ng/mL; Peprotech, Rocky Hill, NJ) and IL-4 (10 ng/mL; Peprotech). On day 6, they were stimulated with or without 10  $\mu\text{g}/\text{mL}$  of lipopolysaccharide (LPS) (Sigma-Aldrich, St Louis, MO) for 24 h.

### 2.9. DC/NK coculture

The coculture experiments of NK cells and DC were performed as described previously [20,21]. In brief, NK cells ( $1.0 \times 10^5$ /well) from healthy individuals were incubated with or without 10% serum of patients with HCC for 48 h. After washing three times, the NK cells were cultured for 24 h with autologous DC ( $1.0 \times 10^5$ /well) and Huh7 cells. In some experiments, anti-NKG2D mAb (1D11) was added at the beginning of the coculture. To analyze the DC phenotype, the cells were stained with PC5-labeled CD11c mAb and PE-labeled CD86 mAb and FITC-labeled CD40 (BD-Biosciences), and subjected to flow cytometric analysis. We set the gate around DC using forward and sidelight scatters and analyzed expression on CD86 and CD40 on CD11c-positive cells. To purify DC from the cocultures, NK cells were depleted from the non-adherent cells using CD56 MicroBeads (Miltenyl). DC were seeded in flat-bottom 96-well plates ( $1.0 \times 10^4$ /well) and then cultured with the responder naive CD4<sup>+</sup> cells isolated from allogeneic donors ( $1.0 \times 10^5$ /well) for 72 h. The cocultured cells were pulsed with 1  $\mu\text{Ci}/\text{well}$  of [ $^3\text{H}$ ] thymidine for 16 h of incubation and collected onto a glass fiber filter. [ $^3\text{H}$ ] Thymidine incorporation was quantified using a beta-plate liquid scintillation counter.



**Table 1**  
**Characteristics of patients with HCC in this study**

Patients	Age/sex	Etiology	AFP (ng/mL)	TNM stage	sMICA (pg/mL)
HCC-1	62/M	HCV	28,800	IV	62
HCC-2	54/M	HBV	58	IV	134
HCC-3	70/F	HCV	312,900	IV	ND
HCC-4	56/M	HCV	660,000	IV	610
HCC-5	52/M	HCV	34,000	IV	ND
HCC-6	57/M	HCV	5,200	IV	620
HCC-7	63/M	HCV	774	IV	98
HCC-8	72/M	HCV	6,300	IV	72
HCC-9	76/F	HCV	189	IV	560
HCC-10	93/F	HCV	776,000	IV	520
HCC-11	68/M	HCV	378	III	68
HCC-12	72/M	HCV	5>	III	132
HCC-13	52/M	HCV	431	III	ND
HCC-14	61/M	HCV	5>	III	ND
HCC-15	72/M	HCV	3,500	II	ND
HCC-16	73/F	HCV	5>	II	124
HCC-17	60/M	HCV	18	II	ND
HCC-18	33/M	HCV	13	II	ND
HCC-19	78/M	HCV	340	II	ND
HCC-20	71/M	HBV	152	II	ND
HCC-21	32/M	HCV	33	II	ND
HCC-22	32/M	HCV	33	I	ND
HCC-23	68/M	HCV	73	I	ND
HCC-24	68/M	HCV	352	I	ND
HCC-25	52/M	HBV	28	I	ND
HCC-26	66/M	HCV	5>	I	ND

ND, not detected.

The results were expressed as the mean counts per minute (cpm) in triplicate cultures. Finally, the cocultured cells were also pretreated with 1  $\mu$ L/mL GolgiPlug (BD-Pharmingen) for 4 h at 37 °C. At the end of the incubation period, CD4<sup>+</sup>T cells were stained with PC5-labeled CD4 mAb (Beckman-Coulter), and then fixed and permeabilized with Cytotfix/Cytoperm buffer (BD-Pharmingen) for 15 min at room temperature. The permeabilized cells were stained with FITC-labeled anti-IFN $\gamma$  mAb (BD-Pharmingen).

### 2.10. Statistical analysis

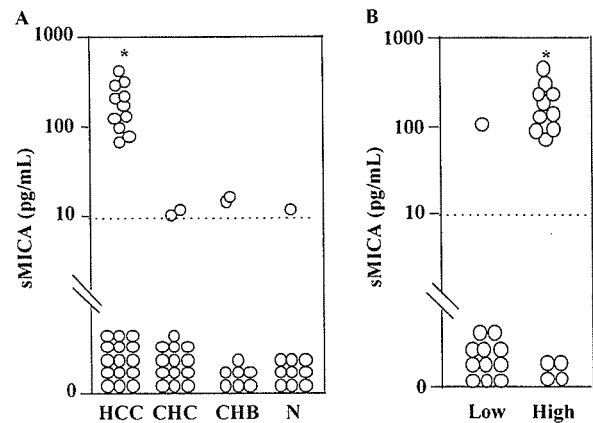
The data are expressed as the mean and SD and compared using ANOVA with Bonferroni's test. Differences were considered significant when the *p* value was <0.01.

## 3. Results

### 3.1. sMICA was preferentially released from patients with advanced HCC

We investigated the serum levels of sMICA in 26 patients with HCC of various progression grades, 24 patients with chronic hepatitis (CH) due to HCV or HBV infection and 10 healthy individuals. Significant amounts of sMICA were detected in sera from 11 of the 26 patients with HCC. In contrast, sMICA was not detected in sera from CH patients or healthy individuals except for five cases with marginal positivity (Table 1 and Fig. 1A). These results suggested that sMICA could be detected in a tumor-specific fashion.

To examine whether the presence of sMICA is associated with the progression of HCC, we divided the 26 HCC patients into low-grade (stages I and II) and high-grade HCC (grade III and IV) groups. The frequency of sMICA positivity was significantly higher for high-grade HCC than low-grade HCC (71 versus 8.4%, respectively; *P* < 0.01).



**Fig. 1.** Serum levels of sMICA. (A) Serum levels of sMICA in patients with HCC (HCC; *n* = 26), those with chronic hepatitis C (CHC; *n* = 15), those with chronic hepatitis B (CHB; *n* = 9) and healthy donors (N; *n* = 10). Dotted line indicates the threshold of detection of the ELISA assay. (B) Serum levels of sMICA in patients with HCC were compared between low-grade HCC (TNM stages I and II) and high-grade HCC (TNM stages III and IV). The difference of each group was analyzed by the Mann–Whitney test. \**P* < 0.01.

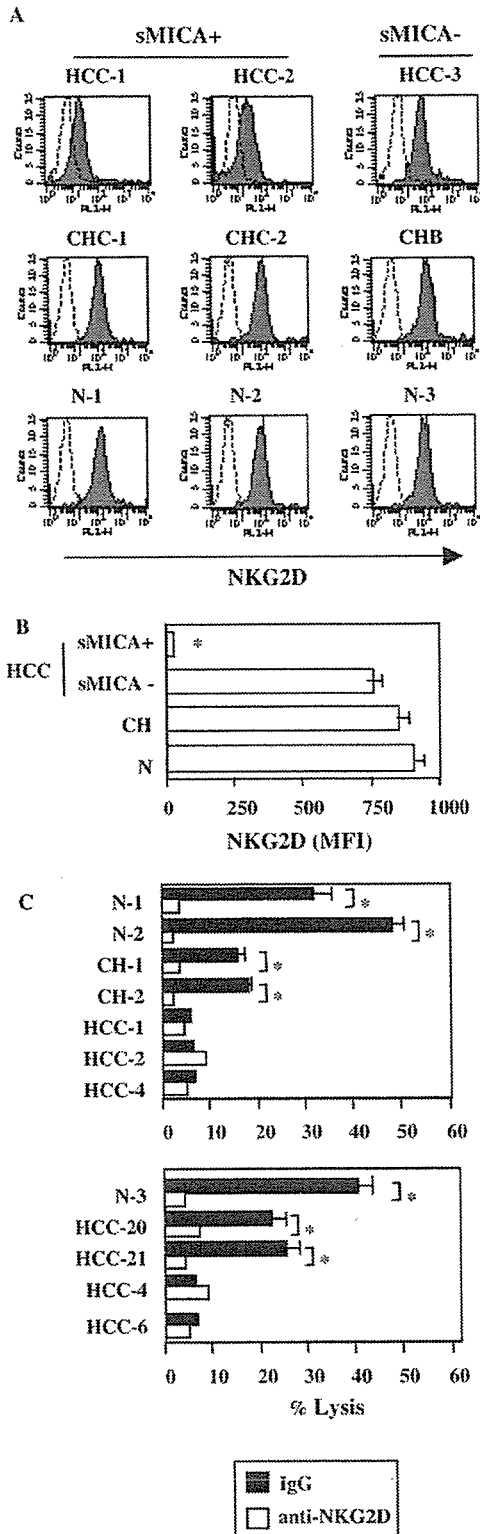


Fig. 2. Expression of NKG2D on NK cells and NKG2D-dependent NK cell activities. (A, B) Surface expression of NKG2D on CD56<sup>+</sup> NK cells. Surface expression of NKG2D on NK cells for patients with HCC with serum sMICA-positive (sMICA+) or negative (sMICA-), chronic hepatitis C (CHC), chronic hepatitis B (CHB) and healthy donors (N).

(Fig. 1B). Therefore, the increase of sMICA was correlated with the disease progression of HCC.

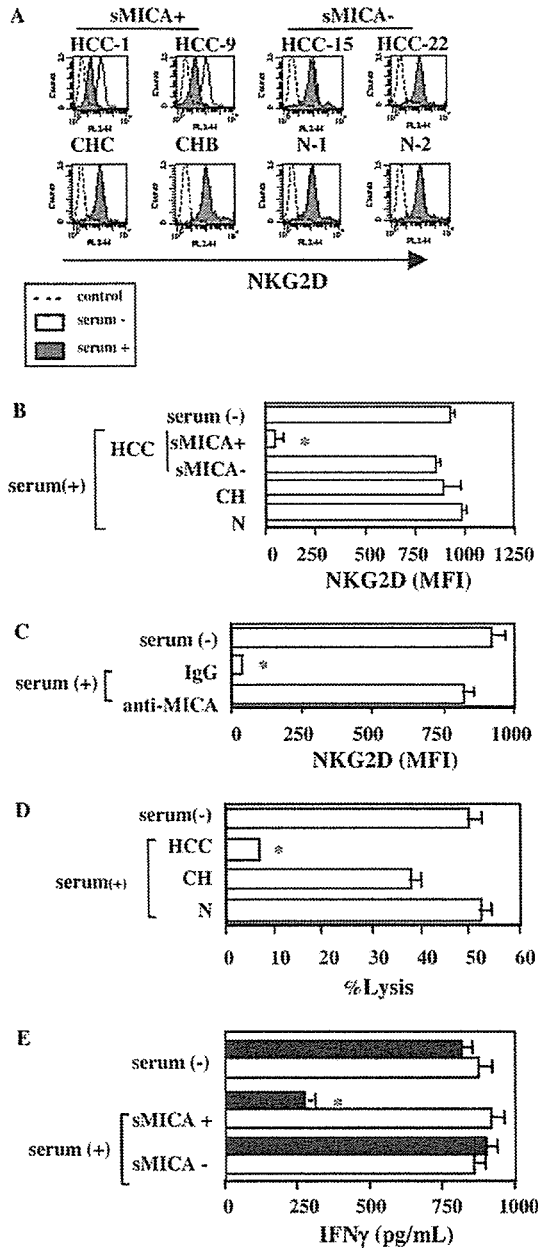
3.2. Impaired NKG2D expression and NKG2D-mediated NK cell activity in sMICA-positive advanced HCC

Given the previous findings of sMICA-induced down-regulation of NKG2D on effector cells [11,13,15], we next compared the surface levels of NKG2D on NK cells from HCC patients having detectable levels of sMICA (sMICA-positive) with those from HCC patients, CH patients or healthy donors having no sMICA (sMICA-negative). As shown in Fig. 2A and B, NKG2D expression on NK cells from sMICA-positive patients was markedly reduced compared to that from sMICA-negative donors. Chromium release assay revealed that NK cells isolated from healthy donors, CH patients, or HCC patients negative for sMICA were capable of killing Huh7 cells in a NKG2D-dependent manner, because the cytotoxicity was completely abolished by the addition of anti-NKG2D mAb (1D11) during the coculture. In contrast, NK cells isolated from HCC patients positive for sMICA were not capable of killing Huh7 cells (Fig. 2C). Therefore, NKG2D expression as well as NKG2D-mediated cytolytic activity of NK cells was impaired in sMICA-positive patients.

3.3. Down-regulation of surface NKG2D expression and NKG2D-mediated activation of normal NK cells by sMICA present in sera of patients with advanced HCC

To further examine the involvement of sMICA in modulated expression of NKG2D and NK cytolytic activity, we cultured NK cells from healthy donors with sMICA-containing serum for 48 h and assessed the NKG2D expression and NK cell functions. The levels of NKG2D expression were reduced on NK cells with the addition of sera from sMICA-positive, but not sMICA-negative, donors (Fig. 3A and B). The reduction of NKG2D was restored when the sera were incubated with anti-MICA mAb (6D4), but not when they were incubated with control IgG (Fig. 3C). NK cells pre-treated with sMICA-containing serum were not capable of efficiently killing Huh7 hepatoma cells, in agreement with their decrease in NKG2D expression (Fig. 3D). In addition, NK cells pre-stimulated

Closed and open histograms represent the staining with anti-NKG2D and control Ab, respectively. Representative results (A) as well as the statistical analysis (B) ( $n=5$  for each group) shown as the mean fluorescence intensity (MFI) of the NKG2D-stained cells are presented.  $*P < 0.01$  vs N. (C) Abrogation of NKG2D-mediated lytic activity of NK cells in sMICA-positive HCC patients. NK cells derived from patients with HCC with serum sMICA-positive (HCC-1, 2, 4, 6), HCC with serum sMICA-negative (HCC-20, 21), chronic hepatitis (CH) or healthy donors (N) were incubated with <sup>51</sup>Cr-labeled Huh7 cells at an effector/target ratio of 20/1. The % specific lysis is compared for the addition of anti-NKG2D mAb 1D11 (open column) and isotype control Ab (closed column) during the incubation period.  $*P < 0.01$ .



**Fig. 3.** Effect of sMICA-containing serum on NKG2D expression of NK cells from healthy donors. (A, B) NK cells from healthy donors were incubated with (serum (+)) or without (serum (-)) serum of HCC patients with sMICA-positive (sMICA+) or sMICA-negative (sMICA-), chronic hepatitis (CH) or healthy donors (N). NKG2D expression was evaluated by FACS analysis. Representative results (A) as well as the statistical analysis (B) ( $n=3$  for each group) are shown as the mean fluorescence intensity (MFI) of the staining cells.  $*P<0.01$ . (C) sMICA is responsible for NKG2D down-modulation on NK cells. NK cells from healthy donors were incubated with (serum (+)) or without (serum (-)) serum of HCC patients with sMICA-positive in the presence of anti-MICA mAb (anti-MICA) or control IgG (IgG) for 48 h. NKG2D expression was then evaluated by FACS analysis. Statistical analysis ( $n=3$  for each group) is shown as the mean fluorescence intensity of the stained cells.  $*P<0.01$ . (D) Effect of sMICA-containing serum on NK cell cytolytic ability against Huh7. Normal NK cells pre-treated with (serum (+)) or without (serum (-))

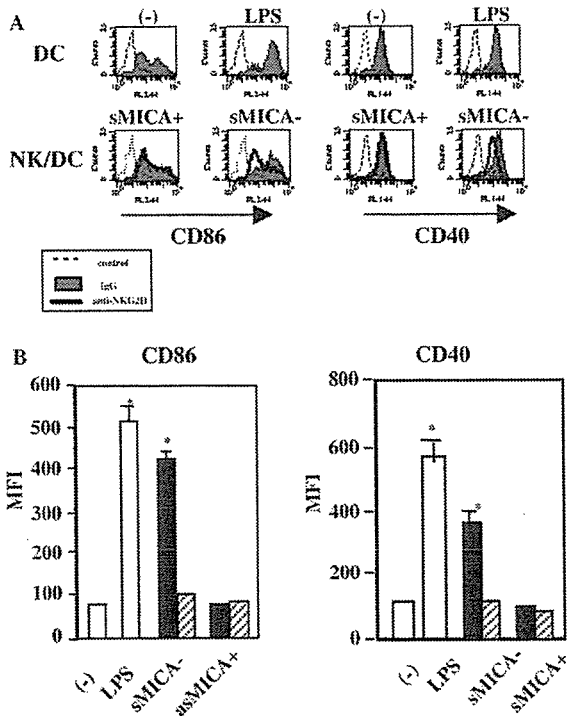
with sMICA-positive serum, upon exposure to Huh7 cells, produced lesser amounts of IFN $\gamma$  than those pre-stimulated with sMICA-negative serum. The inhibitory effect of sMICA-positive serum on IFN $\gamma$  production was not observed when NK cells were exposed to K562 cells lacking MICA expression [12] (Fig. 3E). These results offer evidence for sMICA being a negative regulator of NKG2D expression and NK cell activation upon exposure to human hepatoma cells.

**3.4. Inhibition of NK cell-mediated maturation and activation of DC by sMICA**

It has been increasingly recognized that NK cell-mediated regulation of DC plays an important role in the initiation and extension of adaptive immune responses [5,6,22–24]. These observations led us to examine whether sMICA-mediated down-regulation of NKG2D affects NK cell activation of DC. For this purpose, normal NK cells were treated with sMICA-positive or negative serum of patients with HCC, and then cocultured with both monocyte-derived DC and Huh7 cells for 24 h. Expression of the maturation markers was evaluated on DC by FACS analysis. Neither NK cells nor Huh7 alone had much impact on DC maturation under our experimental conditions (data not shown). DC cultured with both sMICA-negative serum-treated NK cells and Huh7 exhibited a matured phenotype such as with up-regulation of CD86 and CD40 at levels similar to those of LPS-stimulated DC (Fig. 4A). The addition of anti-NKG2D mAb (1D11) during the cocultures completely suppressed NK cell-induced maturation of DC. In contrast, the expression levels of CD86 and CD40 (Fig. 4A and B) were not up-regulated on DC cultured with sMICA-positive serum-treated NK cells and Huh7. The treatment of NKG2D mAb (1D11) had little effect on DC maturation by sMICA-positive serum-treated NK cells (Fig. 4A and B).

We next evaluated whether DC exposed to both NK cells and Huh7 could stimulate naive CD4 $^{+}$ T cells. When DC were cultured with Huh7 and NK cells pre-treated either with or without sMICA-negative sera, they were capable of stimulating proliferation of allogeneic CD4 $^{+}$ T cell at levels similar to those of LPS-stimulated DC. When NKG2D signals were blocked by mAb 1D11 during the cocultures, the ability of DC to stimulate CD4 $^{+}$ T cells was markedly reduced. On the other hand, DC activation of CD4 $^{+}$ T cells was severely impaired when DC were pre-stimulated with sMICA-positive serum-treated NK cells and Huh7 (Fig. 5A). Intracellular cytokine

serum of each group as described above were cultured with  $^{51}\text{Cr}$ -labeled Huh7 at an effector/target ratio of 20/1 for 4 h. Data are presented as a composite of those examined for each group ( $n=3$ ).  $*P<0.01$ . (E) Effect of sMICA-containing serum on IFN $\gamma$  production of NK cells. Normal NK cells pre-treated with (serum (+)) or without (serum (-)) serum of each group were cultured with Huh7 (closed column) or K562 (open column) for 48 h. IFN $\gamma$  in each culture supernatant was measured by ELISA.  $*P<0.01$ .



**Fig. 4.** sMICA-mediated inhibition of NK cell ability on DC maturation. NK cells derived from healthy volunteers were incubated with sMICA-positive (sMICA+) or negative (sMICA-) serum of patients with HCC for 48 h. After extensive washing, NK cells were cocultured with DC from the same healthy donors and Huh7 cells at a ratio of 1:1 (DC/NK) in the presence of anti-NKG2D (solid lined histogram in (A) and hatched bar in (B)) or control IgG (closed histogram in (A) and closed bar in (B)) for 24 h. As negative and positive controls, DCs were unstimulated (-) or were stimulated with LPS for 48 h, respectively. FACS analysis was performed to evaluate the expression of CD86 and CD40 on DC after positively gating the CD11c<sup>+</sup> cell population. Dotted lined histograms represent the staining of control Ab. All experiments were performed three times and representative results (A) as well as statistical analysis ( $n=3$ ) (B) are shown as the mean fluorescence intensity (MFI) of the stained cells. \* $P < 0.01$ .

staining also revealed that CD4<sup>+</sup>T cells substantially produced IFN $\gamma$  upon exposure to DC that had been stimulated with both Huh7 and NK cells pretreated either with or without sMICA-negative sera at similar levels to those of LPS-stimulated DC. NKG2D blockade by mAb 1D11 during the cocultures resulted in the inhibition of DC-mediated IFN $\gamma$  production of CD4<sup>+</sup>T cells. In contrast, DC-mediated IFN $\gamma$  production of CD4<sup>+</sup>T cells was reduced when DC were pre-stimulated with sMICA-positive serum-treated NK cells and Huh7 (Fig. 5B). Taken together, sMICA appears to serve as a negative regulator of the NK cell activation of DC by inhibiting NKG2D-mediated positive signals.

#### 4. Discussion

We previously reported that MICA is expressed in surgically removed tumor tissues with HCC and marks

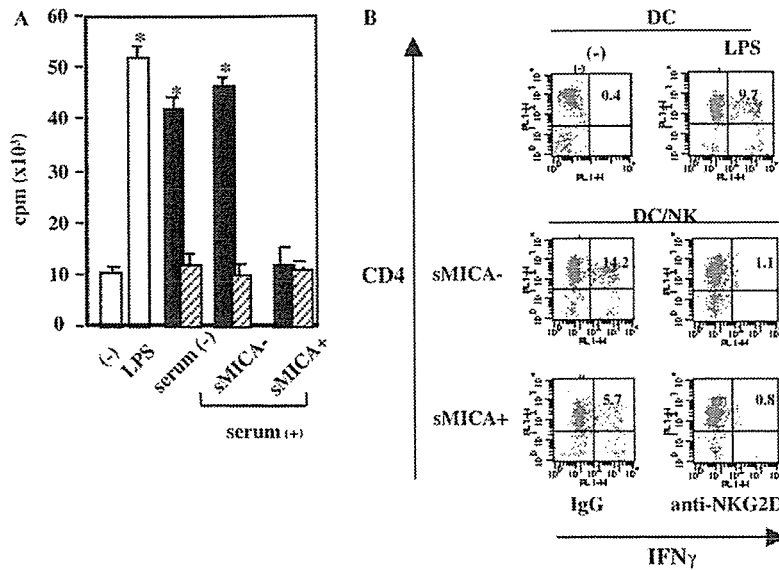
hepatoma cells for recognition of NK cells by activating the immunoreceptor NKG2D [12]. In the present study, significant levels of sMICA were detected in a subset of HCCs. On the other hand, it was only detected at marginal levels in patients with chronic hepatitis C and B, the underlying diseases for HCC development. This clearly is in contrast with the cases of other chronic inflammatory diseases such as rheumatoid arthritis, in which sMICA is released from inflammatory sites [17]. In this regard, sMICA may be potentially useful as a novel tumor marker for HCC.

The rate of positivity for sMICA increased with the grade of HCC. Correlation between serum sMICA and progression of tumor has been shown for prostate cancers but not other diseases, such as hematopoietic malignancy [11,16]. Recent studies have demonstrated that MICA is released as a soluble form from the cell surface of epithelial tumor cells due to the activity of metalloproteinases [14]. Although what determines the shedding of sMICA has not been fully elucidated, the association between sMICA positivity and the grade of HCC suggests that the levels of sMICA in serum are dependent on the amounts of malignant cells releasing sMICA.

The levels of NKG2D expression on NK cells from HCC patients positive for sMICA were significantly lower than those from donors negative for sMICA. In addition, in vitro experiments revealed that sMICA-containing serum of HCC significantly down-regulated NK cell expression of NKG2D. Importantly, the blocking experiment using anti-MICA in the present study proved sMICA to be responsible for the patient serum-mediated down-regulation of NKG2D. It has recently been reported that TGF $\beta$  also has an effect on down-regulation of NKG2D expression [25], and the serum levels of TGF $\beta$  increase in patients with HCC [26]. However, addition of anti-TGF $\beta$  neutralizing Ab to sMICA-containing serum did not reverse NKG2D expression on NK cells (Jinushi M, unpublished observation). These findings indicate that sMICA, but not TGF $\beta$ , in the serum of advanced-stage HCC patients, has a causative effect on down-modulation of NKG2D expression on NK cells.

The sMICA-mediated down-regulation of NKG2D is functionally relevant because NK cells from patients positive for sMICA were less efficient for killing MICA-positive hepatoma cells than those from donors negative for sMICA. NK cells pre-treated with sMICA in HCC serum showed a similar trend. The MICA-NKG2D system should play an important role in immune surveillance for low-grade HCC, because sMICA was rarely detected in these patients. However, at an advanced stage, accumulation of sMICA may serve as an evasion mechanism by which NK cell-mediated immune surveillance against HCC becomes paralyzed.

NK cells not only mediate innate resistance but also affect adaptive immunity via modulation of DC, although



**Fig. 5.** Impaired NKG2D-mediated NK cell activation of DC allostimulatory capacity by sMICA. NK cells from healthy donors were cultured for 48 h with (serum (+)) or without (serum (-)) sera positive for sMICA (sMICA+) or negative for sMICA (sMICA-). After extensive washing, NK cells were cocultured with DC and Huh7 in the presence of anti-NKG2D mAb (hatched bar) or control IgG (closed bar) for 24 h. Allogeneic naive CD4<sup>+</sup>T cells ( $1 \times 10^5$ /well) were cultured for 72 h with DC pre-treated as described above. As negative and positive controls, DC were stimulated with (LPS) or without (-) LPS. (A) Proliferation of CD4<sup>+</sup>T cells was examined by [<sup>3</sup>H]thymidine incorporation. \* $P < 0.01$  (B) Intracellular expression of IFN $\gamma$  in CD4<sup>+</sup>T cells was determined by FACS analysis. The number in each quadrant represents the percentage of IFN $\gamma$ -positive CD4<sup>+</sup>T cells. Similar results were obtained in two independent experiments, and representative data are shown.

the underlying mechanisms are not fully understood [5,6]. Here, we demonstrated for the first time that NK cells augment the immune stimulatory capacity of DC in an NKG2D-dependent fashion and that sMICA-mediated down-modulation of NKG2D results in impairment of DC functions. We previously reported that HLA-E-NKG2A interaction between hepatoma cells and NK cells negatively regulated DC functions [24]. Therefore, DC functions may be modulated by a balance between NK cell inhibitory and activating receptors in the presence of third party cells such as hepatoma cells. Since DC functions as a sentinel between innate and adaptive immunity, cognate interaction of DC and NK cells in HCC should regulate tumor-specific adaptive immune responses. sMICA inhibits activation signals towards NK cells via down-modulation of NKG2D, where inhibitory signals dominate DC-regulation. Therefore, sMICA could inhibit not only NK cell-mediated innate resistance but also adaptive tumor immunity in advanced-stage HCC.

In conclusion, sMICA may not only serve as a novel biomarker of HCC, but also function as a tumor evasion mechanism at an advanced stage of HCC. Given recent studies suggesting that some stimulatory cytokines, such as IL-2 and IL-15, restored NKG2D expression on NK cells and CD8<sup>+</sup>T cells in patients with prostate cancer [11], strategies that reverse sMICA-mediated down-regulation of NKG2D may serve as attractive options for provoking both innate and adaptive immune responses against HCC.

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## Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication

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**Background/Aims:** The lack of small animal models supporting chronic hepatitis B virus (HBV) infection impedes the assessment of anti-viral drugs in the whole animal. Although transgenic mice have been used for this purpose, these models are clearly different from natural infection, because HBV is produced from the integrated HBV sequence harbored in all hepatocytes.

**Methods:** Balb/cA nude mice were hydrodynamically injected with a plasmid having 1.5-fold over-length of HBV DNA and analyzed for HBV replication.

**Results:** Hydrodynamically injected mice showed substantial levels of antigenemia and viremia for more than 1 year. Covalently closed circular DNA (cccDNA), the template of viral replication in natural infection, was produced in the livers and was critically involved in the long-term HBV production, because disruption of the *pol* gene of the inoculated DNA resulted in transient expression of HBV genes for less than 2 months. Administration of the IFN $\alpha$  gene transiently suppressed HBV DNA replication, but was not capable of eliminating HBV in this model.

**Conclusions:** In vivo gene transfer of a plasmid encoding HBV DNA can establish chronic viral replication in mice, which involves, at least in part, new synthesis of the HBV cccDNA episome, thus recapitulating a part of human HBV infection.

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**Keywords:** HBV; Liver; DNA; Hydrodynamics; Naked; Gene; Transfer; Transfection; IFN; Mouse

### 1. Introduction

Hepatitis B virus (HBV) causes both transient and persistent infection in the human liver [1,2]. When healthy adults are exposed to this virus, they usually develop acute transient infection with various degrees of liver injury, and, in most cases, have favorable outcomes. In contrast, when immunocompromised hosts such as newborn babies, drug abusers, and patients receiving immunosuppressive drugs, are infected with HBV, they cannot eliminate it and often suffer from chronic liver injury and hepatocellular

carcinoma. Chronic carriage of this virus is a major health problem in many countries. Patients with chronic HBV infection are currently treated with interferon (IFN) or nucleotide analogs such as lamivudine and adefovir. However, the limited success and frequent recurrence after cessation of therapy require new strategies for terminating this viral infection.

Study of HBV replication in vivo is hampered by the lack of suitable small and well-characterized animal models; thus far, only chimpanzees and the tree shrew (*Tupaia*), a relatively uncharacterized animal, appear to support HBV infection [3]. Several lines of transgenic mice have been established but HBV replication is generated from the integrated HBV sequence harbored in all hepatocytes, which is clearly different from the natural infection [4,5]. An alternative strategy is in vivo gene transfer of HBV DNA. Takahashi et al. [6] previously reported that

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intrahepatic injection of naked HBV DNA with cationic liposome can cross the species barrier and leads to HBV replication in rats. We and others have reported that hydrodynamics-based delivery of HBV DNA efficiently transduces murine livers and leads to HBV replication [7,8]. However, HBV replication in these models is terminated within a couple of weeks, presumably resulting from immunological elimination of HBV-expressing hepatocytes. Very recently, there have been reports of these models being applied for the assessment of anti-viral drugs [9–11]. However, the analysis may be hampered because this is a model of acute transient infection and would not allow observation of the long-term outcome.

In an attempt to develop a better long-term model, we hydrodynamically injected a plasmid encoding replication competent HBV DNA into immunocompromised mice and examined the kinetics of expression and replication of HBV. The mice produced HBV-related proteins for over 1 year, which appeared to be dependent on episomal HBV DNA replication in the liver, because the introduction of replication-incompetent HBV DNA led to transient expression of HBV genes. IFN $\alpha$  treatment of these mice showed transient repression of HBV replication but could not terminate it. These mice mimic a part of human HBV infection in terms of the template of viral replication and should be useful for analyzing the long-term outcome of anti-HBV therapy.

## 2. Materials and methods

### 2.1. Plasmids and mutagenesis

Plasmid pHBV1.5 containing an overlength (1.5-mer) copy of HBV DNA (GenBank accession no. AF305422) has been described previously [7]. A plasmid containing mutant HBV DNA carrying a stop codon instead of 54Trp of the *pol* gene was generated from pHBV1.5 by a GeneTailor Site-Directed Mutagenesis system (Invitrogen, Carlsbad, CA) and verified by sequencing. The site of the mutation was designed not to affect the expression of any HBV-related genes except for the *pol* gene. A plasmid coding the murine IFN $\alpha$ 1 gene, pCMV-IFN $\alpha$ 1, was generously provided by Dr Daniel J.J. Carr (University of Oklahoma, Health Science Center) [12].

### 2.2. Mice

Specific pathogen-free female Balb/cA nude mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at the age of 5 to 6 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and study protocol complied with the institution's guideline.

### 2.3. Injection of naked plasmid DNA

Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed according to previous reports [13,14]. In brief, 25  $\mu$ g of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and

injected into the tail vein using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 s.

### 2.4. Northern blot

Total tissue RNA was isolated with Isogen (Nippon Gene, Toyama, Japan), and then 30  $\mu$ g of total RNA was analyzed by Northern blotting with the HBV *adw2* probe, as described previously [15].

### 2.5. Immunohistochemistry

For immunohistochemical detection of HBc protein, tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. After being deparaffinized, sections 4  $\mu$ m thick were incubated with anti-HBc antibody (Dako, Denmark), followed by immunoperoxidase staining using the ABC procedure (Vector Laboratories, Burlingame, CA) and counterstaining with hematoxylin.

### 2.6. Detection of hepatitis B antigens in serum

Under light anesthesia using sevoflurane, animals were bled from the retro-orbital vessels. Serum HBs antigen and HBe antigen were measured by chemiluminescent immunoassay (CLIA system, Abbott Laboratories, North Chicago, IL).

### 2.7. Real-time detection of HBV DNA in serum

Serum was treated with DNase I (Takara, Tokyo, Japan) and then proteinase K. DNA was extracted from the sera by a QIAamp DNA blood isolation system (Qiagen). HBV DNA was quantified by using real-time polymerase chain reaction (PCR) technology (Applied Biosystems, Foster City, CA) as described previously [16]. Primers and fluorescent probes are as follows: sense (nucleotides 168–188), 5'-CACATCAGGATTCCTAG-GACC-3'; antisense (nucleotides 341–321), 5'-GGTGAGTGATTG-GAGGTTGG-3'; probe (nucleotides 244–269), 5'-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-3'.

### 2.8. Density analysis of HBV particles in serum

DNase I-treated serum was clarified by centrifugation at 15,000 rpm for 15 min using a 0.45  $\mu$ m membrane filter. The clarified serum was layered on top of a 10–60% discontinuous sucrose gradient. Centrifugation was carried out at 141,000 *g* for 48 h. Fractions were collected from the bottom of the tube. After treatment with proteinase K, DNA was isolated from each fraction and applied for analysis of HBV DNA by PCR [7]. In an additional experiment, DNase I-treated serum was incubated with 1% Nonidet P-40 and 0.3% 2-mercaptoethanol for 16 h at 37 °C, and then used for density analysis.

### 2.9. Detection of HBV covalently closed circular DNA (cccDNA)

DNA was isolated from liver tissues by using a DNeasy Tissue kit (Qiagen). PCR detection of cccDNA was performed according to the procedure of Jun-Bin et al. [17] with some modification (Fig. 1). The PCR product was analyzed on a 1.2% agarose gel by electrophoresis. In some experiments, cccDNA was quantified using real-time PCR. To calculate the number of cccDNA per HBcAg-positive hepatocyte, the total number of hepatocytes was estimated from the genomic DNA content in the murine liver under the assumption that the liver is about 70% hepatocytes. In addition, ampicillin resistance gene in the plasmids was amplified by using a sense primer (5'-TATGGCTTCATCAGCTCCG-3') and an antisense primer (5'-TCGAAGTGGATCTCAACAGC-3').

### 2.10. IFN $\alpha$ gene therapy

At 70 days after pHBV1.5 injection, nude mice were hydrodynamically injected with either pCMV-IFN $\alpha$ 1 or pCMV mock plasmid and examined



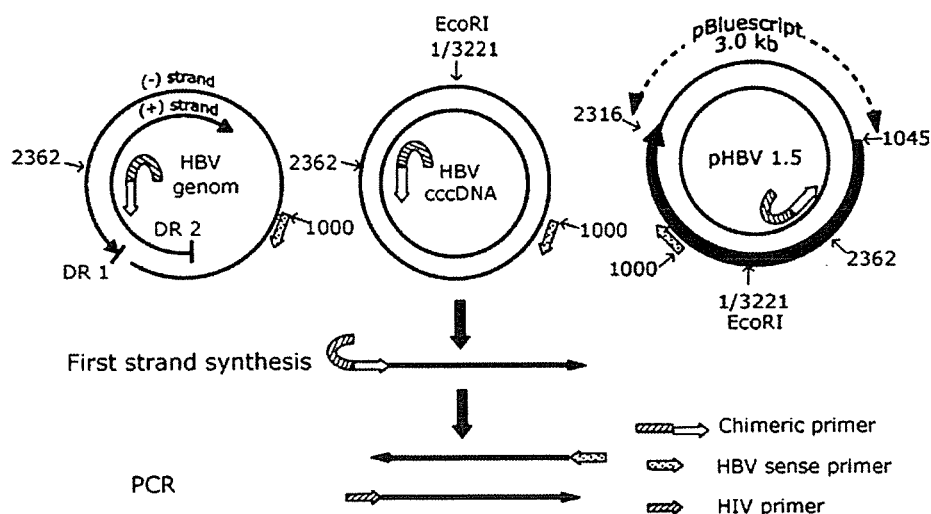


Fig. 1. Principle of PCR detection of HBV cccDNA. Three forms of HBV sequences which could have been present in our samples are shown: HBV genome, HBV cccDNA, and pHBV1.5. The number of nucleotides starts at the EcoRI site. A chimeric primer (5'-TCGCTTCGGGTCCTCCCTGGTCCCGTCGTC-3') is composed of two segments: the segment A sequence near the 5' end is HIV-specific and the segment B sequence near the 3' end is complementary to the HBV DNA plus strand from nucleotide 2362 to 2351. With DNA polymerase activity, the chimeric primer extends and produces a new single DNA strand. Since the HBV plus strand has a gap, nucleotide extension will be stopped at the DR2 gap. On the other hand, an extremely long strand will be generated if pHBV1.5 acts as a template DNA. One-twentieth volume of the elongated strand was used as a template in the next PCR amplification in the presence of one primer, identical to the chimeric primer segment A (HIV primer; 5'-TCGCTTCGGGTCCTCCCT-3') and another primer complementary to the HBV DNA minus strand from nucleotide 1000 to 1016 (HBV sense primer 5'-TTGTGGGTCTTTGGG-3'), cycled 35 times through a program of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. The 1352 bp products will be amplified only from cccDNA. In real-time PCR, the DNA samples were digested with EcoRI and ScaI and subjected to the elongation reaction followed by PCR using a fluorescent probe (5'-FAM-GAGACCACCGTGAACGCCCATCAGAT-3' (nucleotides 1444–1469)). ScaI site is located in the ampicillin resistance gene of the pBluescript but not in HBV DNA sequence.

for HBV replication. IFN $\alpha$  production was assessed using a commercially available mouse IFN $\alpha$  ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

### 3. Results

#### 3.1. Intravenous injection of pHBV1.5 leads to hepatitis B antigenemia as well as hepatic expression of HBcAg for more than 1 year

We injected 25  $\mu$ g of pHBV1.5, which contains 1.5-fold overlength HBV DNA, into the tail veins of nude mice with acute circulatory overload. To investigate the expression of HBV, the presence of HBV transcripts was analyzed by Northern blot in various organs from mice sacrificed at 3 days after the injection (Fig. 2A). Two major bands corresponding to 3.5 and 2.4/2.1 kb transcripts were detected in the liver but not in other tissues including the kidney, spleen, thymus, lung, heart, and brain. The levels of HBsAg and HBeAg in the serum were serially determined by a quantitative CLIA method (Fig. 2B). Although the levels of HBsAg rapidly decreased 1.5 log within the first 2 weeks, all mice were persistently positive for HBsAg and HBeAg for more than 1 year. Immunohistochemical analysis revealed that around 4% of the hepatocytes were positive for HBc at 3 days after injection (Fig. 2C). HBcAg-positive cells

gradually decreased in number but were still detected at one year after the injection (Fig. 2D). Although data are not shown, hepatic damage could not be detected, as evidenced by biochemical and histological analysis, throughout the course, except during the first week; it resulted from hemorrhagic destruction of the liver due to hydrodynamic pressure. Taken together, these results indicated that hydrodynamics-based delivery of a plasmid encoding replication-competent HBV DNA can establish specific expression of HBV genes in the liver and persistent expression without significant liver injury for a period of more than 1 year.

#### 3.2. Long-term productive replication of HBV DNA

To examine if viral particles are produced into the circulation, sera obtained at 3 days after pHBV1.5 injection was treated with DNase I and fractionated by sucrose density gradient centrifugation. As shown in Fig. 3A, when each fraction was assayed in PCR for the presence of HBV DNA, the strongest signal was observed in the fraction with a density of 1.21 g/ml, corresponding to the density of HBV particles derived from human sera [18]. In addition, when serum was pre-treated with detergent before the centrifugation, the positive fraction shifted to a density of 1.28 g/ml, suggesting that detergent treatment releases core particles from HBV particles by removing the envelope.

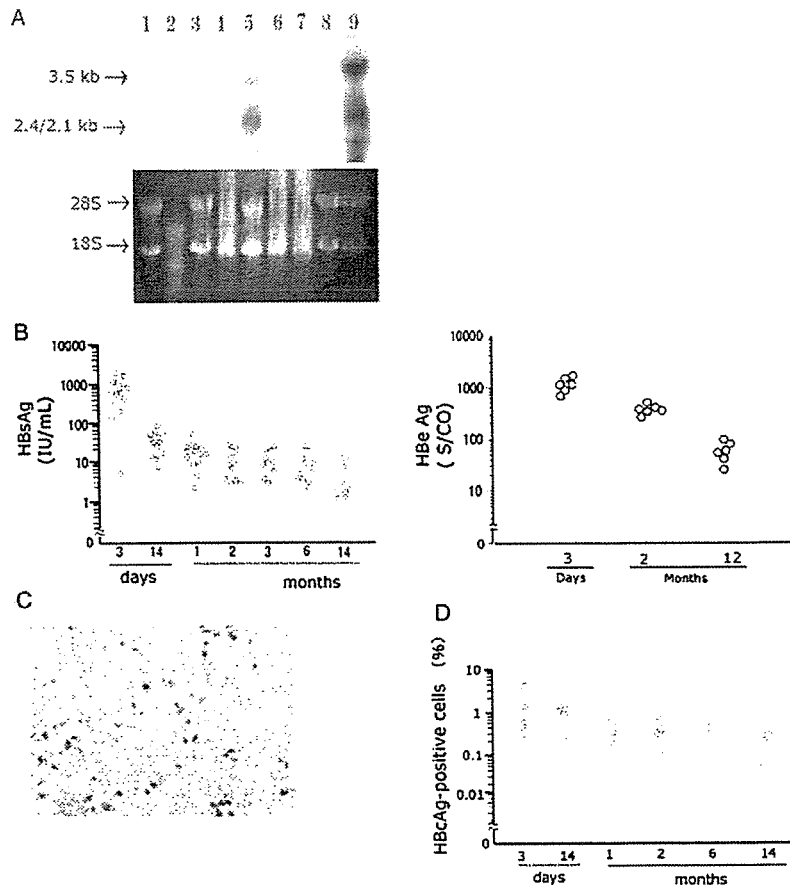


Fig. 2. Expression of HBV in hydrodynamically-transfected nude mice. (A) HBV RNA expression in various organs. Total RNA isolated from the indicated organs was analyzed for the presence of HBV-specific sequence 3 days after transfection (upper panel). Huh7 transfected with pHBV1.5 or pCMV were included as positive and negative controls, respectively. Arrows indicate 3.5- and 2.4/2.1-kb transcripts. A photograph of the ethidium bromide-stained gel is also shown in the lower panel. Lane 1, brain; lane 2, heart; lane 3, thymus; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, spleen; lane 8, pCMV-transfected Huh7; lane 9, pHBV1.5-transfected Huh7. (B) HBsAg and HBeAg in serum. The levels of HBsAg and HBeAg were serially determined in a cohort of mice hydrodynamically transfected with pHBV1.5. (C) Immunohistochemical detection of HBc Ag. Representative data for nude mice 3 days after pHBV1.5 injection. (D) Frequency of HBcAg-positive hepatocytes in the livers.

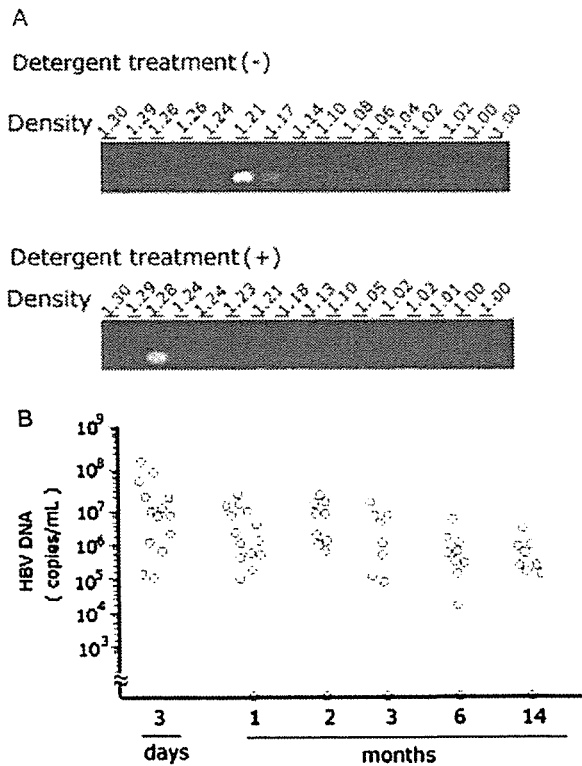
To examine the kinetics of viremia, we examined the levels of DNase I-resistant HBV DNA in serum by real-time PCR analysis (Fig. 3B). The levels of HBV DNA were as high as  $1 \times 10^7$  copies/ml at 3 days after the injection and gradually decreased by 1.5 log over 1 year.

### 3.3. Long-term expression of HBV is dependent on HBV replication

The extremely long-term expression and carriage of HBV in this system led us to examine whether episomal replication could affect the kinetics of expression of HBV-related genes. Toward this goal, we introduced point mutation in the *pol* gene of pHBV1.5 which could produce the truncated form of the HBV polymerase without affecting the expression of any other HBV-related proteins. Mice hydrodynamically injected with mutant pHBV1.5 produced HBsAg as well as HBcAg at levels similar to those of wild-type pHBV1.5-injected mice 3 days after injection (Fig. 4A

and B). However, mutant pHBV1.5-induced expression of HBsAg, HBeAg and HBcAg was terminated within 2 months, in striking contrast to wild-type pHBV1.5-induced gene expression (Fig. 1B and D). Northern blot analysis confirmed the transient expression of HBV genes after injection of mutant pHBV1.5 (Fig. 4C).

HBV DNA polymerase binds to the 5' end of its own mRNA template, and the complex is then packaged into nucleocapsids, where viral DNA synthesis occurs [19]. HBV genomic DNA produced via the reverse transcription pathway predominantly consists of relaxed-circular DNA with a complete minus strand and a partially synthesized plus strand. In natural HBV infection in humans, part of the nucleocapsids migrates to the nucleus where relaxed-circular DNA is converted to cccDNA that serves as a template for transcription [19]. The finding in the present model of long-term expression of HBV involving HBV DNA replication suggested that viral cccDNA may be produced in murine livers and work as a transcriptional



**Fig. 3.** Production of HBV in hydrodynamically-transfected mice. (A) Density of HBV. Sera from mice injected 3 days earlier with pHBV1.5 were treated with or without *detergent* then fractionated through sucrose density gradient. DNA was isolated from each fraction after treatment with DNase I and then the presence of the HBV DNA sequence was checked by PCR. The density ( $\text{g}/\text{cm}^3$ ) of each fraction is indicated above each lane. (B) Viral titers in serum. The levels of HBV DNA were serially determined by real-time PCR.

template for HBV expression, in addition to the inoculated plasmid. To examine the presence of cccDNA in the liver, we used a PCR procedure which selectively detects cccDNA (Fig. 1). We also checked for the presence of inoculated plasmids by amplifying the ampicillin resistance gene by PCR. The authenticity of the cccDNA detection was confirmed by the detection of a specific signal from liver tissues of patients with chronic hepatitis B, but not from the serum of patients or pHBV1.5 (Fig. 5A). Viral cccDNA was clearly detected in wild-type pHBV1.5-injected livers at 3 days as well as 3 months after the injection (Fig. 5B). As expected, cccDNA was not detected in mutant pHBV1.5-injected livers. The levels of cccDNA were measured by real-time PCR ( $n=5$  for each time point) and results were  $2.4 \times 10^7$  and  $6.0 \times 10^5$  copies per gram of liver tissue at 3 days and 2 months after the injection, respectively. Since the liver approximately contains  $1.1 \times 10^8$  of hepatocytes, the average copy numbers of HBV cccDNA per core Ag-positive hepatocyte could be estimated to be 1 or 4. Ampicillin resistance gene was similarly amplified from both wild-type pHBV1.5- and mutant pHBV1.5-injected livers. The fact that HBV gene expression was terminated

within 2 months upon injection of mutant pHBV1.5 clearly indicates that the presence of residual plasmids in the livers at later time points is not sufficient for the expression of detectable levels of HBV genes; this is consistent with a previous report [20] demonstrating that transgene expression is rapidly terminated after hydrodynamic gene delivery despite the persistence of plasmid DNA in the livers. These results support the idea that viral cccDNA is critically involved in the long-term expression and carriage of HBV in this model.

### 3.4. Administration of IFN $\alpha$ gene transiently suppressed HBV DNA replication and failed to eradicate viral template

We next sought to examine the potential usefulness of this model for the assessment of anti-viral drugs. To examine the effect of IFN $\alpha$  in the phase of cccDNA-dependent HBV replication, we injected either pCMV-IFN $\alpha$ 1 or pCMV at 70 days after pHBV1.5 injection. Injection of pCMV-IFN $\alpha$ 1 led to substantial IFN $\alpha$  production at day 1 (Fig. 6A), although IFN $\alpha$  could not be detected in the mock-injected mice (data not shown). The levels of IFN $\alpha$  after pCMV-IFN $\alpha$ 1 injection rapidly declined at day 3 and could not be detected at day 28. Injection of pCMV-IFN $\alpha$ 1 significantly suppressed viral production at day 3 but did not affect HBs production (Fig. 6B and C); this is consistent with previous findings [15,21] that IFN $\alpha$  suppressed HBV replication at a step of reverse transcription. In spite of the substantial suppression of HBV production at day 3, the levels of viral titers of mice injected with pCMV-IFN $\alpha$ 1 increased to levels similar to those of pCMV-injected mice at day 14 and later. These results indicate that IFN treatment substantially suppressed viral replication, but could not eliminate the viral template from the infected host. This model should be useful for assessing anti-viral therapy aimed at eradication of the viral template.

## 4. Discussion

In the present study, we demonstrated that hydrodynamic injection of a plasmid encoding an overlength of HBV DNA into nude mice established long-term replication of HBV in the liver. Since hepatic damage was not observed, this model mimics the chronic carrier-like state of human HBV infections. This model reminds us of a 1988 report by Feitelson et al., [22] in which they stated that intrahepatic injection of replication competent HBV DNA led to persistent HBs antigenemia as well as chronic liver injury in nude mice. They had no evidence of HBV replication such as production of Dane particles in the circulation. In a preliminary experiment, we intrahepatically injected pHBV1.5 into nude mice and monitored viral production in the serum. DNase I-resistant HBV DNA could not be detected in most mice tested; a small number of mice

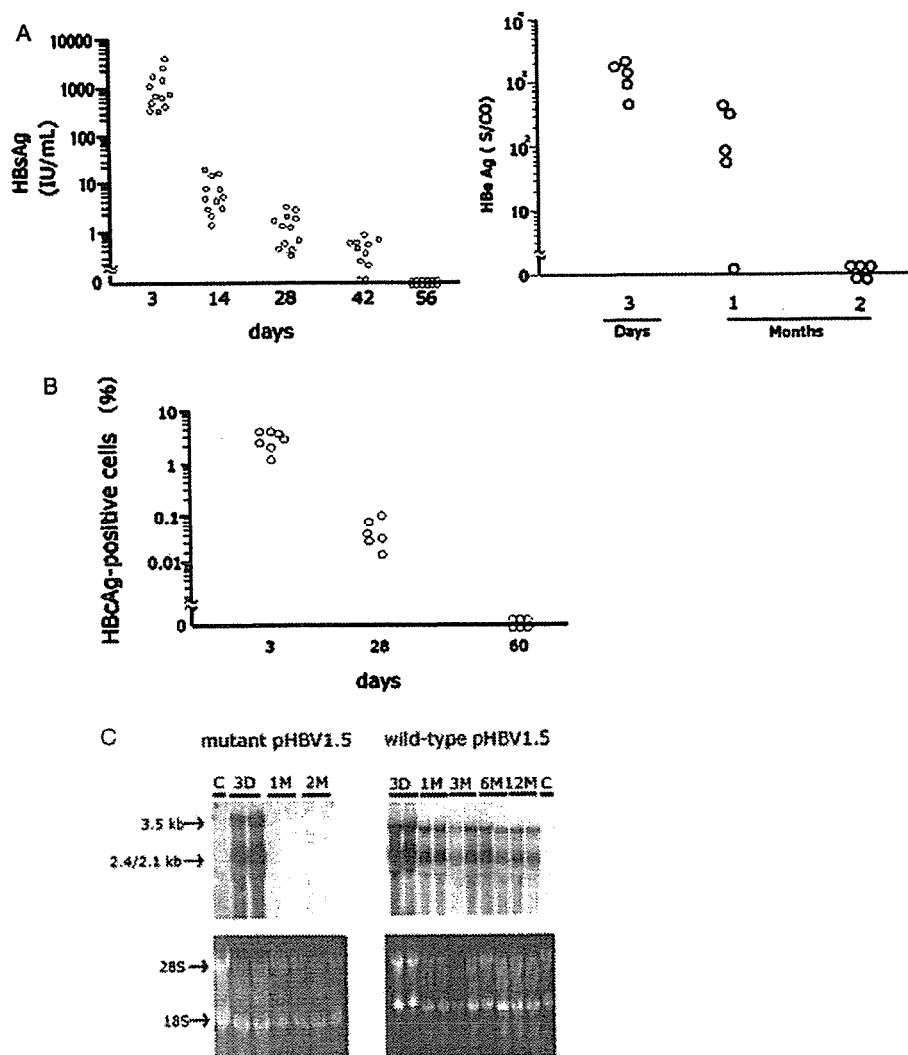


Fig. 4. Expression of HBV genes in nude mice injected with replication-incompetent pHBV1.5. (A) Serial detection of HBsAg and HBeAg in serum. (B) Frequency of HBcAg-positive hepatocytes in the livers. (C) Time-course of HBV RNA expression in the livers transfected with mutant pHBV1.5 or wild-type pHBV1.5 determined by Northern blot. C, control livers; 3D, 1M, 3M, 6M, and 12M, liver samples obtained at 3 days, 1 month, 3, 6, and 12 months after hydrodynamic injection, respectively.

produced low levels of virus at 3 days after injection but not at later time points (our unpublished data). Thus, the transfection efficiency of hydrodynamic injection of HBV DNA appeared to be higher than that of intrahepatic injection. Despite the difference in liver damage observed among these studies, we considered the absence of hepatitis in the present model reasonable, since the T-cell immune response towards HBV-related antigens could not occur. Furthermore, it should be noted that the antigenemia as well as HBV production achieved by the hydrodynamic procedure was very reproducible, which is critically important when applying this model to evaluate the efficacy of anti-viral drugs.

The duration of hydrodynamics-based gene expression varies among reports from days to months [19,23,24]. The plasmid-based gene expression of our model terminated

within 2 months, as demonstrated by the injection of replication-incompetent HBV DNA (mutant pHBV1.5). Replication-competent HBV DNA (wild-type pHBV1.5)-injected mice displayed a rapid decline of HBsAg production followed by relatively stable antigenemia for more than 1 year (Fig. 2B). Although the rapid decline observed in the first 2 weeks may reflect the plasmid-based gene expression, stable expression of HBsAg at later time points did not depend on residual plasmids in the livers, but required intracellular reproduction of HBV DNA. These results indicate that HBV replication in addition to immunological tolerance is critically important for long-term HBV expression in this system. Previous research on *in vivo* gene transfer [8] and transgenic mice [5] has indicated that HBV cccDNA, the template of HBV replication in natural infection, could not be detected in