一方，チンパンジーへの感染実験では，樹状細胞の機能低下は認められないとする結果や，C型慢性肝炎患者の検討で樹状細胞の成熟化やアロ刺激能は正常であることも報告されており，樹状細胞のC型肝炎への関与は今後さらなる検討が必要である．

5. HCV蛋白による細胞に対するその他の影響

HCVトランスジェニックマウスの系で，HCV蛋白がインターフェロンによりもたらされる細胞内伝達シグナル(Jak-STAT系)を抑制することが示唆され，これがインターフェロン不応性の一因となることが想定されている[10]．また，HCV蛋白は感染した肝細胞のFasを介したアポトーシスを抑制しており，これも持続感染を誘導するのに重要であると指摘している．一方，HCVコア蛋白はJurkat細胞に対しFasを介したアポトーシスの経路を活性化させるとの報告もある[11]．細胞内コア蛋白は，TNFレセプターの細胞内ドメイン(TNFRI)あるいはFasと結合し，細胞にアポトーシスを誘導することが証明され，肝細胞やリンパ球のアポトーシスに関与する可能性も考えられている．

V．HCV特異的調節性T細胞の関与

生体の免疫応答を抑制する要因の一つとして，抗原を特異的に認識してIL-10やTGF-βを産生する調節性T細胞(Tr)が注目されている．C型慢性肝炎患者においては，Tr細胞と考えられる$CD4^+$ $CD25^+$ T細胞のfrequencyが高く，この細胞集団は直接T細胞の機能を抑制し，これがHCV特異的細胞性免疫の質的，量的な抑制を引き起こして，肝炎の持続化に寄与していると想定されている[12]．HCVコア蛋白に特異的なTr細胞がC型慢性肝炎の末梢血から分離誘導され，この細胞が産生するIL-10がHCV感染の持続化に関与すると報告された[13]．また，C型肝炎患者の肝内にはIL-10を産生するHCV特異的$CCR7^-$ $CD8^+$調節性T細胞が存在し，肝内に多数集積しているHCV特異的$CCR7^-$ $CD8^+$メモリーT細胞の機能を抑制することも報告された[14]．さらに，HCV NS4蛋白は，C型肝炎患者のみならず，健常者の末梢単核球からもIL-10の産生を促しIL-12の分泌を抑制させ，さらに樹状細胞の分化成熟化を抑制するとの報告もあり，細胞性免疫の活性化抑制の一つの機序として興味深い[15]．

以上のようにTr細胞のHCV感染持続化への関与が強く示唆されているが，まだ不明な点も多くさらなる検討が必要である．

VI．その他のHCV持続感染の機序

非構造タンパク領域であるNS5AのC末端側に存在する40個のアミノ酸からなる部分(IFN-sensitivity determining region)の変異は，IFNにより誘導され抗ウイルス効果を発揮するプロテインキナーゼと結合しその活性化を阻害することによりIFN治療抵抗性を得るとされている．また，HCV NS3/4のセリンプロテアーゼは，細胞が抗ウイルス効果を発揮するうえで重要なinterferon regulatory factor-3を抑制するといわれている．また，分子レベルではウイルスが感染細胞に，ウイルス遺伝子発現抑制，抗原のプロセッシング抑制といった影響を与えていることも想定されている．

おわりに

肝炎動物モデルやの臨床検体の解析などにより，ウイルス肝炎における免疫応答が長年にわたり研究されてきた．肝細胞障害にはCTLを中心とした細胞性免疫応答の関与が明らかになり，その障害機序や生体免疫応答の抑制機序も徐々に解明されてきている．免疫応答を適切にコントロールすることは肝炎ウイルス排除あるいは肝炎の鎮静化に重要であるが，これから解明していかなければならない問題も多々ある．これらの問題点を

生体免疫反応のみならず，ウイルス側からも詳細に解明していくことで，将来，肝炎ウイルスを完全に生体から排除できる治療法の開発が可能になると考えられる．今後のウイルス学，免疫学のさらなる発展を期待する．

文　献

1) Wertheimer AM, Miner C, Lewinsohn DM, et al：Novel CD4$^+$ and CD8$^+$ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. Hepatology 37 (3)：577-589, 2003.
2) Hiroishi K, Kita H, Kojima M, et al：Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. Hepatology 25(3)：705-712, 1997.
3) Ando K, Hiroishi K, Kaneko T, et al：Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. J Immunol 158 (11)：5283-5291, 1997.
4) Wang JH, Layden TJ, Eckels DD：Modulation of the peripheral T-Cell response by CD4 mutants of hepatitis C virus：transition from a Th1 to a Th2 response. Hum Immunol 64 (7)：662-673, 2003.
5) Tseng CT, Klimpel GR：Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. J Exp Med 195(1)：43-49, 2002.
6) Francavilla V, Accapezzato D, De Salvo M, et al：Subversion of effector CD8$^+$ T cell differentiation in acute hepatitis C virus infection:exploring the immunological mechanisms. Eur J Immunol 34(2)：427-437, 2004.
7) Konan KV, Giddings THJr, Ikeda M, et al：Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. J Virol 77 (14)：7843-7855, 2003.
8) Mehal WZ, Azzaroli F, Crispe IN：Immunology of the healthy liver：old questions and new insights. Gastroenterology 120(1)：250-260, 2001.
9) Jinushi M, Takehara T, Tatsumi T, et al：Autocrine/paracrine IL-15 that is required for type Ⅰ IFN-mediated dendritic cell expression of MHC class Ⅰ-related chain A and B is impaired in hepatitis C virus infection. J Immunol 171(10)：5423-5429, 2003.
10) Blindenbacher A, Duong FH, Hunziker L, et al：Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology 124 (5)：1465-1475, 2003.
11) Moorman JP, Prayther D, McVay D, et al：The C-terminal region of hepatitis C core protein is required for Fas-ligand independent apoptosis in Jurkat cells by facilitating Fas oligomerization. Virology 312(2)：320-329, 2003.
12) Sugimoto K, Ikeda F, Stadanlick J, et al：Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. Hepatology 38(6)：1437-1448, 2003.
13) MacDonald AJ, Duffy M, Brady MT, et al：CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. J Infect Dis 185(6)：720-727, 2002.
14) Accapezzato D, Francavilla V, Paroli M, et al：Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 113(7)：963-972, 2004.
15) Brady MT, MacDonald AJ, Rowan AG, et al：Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. Eur J Immunol 33 (12)：3448-3457, 2003.

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Alteration in Gene Expression Profile by Full-Length Hepatitis B Virus Genome

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Key Words
Hepatitis B virus · Hepatitis B virus-expressing cell · DNA array assay · Corroboration assay · Hepatocarcinogenesis

Abstract
Persistent expression of hepatitis B virus (HBV) proteins is thought to be involved in virus-related hepatocarcinogenesis. Here, we compared the gene expression profile of cells persistently expressing the full-length HBV with that of negative control cells to comprehensively investigate virus-mediated changes in the gene expression of the host cells. RNA samples from both virus-expressing and negative control cells were used for the DNA array assay. DNA array assay and subsequent corroboration assays revealed that expression of 14 of 1,176 genes (1.2%) was altered in response to virus expression. The upregulated genes included CD44, high mobility group protein-I, thymosin beta-10 and 27-kD heat shock protein, while the downregulated genes included NM23-H1, all of which are thought to be associated with the development or progression of carcinoma in the liver or other organs. Furthermore, virus expression resulted in the decrease of two apoptosis-inducing molecules, caspase-3 and BAX, which may also contribute to carcinogenesis through prolonged survival of the host cell. Thus, expression of the virus genome caused carcinogenesis-related changes in host cell gene expression. HBV expression may change the host cell to a malignant phenotype through alterations in the expression levels of a set of genes.

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Introduction

Hepatitis B virus (HBV) is a major causative agent of acute and chronic liver diseases. Chronic HBV infection eventually results in more serious liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. HBV is a circular, partially double-stranded DNA virus of approximately 3.2 kb in length that encodes four kinds of viral proteins, i.e. preS/S, precore/core, polymerase and X proteins. Among these HBV proteins, the role of the X protein (HBx) in HBV-mediated pathogenesis has been studied most extensively. HBx displays tumorigenic transforming activity in vitro [3] and in vivo [4]. Also, HBx is thought to considerably modify cellular apoptotic processes under various apoptosis-inducing stimuli [5–7]. HBx acts as a transcription activator for many cellular and virus promoters and enhancers [reviewed in ref. 8]. In

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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. Matsubara (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_2-95% 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 $(dT)_{20}$ 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-

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

Genes		Primer sequence (5′ to 3′)
CD44	sense	CATCTACCCCAGCAACCCTA
	antisense	CTTCTGCCCACACCTTCTTC
HMG-I	sense	AGTGAGTCGAGCTCGAAGTC
	antisense	GTCTCTTAGGTGTTGGCACT
TB-10	sense	GGAAATCGCCAGCTTCGATA
	antisense	AATCCCTCCAGGATCTTAGG
α_1-AT	sense	GGGTCAACTGGGCATCACTA
	antisense	CCATGAAGAGGGGAGACTTG
α_1-AG	sense	AGAGTACCAGACCCGACAGG
	antisense	CTCTCCTTCTCGTGCTGCTT
IGFBP-1	sense	GAGAGCACGGAGATAACTGAGG
	antisense	AACCACTGTACCTCTCGGAAGC
BTEB2	sense	ACTTACTTTCCCCCGTCACC
	antisense	CAGCCTTCCCAGGTACACTT
TOPO-IIα	sense	TGTCACCATTGCAGCCTGTA
	antisense	GTCGAGAAGGGTATAATAGG
HIF-1α	sense	TGTAATGCTCCCCTCACCCAACGAA
	antisense	GTGACCCTGATAATCCGAGTCCACT
ROR1	sense	CCTCATGACAGAGTGCTGGA
	antisense	GAGGACCTGTTGGCTGGTAG
β-Actin	sense	ACACTGTGCCCATCTACGAGG
	antisense	AGGGGCCGGACTCGTCATACT

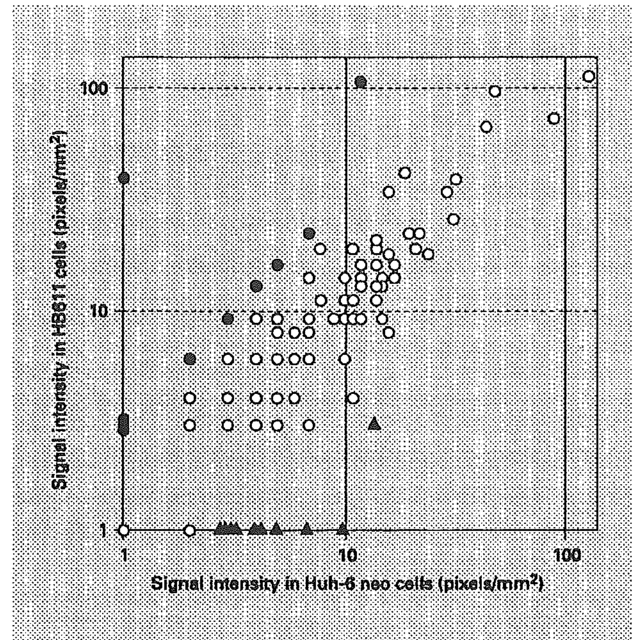

Fig. 1. Comparison of gene expression levels between Huh-6 neo and HBV-expressing HB611 cells in DNA array analysis. The signal intensities of each gene were quantitated with Atlas Image software (Clontech Laboratories). ● = Genes upregulated by HBV expression, with a HB611 to Huh-6 neo signal intensity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

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 (α_1-AT), alpha-1-acid glycoprotein 1 (α_1-AG), insulin-like growth factor-binding protein 1 (IGFBP-1) and basic transcription element-binding protein 2 (BTEB2), were upregulated, whereas 3 genes, i.e. DNA topoisomerase II alpha (TOPO-IIα), hypoxia-inducible factor 1 alpha (HIF-1α) 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.

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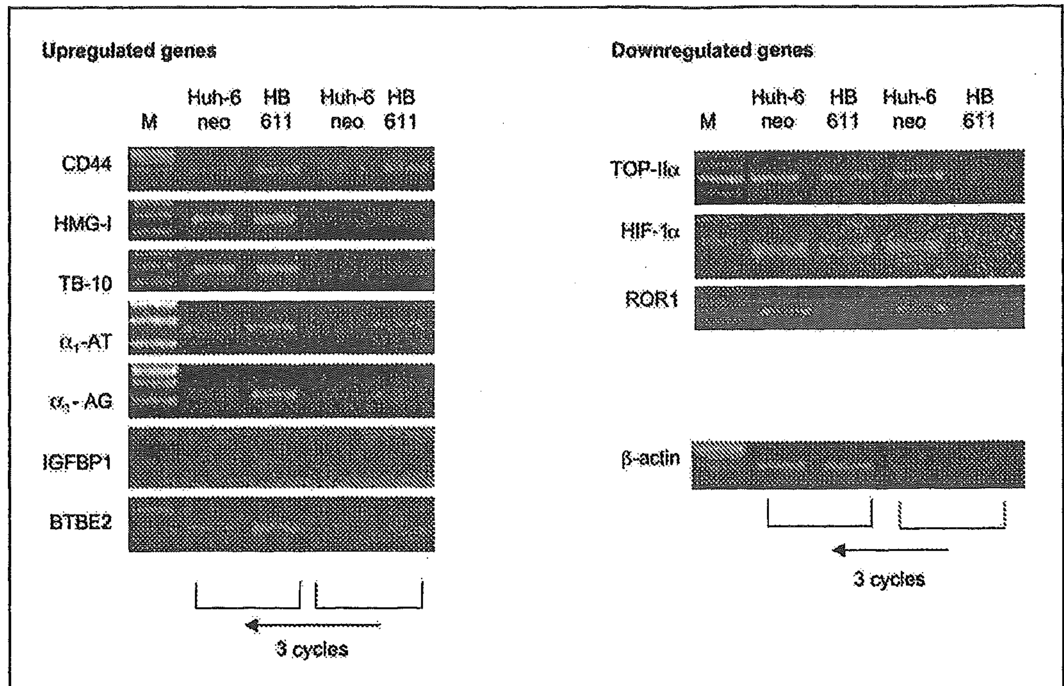

Fig. 2. RT-PCR assay for the detection of HBV-responsive genes. Total RNA samples were extracted from Huh-6 neo and HB611 cells and used for RT-PCR assay. The left panel represents upregulated genes (8 genes), whereas the right panel represents downregulated genes (6 genes). The right bottom panel shows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

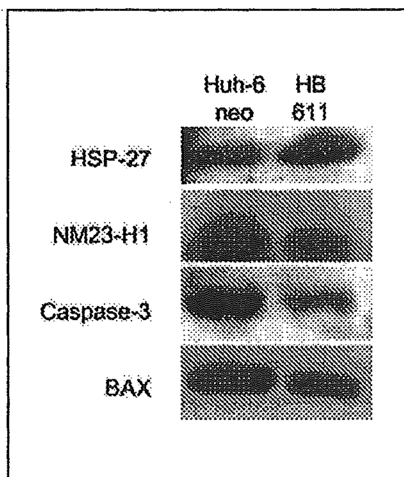

Fig. 3. Western blot analysis for the detection of HBV-responsive genes. Total cellular lysates were extracted from Huh-6 neo and HB611 cells and subjected to Western blot analysis to examine HSP-27, NM23-H1, caspase-3 and BAX expression levels.

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-

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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 β 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, α_1-AT and α_1-AG, were also increased by HBV expression in the present study. Serum α_1-AT and α_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 α_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, α_1-AT and α_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 cellular apoptosis under various stimuli [7]. Inthe 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-IIα is a nuclear enzyme that changes the topology of DNA and is essential for chromosome segregation at mitosis. High expression levels of TOPO-IIα are reported in lung cancer [43], in contrast to the suppression of TOPO-IIα by HBV expression observed in the present study. HIF-1α is involved in the transcriptional regulation of a variety of genes related to angiogenesis [44]. HBV expression, however, sup-

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 ≥ 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 ≤ 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.

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presses HIF-1α 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.

References

1 Beasley RP, Hwang LY, Lin CC, Chien CS: Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet 1981;ii:1129–1133.
2 Weissberg JI, Andres LL, Smith CI, Weick S, Nichols JE, Garcia G, Robinson WS, Merigan TC, Gregory PB: Survival in chronic hepatitis B: An analysis of 379 patients. Ann Intern Med 1984;101:613–616.
3 Hohne M, Schaefer S, Seifer M, Feitelson MA, Paul D, Gerlich WH: Malignant transformation of immortalized transgenic hepatocytes after transfection with hepatitis B virus DNA. EMBO J 1990;9:1137–1145.
4 Kim CM, Koike K, Saito I, Miyamura T, Jay G: HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351:317–320.
5 Wang XW, Gibson MK, Vermeulen W, Yeh H, Forrester K, Sturzbecher HW, Hoeijmakers JH, Harris CC: Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. Cancer Res 1995;55:6012–6016.
6 Su F, Schneider RJ: Hepatitis B virus HBx protein sensitizes cells to apoptotic killing by tumor necrosis factor alpha. Proc Natl Acad Sci USA 1997;94:8744–8749.
7 Gottlob K, Fulco M, Levrero M, Graessmann A: The hepatitis B virus HBx protein inhibits caspase 3 activity. J Biol Chem 1998;273:33347–33353.
8 Andrisani OM, Barnabas S: The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis (review). Int J Oncol 1999;15:373–379.
9 Benn J, Schneider RJ: Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. Proc Natl Acad Sci USA 1994;91:10350–10354.
10 Klein NP, Schneider RJ: Activation of Src family kinases by hepatitis B virus HBx protein and coupled signaling to Ras. Mol Cell Biol 1997;17:6427–6436.
11 Kekule AS, Lauer U, Weiss L, Luber B, Hofschneider PH: Hepatitis B virus transactivator HBx uses a tumour promoter signalling pathway. Nature 1993;361:742–745.
12 Benn J, Su F, Doria M, Schneider RJ: Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases. J Virol 1996;70:4978–4985.
13 Chirillo P, Falco M, Puri PL, Artini M, Balsano C, Levrero M, Natoli G: Hepatitis B virus pX activates NF-kappa B-dependent transcription through a Raf-independent pathway. J Virol 1996;70:641–646.
14 Maguire HF, Hoeffler JP, Siddiqui A: HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science 1991;252:842–844.
15 Cheong JH, Yi M, Lin Y, Murakami S: Human RPB5, a subunit shared by eukaryotic nuclear RNA polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. EMBO J 1995;14:143–150.
16 Lin Y, Nomura T, Cheong J, Dorjsuren D, Iida K, Murakami S: Hepatitis B virus X protein is a transcriptional modulator that communicates with transcription factor IIB and the RNA polymerase II subunit 5. J Biol Chem 1997;272:7132–7139.
17 Qadri I, Conaway JW, Conaway RC, Schaack J, Siddiqui A: Hepatitis B virus transactivator protein, HBx, associates with the components of TFIIH and stimulates the DNA helicase activity of TFIIH. Proc Natl Acad Sci USA 1996;93:10578–10583.
18 Qadri I, Maguire HF, Siddiqui A: Hepatitis B virus transactivator protein X interacts with the TATA-binding protein. Proc Natl Acad Sci USA 1995;92:1003–1007.
19 Feitelson MA, Zhu M, Duan LX, London WT: Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene 1993;8:1109–1117.
20 Sitterlin D, Lee TH, Prigent S, Tiollais P, Butel JS, Transy C: Interaction of the UV-damaged DNA-binding protein with hepatitis B virus X protein is conserved among mammalian hepadnaviruses and restricted to transactivation-proficient X-insertion mutants. J Virol 1997;71:6194–6199.
21 Rahmani Z, Huh KW, Lasher R, Siddiqui A: Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. J Virol 2000;74:2840–2846.
22 Ono M, Morisawa K, Nie J, Ota K, Taniguchi T, Saibara T, Onishi S: Transactivation of transforming growth factor alpha gene by hepatitis B virus preS1. Cancer Res 1998;58:1813–1816.
23 Kekule AS, Lauer U, Meyer M, Caselmann WH, Hofschneider PH, Koshy R: The preS2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. Nature 1990;343:457–461.
24 Tsurimoto T, Fujiyama A, Matsubara K: Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. Proc Natl Acad Sci USA 1987;84:444–448.
25 Haramaki M, Yano H, Fukuda K, Momosaki S, Ogasawara S, Kojiro M: Expression of CD44 in human hepatocellular carcinoma cell lines. Hepatology 1995;21:1276–1284.
26 Mathew J, Hines JE, Obafunwa JO, Burr AW, Toole K, Burt AD: CD44 is expressed in hepatocellular carcinomas showing vascular invasion. J Pathol 1996;179:74–79.
27 Lara-Pezzi E, Serrador JM, Montoya MC, Zamora D, Yanez-Mo M, Carretero M, Furthmayr H, Sanchez-Madrid F, Lopez-Cabrera M: The hepatitis B virus X protein (HBx) induces a migratory phenotype in a CD44-dependent manner: Possible role of HBx in invasion and metastasis. Hepatology 2001;33:1270–1281.
28 Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JB, Liotta LA, Sobel M: Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 1988;80:200–204.
29 Iizuka N, Oka M, Noma T, Nakazawa A, Hirose K, Suzuki T: NM23-H1 and NM23-H2 messenger RNA abundance in human hepatocellular carcinoma. Cancer Res 1995;55:652–657.

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30 Boix L, Bruix J, Campo E, Sole M, Castells A, Fuster J, Rivera F, Cardesa A, Rodes J: nm23-H1 expression and disease recurrence after surgical resection of small hepatocellular carcinoma. Gastroenterology 1994;107:486–491.
31 Chiappetta G, Tallini G, De Biasio MC, Manfioletti G, Martinez-Tello FJ, Pentimalli F, de Nigris F, Mastro A, Botti G, Fedele M, Berger N, Santoro M, Giancotti V, Fusco A: Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. Cancer Res 1998;58:4193–4198.
32 Abe N, Watanabe T, Sugiyama M, Uchimura H, Chiappetta G, Fusco A, Atomi Y: Determination of high mobility group I(Y) expression level in colorectal neoplasias: A potential diagnostic marker. Cancer Res 1999;59:1169–1174.
33 Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, Fujioka Y, Chiappetta G, Fusco A, Atomi Y: Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. Cancer Res 2000;60:3117–3122.
34 Reeves R, Edberg DD, Li Y: Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol Cell Biol 2001;21:575–594.
35 Santelli G, Califano D, Chiappetta G, Vento MT, Bartoli PC, Zullo F, Trapasso F, Viglietto G, Fusco A: Thymosin β-10 gene overexpression is a general event in human carcinogenesis. Am J Pathol 1999;155:799–804.
36 Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke Y, Foster CS: Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res 2000;60:7099–7105.
37 Love S, King RJ: A 27 kDa heat shock protein that has anomalous prognostic powers in early and advanced breast cancer. Br J Cancer 1994;69:743–748.
38 Han J, Yoo HY, Choi BH, Rho HM: Selective transcriptional regulations in the human liver cell by hepatitis B viral X protein. Biochem Biophys Res Commun 2000;272:525–530.
39 Fabris C, Pirisi M, Soardo G, Toniutto P, Falleti E, Vitulli D, Pezzetta F, Gonano F, Bartoli E: Diagnostic usefulness of acute-phase protein measurement in hepatocellular carcinoma. Cancer Invest 1996;14:103–108.
40 Tzonou A, Sparos L, Kalapothaki V, Zavitsanos X, Rebelakos A, Trichopoulos D: α_1-antitrypsin and survival in hepatocellular carcinoma. Br J Cancer 1990;61:72–73.
41 Beerheide W, Tan YJ, Teng E, Ting AE, Jedpiyawongse A, Srivatanakul P: Downregulation of proapoptotic proteins Bax and Bcl-Xs in p53 overexpressing hepatocellular carcinomas. Biochem Biophys Res Commun 2000;273:54–61.
42 Shimasaki S, Ling N: Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). Prog Growth Factor Res 1991;3:243–266.
43 Mirski SEL, Voskoglou-Nomikos T, Young LC, Deeley RG, Campling BG, Gerlach JH, Cole SPC: Simultaneous quantitation of topoisomerase II α and β isoform mRNAs in lung tumor cells and normal and malignant lung tissue. Lab Invest 2000;80:787–795.
44 Wang GL, Semenza GL: General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 1993;90:4304–4308.
45 Masiakowski P, Carroll RD: A novel family of cell surface receptors with tyrosine kinase-like domain. J Biol Chem 1992;267:26181–26190.
46 Watanabe N, Kurabayashi M, Shimomura Y, Kawai-Kowase K, Hoshino Y, Manabe I, Watanabe M, Aikawa M, Kuro-o M, Suzuki T, Yazaki Y, Nagai R: BTEB2, a Kruppel-like transcription factor, regulates expression of the SMemb/nonmuscle myosin heavy chain B (SMemb/NMHC-B) gene. Circ Res 1999;85:182–191.

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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 $CD56^+CD3^-$ lymphocytes. In some cases, enriched NK cells were cultured in 24-well-culture plates (5.0×10^5/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 λ (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}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 γ-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) × 100/(maximum release − spontaneous release). The spontaneous release in all assays was less than 20% of the maximum release.

2.7. Measurements of IFNγ production of NK cells

NK cells were cultured with Huh7 or K562 at a ratio of 1:1 for 24 h. Interferon γ (IFNγ) 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 μg/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 (1.0×10^5/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 (1.0×10^5/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 (1.0×10^4/well) and then cultured with the responder naive $CD4^+$ cells isolated from allogeneic donors (1.0×10^5/well) for 72 h. The cocultured cells were pulsed with 1 μCi/well of [^{3}H] thymidine for 16 h of incubation and collected onto a glass fiber filter. [^{3}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 μl/mL GolgiPlug (BD-Pharmingen) for 4 h at 37 °C. At the end of the incubation period, $CD4^{+}T$ cells were stained with PC5-labeled CD4 mAb (Beckman-Coulter), and then fixed and permeabilized with Cytofix/Cytoperm buffer (BD-Pharmingen) for 15 min at room temperature. The permeabilized cells were stained with FITC-labeled anti-IFNγ 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; $P<0.01$)

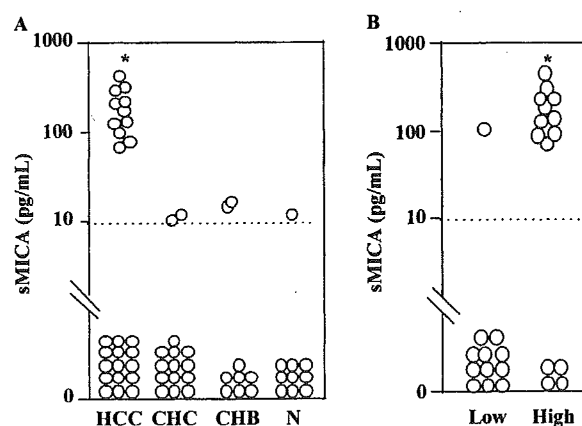

Fig. 1. Serum levels of sMICA. (A) Serum levels of sMICA in patients with HCC (HCC; $n=26$), those with chronic hepatitis C (CHC; $n=15$), those with chronic hepatitis B (CHB; $n=9$) and healthy donors (N; $n=10$). Dotted line indicates the threshold of detection of the ELISA assay. (B) Serum levels 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. 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 cytolysis 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

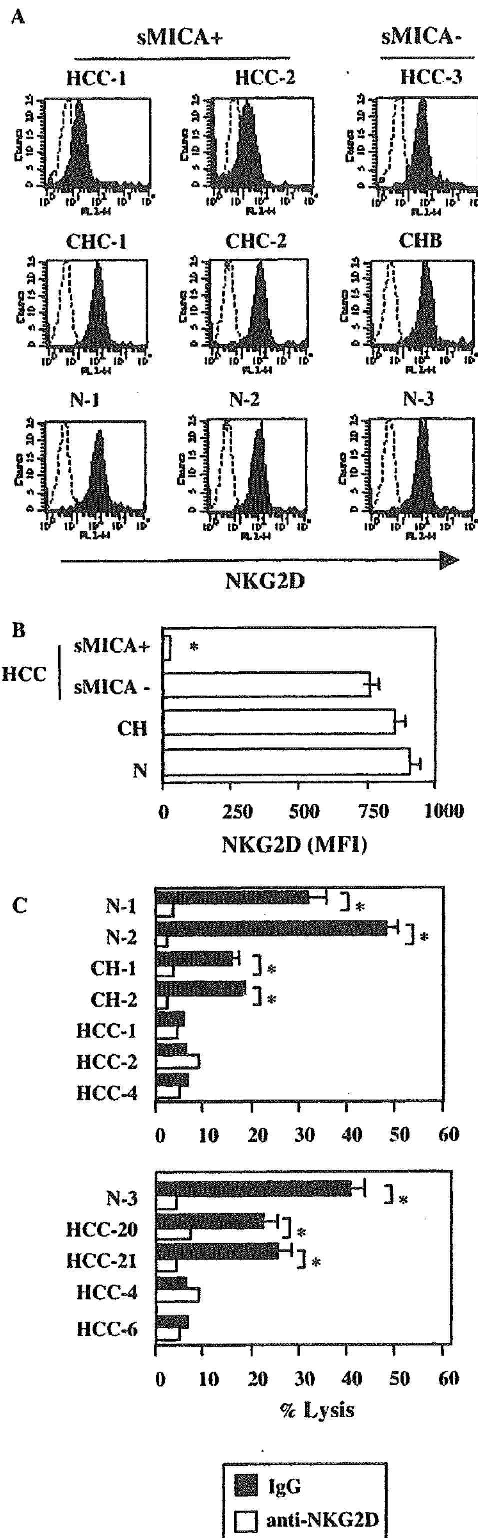

Fig. 2. Expression of NKG2D on NK cells and NKG2D-dependent NK cell activities. (A, B) Surface expression of NKG2D on CD56$^+$ NK cells. Surface expression of NKG2D on NK cells for patients with HCC with serum sMICA-positive (sMICA+) or negative (sMICA−), chronic hepatitis C (CHC), chronic hepatitis B (CHB) and healthy donors (N). 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 ^{51}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. *$P<0.01$.

with sMICA-positive serum, upon exposure to Huh7 cells, produced lesser amounts of IFNγ than those pre-stimulated with sMICA-negative serum. The inhibitory effect of sMICA-positive serum on IFNγ 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

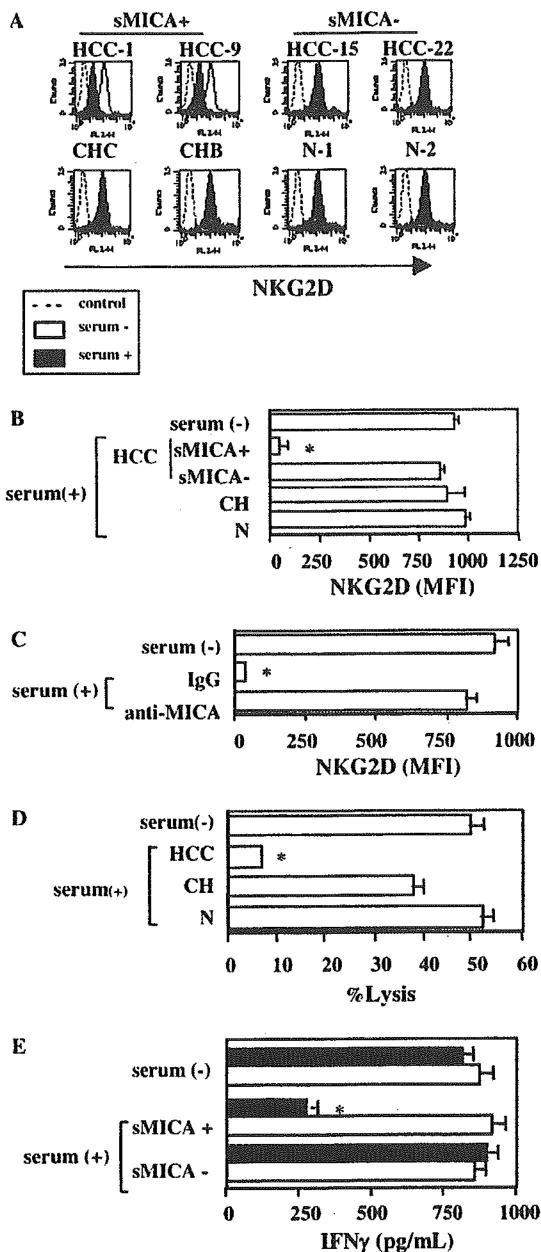

Fig. 3. Effect of sMICA-containing serum on NKG2D expression of NK cells from healthy donors. (A, B) NK cells from healthy donors were incubated with (serum (+)) or without (serum (−)) serum of HCC patients with sMICA-positive (sMICA+) or sMICA-negative (sMICA−), chronic hepatitis (CH) or healthy donors (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 (−)) serum of each group as described above were cultured with ^{51}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 IFNγ 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. IFNγ in each culture supernatant was measured by ELISA. *$P<0.01$.

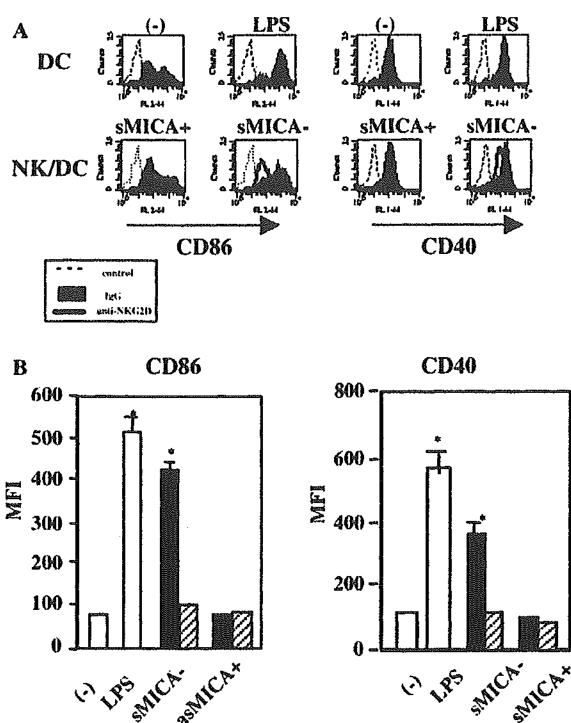

Fig. 4. sMICA-mediated inhibition of NK cell ability on DC maturation. NK cells derived from healthy volunteers were incubated with sMICA-positive (sMICA+) or negative (sMICA−) serum of patients with HCC for 48 h. After extensive washing, NK cells were cocultured with DC from the same healthy donors 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$^+$ 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$^+$T cells substantially produced IFNγ 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 IFNγ production of CD4$^+$T cells. In contrast, DC-mediated IFNγ production of CD4$^+$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 TGFβ also has an effect on down-regulation of NKG2D expression [25], and the serum levels of TGFβ increase in patients with HCC [26]. However, addition of anti-TGFβ 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 TGFβ, 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

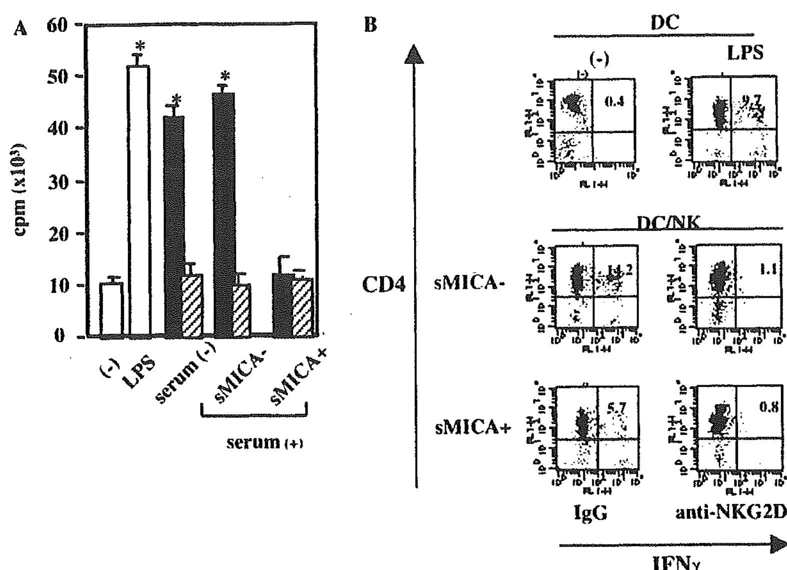

Fig. 5. Impaired NKG2D-mediated NK cell activation of DC allostimulatory capacity by sMICA. NK cells from healthy donors were cultured for 48 h with (serum (+)) or without (serum (−)) sera positive for sMICA (sMICA+) or negative for sMICA (sMICA−). After extensive washing, NK cells were cocultured with DC and Huh7 in the presence of anti-NKG2D mAb (hatched bar) or control IgG (closed bar) for 24 h. Allogeneic naive $CD4^{+}T$ cells (1×10^{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 $CD4^{+}T$ cells was examined by [^{3}H]thymidine corporation. *$P<0.01$ (B) Intracellular expression of IFNγ in $CD4^{+}T$ cells was determined by FACS analysis. The number in each quadrant represents the percentage of IFNγ-positive $CD4^{+}T$ 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 $CD8^{+}T$ 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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References

[1] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;62:1907–1917.

[2] Crispe IN. Hepatic T cells and liver tolerance. Nat Rev Immunol 2002;3:51–62.

[3] Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural killer cell surveillance and therapy of cancer. Nat Rev Cancer 2002;2: 850–861.

[4] Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather P. Natural killer cells in antiviral defence: function and regulation by innate cytokines. Ann Rev Immunol 1999;17:189–220.

[5] Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol. 2003;2:957–967.

[6] Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: Dendritic cells and NK cells take centre stage. Nat Rev Immunol. 2005;5:112–124.

[7] Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat Rev Immunol 2001;1:41–49.

[8] Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003;3:781–790.

[9] Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285:727–729.
[10] Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived γδT cells of MICA and MICB. Proc Natl Acad Sci USA 1999;96:6879–6884.
[11] Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K, Plymate SR. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. J Clin Invest 2004;114:560–568.
[12] Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, et al. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. Int J Cancer. 2003; 104:354–361.
[13] Groh V, Wu J, Yee C, Spies T. Tumor-derived soluble MIC ligands impair expression of NKG2D and T cell activation. Nature 2002;419:734–738.
[14] Salih HR, Rammensee HG, Steinle A. Down-regulation of MICA on human tumors by proteolytic shedding. J Immunol 2002;169: 4098–4102.
[15] Doubrovina ES, Doubrovin MM, Vider E, Sisson RB, O'Reilly RJ, Dupon B, et al. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. J Immunol 2003;171:6891–6899.
[16] Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 2003;102:1389–1396.
[17] Groh V, Bruhl A, El-Gabalawy H, Nelson JL, Spies T. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. Proc Natl Acad Sci USA 2003;100: 9452–9457.
[18] Kudo M, Chung H, Haji S, Osaki Y, Oka H, Seki T, Kasugai H, Sasaki Y, Matsunaga T. Validation of a new prognostic staging system for hepatocellular carcinoma: the JIS score compared with the CLIP score. Hepatology 2004;40:1396–1405.
[19] Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med 2002;195:625–636.
[20] Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T, et al. Critical role of MHC class I-related chain A and B expression on IFNα-stimulated dendritic cells in NK cell activation: Impairment in chronic hepatitis C infection. J Immunol 2003;170:1249–1256.
[21] Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, et al. Autocrine/Paracrine IL-15 that is required for type I IFN-mediated dendritic cell expression of MHC class I-related chain A and B is impaired in hepatitis C virus infection. J Immnunol 2003;171: 5423–5429.
[22] Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, et al. Natural killer cells activated by MHC^{LOW} targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 2003;19: 561–569.
[23] Mailliard RB, Son YI, Redlinger R, Coates PT, Giermasz A, Morel P A, et al. Dendritic cells mediate NK cell help for Th1 and CTL responses; Two signal requirement for induction of NK cell helper function. J Immunol 2003;171:2366–2372.
[24] Jinushi M, Takehara T, Tatsumi T, Kanto T, Miyagi T, Suzuki T, et al. The negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to the altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. J Immunol 2004;173:6072–6081.
[25] Castriconi R, Cantoni C, Della-Chiesa M, Vitale M, Marcenaro E, Conte R, et al. Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. Proc Natl Acad Sci USA 2003;100: 4120–4125.
[26] Bedossa P, Peltier E, Terris B, Franco D, Poynard T. Transforming growth factor-beta 1 and TGF-beta 1 receptors in normal, cirrhotic and neoplastic human livers. Hepatology 1995;21:760–766.

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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 IFNα 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. IFNα 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 IFNa1 gene, pCMV-IFNa1, was generously provided by Dr Daniel J.J. Carr (University of Okulahoma, 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 μg 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. Nothern blot

Total tissue RNA was isolated with Isogen (Nippon Gene, Toyama, Japan), and then 30 μg 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 μm 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′-CACATCAGGATTCCTAGGACC-3′; antisense (nucleotides 341–321), 5′-GGTGAGTGATTGGAGGTTGG-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 μm 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-positeve 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′-TATGGCTTCATTCAGCTCCG-3′) and an antisense primer (5′-TCGAACTGGATCTCAACAGC-3′).

2.10. IFNα gene therapy

At 70 days after pHBV1.5 injection, nude mice were hydrodynamically injected with either pCMV-IFNa1 or pCMV mock plasmid and examined

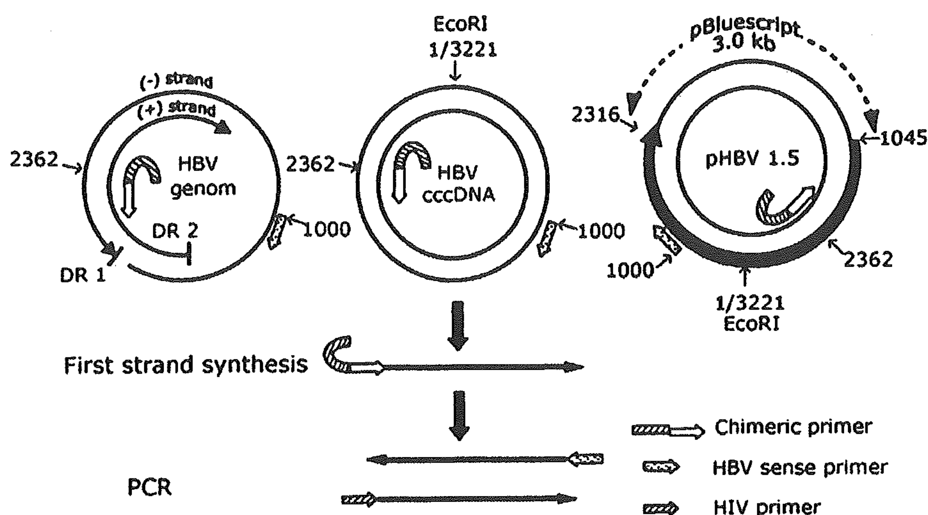

Fig. 1. Principle of PCR detection of HBV cccDNA. Three forms of HBV sequences which could have been present in our samples are shown: HBV genome, HBV cccDNA, and pHBV1.5. The number of nucleotides starts at the EcoRI site. A chimeric primer (5′-TCGCTTTCGGGTCCCTGGTCCCGTCGTC-3′) is composed of two segments: 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′-TCGCTTTCGGGTCCCT-3′) and another primer complementary to the HBV DNA minus strand from nucleotide 1000 to 1016 (HBV sense primer 5′-TTGTGGGTCTTTTGGG-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. IFNα production was assessed using a commercially available mouse IFNα 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 μg 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.