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Improvement of Doxorubicin Efficacy Using Liposomal Anti-Polo-like Kinase 1 siRNA in Human Renal Cell Carcinomas

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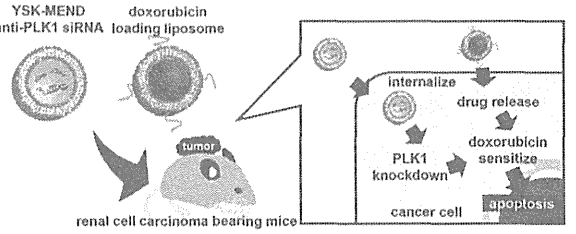
Supporting Information

ABSTRACT: It is well-known that renal cell carcinomas (RCCs) are resistant to classical cytotoxic anticancer drugs. Therefore, facilitating the impact of anticancer drugs by altering the cell phenotype should be a useful strategy for circumventing this. We developed a multifunctional envelope-type nanodevice (MEND) as an *in vivo* carrier of siRNA to tumor tissues. We previously reported that a MEND containing YSK05 (YSK-MEND) efficiently delivered siRNA in RCC-bearing mice. We herein report on a combination therapy involving the use of siRNA-mediated specific gene knockdown and cytotoxic drug doxorubicin (DOX), and an advantage of YSK-MEND as an investigation tool for *in vivo* function of a gene. si-*PLK1* encapsulated within YSK-MEND was prepared using the tertiary butanol dilution method. The *in vitro* cellular viability under the exposure of DOX was compared between OS-RC-2 cells with and without si-*PLK1* transfection. In an *in vivo* study, tumor-bearing mice were systemically injected with YSK-MEND and DOX-loaded liposomes. The combination of DOX and si-*PLK1* drastically reduced tumor growth rate, and apoptotic cells were observed. In an *in vitro* study, *PLK1* knockdown increased G2/M cell population and reduced the expression of cyclin B1 (*CCNB1*) mRNA. *CCNB1* suppression by si-*PLK1* encapsulated in YSK-MEND was also observed in the *in vivo* experiments. A combination of DOX and anti-polo-like kinase 1 siRNA (si-*PLK1*) resulted in a measurable delay in OS-RC-2 tumor growth. This result suggests that the combination of si-*PLK1* delivery and doxorubicin by YSK-MEND holds potential for RCC therapy via cell *CCNB1* regulation.



KEYWORDS: *liposomes, siRNA, combination therapy, renal cell carcinomas, EPR effect, doxorubicin, cell cycle*

INTRODUCTION

Renal cell carcinomas (RCCs) are one of the major forms of cancer, and there are estimated to be 61,000 new patients suffering from RCCs in the U.S.A.[1] Cytokines, such as interleukin-2 and interferon-α, are currently used for the treatment of RCCs because RCCs do not respond to typical anticancer drugs.[2] Although an anti-vascular endothelial cell growth factor (*VEGF*) antibody, multikinase inhibitors, and mTOR inhibitors are currently approved for metastatic RCCs, developing a new remedy for RCCs still remains a significant issue.[3]

Small interfering RNA (siRNA) holds great promise as a therapeutic strategy due to its ability to specifically and efficiently inhibit disease-related genes.[4] However, *in vivo* applications of siRNA are severely limited by their instability in the circulation and poor internalization through the plasma membrane.[5] We recently developed a liposomal siRNA delivery system (MEND; multifunctional envelope-type nanodevice) for efficiently delivering nucleic acids to target cells.[6,7] We previously demonstrated that a MEND containing our original cationic lipid, YSK05 (YSK-MEND), when intravenously administered to tumor-bearing mice, strongly suppressed target gene expression in tumor tissue and the tumor endothelium.[8,9] YSK05 has high membrane fusogenic properties specifically at an acidic pH, because YSK consists of a tertiary amine group in the hydrophilic moiety, which becomes protonated in acidic circumstance.[10] This positively charged headgroup of YSK05 is associated with the endosomal membrane after internalization into target cells, and, hence, siRNA can be released from endosomes via membrane fusion through an inverted hexagonal phase structure between endosomal lipids and YSK05. Since YSK-MEND had no charge on the surface at a neutral pH, YSK-MEND is stable due to lower absorption of serum protein compared to MEND containing normal cationic lipid. The biocompatibility of YSK05 enabled systemically injected YSK-MEND to deliver siRNA into the cytosol of cancer cells, and induce RNAi-mediated silencing even in the *in vivo* circumstance.

In the current report, we propose the use of a combination therapy using DOX and a MEND containing siRNA which improves the sensitivity of cancer cells against DOX. In other words, YSK-MEND could be a useful strategy for an evaluation of a gene of interest under *in vivo* conditions.

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MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-*sn*-glycerophosphoethanolamine (POPE), 1,2-distearoyl-*sn*-glycerophosphocholine (DSPC), dimyristoyl-*sn*-glycerol, methoxypoly(ethylene glycol)$_{2,000}$ (PEG-DMG) and 1,2-distearoyl-*sn*-glycerol, methoxypoly(ethylene glycol)$_{2,000}$ (PEG-DSG) were purchased from the NOF CORPORATION (Tokyo, Japan). YSK05 was synthesized as previously reported.[10] Cholesterol (chol) and RPMI-1640 medium were obtained from SIGMA Aldrich (St. Louis, MO). 1,2-Disteroyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol))-2000] (PEG-DSPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Anti-luciferase siRNA (si-*luc*; sense, 5′-CCG UCG UAU UCG UGA GCA AdTdT-3′; antisense, 5′-UUG CUC ACG AAU ACG ACG GdTdT-3′) and Anti-PLK1 siRNA (sense, 5′-AGA uCA CCC uCC UuA AAu AUU-3′; antisense, 5′-UAU UUA AgG AGG GUG AuC UUU-3′, 2′-OMe-modified nucleotides are in lowercase) were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). RiboGreen was purchased from Molecular Probes (Eugene, OR, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). OS-RC-2 (human renal cell carcinoma cells) were generously provided by Dr. Kyoko Hida (Hokkaido University, Sapporo, Hokkaido, Japan). The CDK1/CCNB1 inhibitor was purchased from Calbiochem (San Diego, CA, USA)

MEND Preparation. YSK-MEND was assembled as previously reported.[8,9] In brief, 0.8 mg/mL of siRNA in 2 mM filter-sterilized citrate buffer (pH 4.0) was gradually added to 7.5 mM lipid dissolved in *tert*-butanol and then subjected to ultrafiltration through an Amicon Ultra-15 (MWCO 100 kDa, Millipore, Billerica, MA) to remove *tert*-butanol from the solution. The lipid compositions were YSK05/chol/POPE/PEG-DMG (50/25/25/3) for *in vitro* study and YSK05/chol/PEG-DSG (70/30/3) for *in vivo* study. The size distribution and zeta-potential of YSK-MEND were determined by a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worchestershire, U.K.). siRNA encapsulation efficiency and recovery ratio were calculated using RiboGreen. All MENDs in the manuscript are 85 ± 8 nm (size), +3 ± 2 mV (zeta-potential), and 93 ± 5% (siRNA encapsulation ratio).

Preparing Doxorubicin (DOX)-Loaded Liposomes (DOX-lip). DOX-loaded liposomes (DOX-lip) (composed of DSPC/chol/PEG-DSPE 70/30/5) were prepared by the lipid thin layer hydration method. 50 mM DSPC (140 μL), 50 mM chol (60 μL), and 10 mM PEG-DSPE (50 μL) in ethanol were added to glass tubes, and the organic solvents were then removed under reduced pressure. The residual lipid films in glass tubes were hydrated with 20 mM ammonium sulfate buffered saline (pH 5.5). The suspension was sonicated with bath-type sonicator for 30 s, and then sonicated with a probe-type sonicator (S3000, Misonix, NY, USA) for 10 min. The liposomal suspension was centrifuged at 20000*g* for 10 min, and supernatant was moved to a new tube. This procedure was repeated three times to completely remove all titan debris. The supernatant was centrifuged in an Amicon 14-Ultra (Millipore, MWCO 100 kDa) at 2500*g* for 90 min with PBS (−), and the concentrate was then centrifuged with PBS (−) again. Free DOX in water was incubated with the outer buffer-exchanged liposomes at 60 °C for 1 h (1:10 wt/wt), and the DOX that was not loaded was removed by ultrafiltration with an Amicon 14-Ultra. The loading efficiency of DOX into liposomes was quantified by measuring the fluorescence of DOX (excitation = 450 nm, emission = 590 nm, ε = 10,840 (M^{-1} cm^{-1})) after lysing liposomes with methanol. The characteristics of prepared liposomes were determined as mentioned above in MEND Preparation.

Cell Culture. OS-RC-2 cells were cultured in RPMI-1640. The media were supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Animal Studies. BALB/cAJcl-nu/nu (male, 4-week-old) were purchased from CLEA (Tokyo, Japan). For preparing OS-RC-2-bearing mice, BALB/cAJcl-nu/nu mice were inoculated with 1.0 × 10^6 OS-RC-2 cells in 75 μL of sterilized PBS on the right flank under ether anesthesia. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

Gene Expression Evaluation by Quantitative RT-PCR. Cells seeded on a 6-well plate were lysed by treatment with 350 μL of TRIzol. For the *in vivo* experiment, approximately 50 mg of collected tissue was homogenized by PreCellys (Bertin Technologies, Montigny-Le-Bretonneux, France) in 500 μL of TRIzol, and then centrifuged at 12000*g* at 4 °C for 15 min. The supernatant was used as an RNA extraction sample. RNA was extracted from lysates and purified according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Reverse transcription conditions were 65 °C for 5 min, 42 °C for 60 min.

Fifty-fold diluted cDNA was subjected to qPCR with Fast SYBR Green Master Mix (Applied Biosystems) using Light-Cycler-480 (Roche Diagnostics, Germany) according to the manufacturer's protocol. The primer sets used in the study were as follows: *PLK1* (forward, CTC CTT GAT GAA GAA GAT CAC C; reverse, GAA GAA GTT GAT CTG CAC GC), *CCNB1* (forward, GGC CAA ATA CCT GAT GG; reverse, TGC TGC ATA ACT GGA AGA AG), and *GAPDH* (forward, CCT CTG ACT TCA ACA GCG AC; reverse, CGT TGT CAT ACC AGG AAA TGA G). PCR was performed as follows: 95 °C for 20 s (1 cycle), 95 °C for 3 s, and 60 °C for 30 s (40 cycles). A melting curve analysis was done to verify whether a single, specific amplicon was obtained.

Cytotoxicity Assay with the Combination Therapy. Cells were transfected with si-*PLK1* or si-*luc* at 20 nM using the YSK-MEND for *in vitro* transfection[10] (YSK05/POPE/chol/PEG-DMG = 50/25/25/3) in a 10 cm dish. Cells were trypsinized 24 h after transfection, and 2.0 × 10^4 cells were then reseeded onto a 24-well plate. Cells were cultured in medium containing free DOX at various concentrations 24 h after reseeding, and the DOX-containing medium was removed, the cells were washed with PBS (−) 8 h after DOX adding, and the cells were finally incubated in fresh medium for 16 h. To evaluate cell viability, 500 μL of 1× Reporter Lysis Buffer (Promega) was added, and the protein concentration of the cell lysate 10-fold diluted with DDW was determined by BCA Protein Assay Reagent Kit (Thermo Scientific). To investigate the involvement of *CCNB1* on the improvement of DOX sensitivity, the *CCNB1* inhibitor was added to DOX-containing medium, and the subsequent procedures were the same as that used in the DOX only experiment.

Apoptosis Detection in Tumor Tissue. Mice bearing OS-RC-2 tumors (~150 mm^3) were intravenously injected with 1.5 mg/kg of DOX-lip and/or 3.0 mg/kg of siRNA-encapsulating YSK-MEND daily for 3 days. At day 4, 200 μL of APO-TRACE

solution (SIGMA Aldrich) was injected via the tail vein; 1.5 h after injection, the tumors were collected from anesthetized mice. Tumor tissue was fixed in 4% paraformaldehyde overnight, and then dehydrated in a series of 10, 30, and 50% sucrose solutions. A fixed tumor was embedded in OCT compound, and the embedded tissue was sectioned at a thickness of 10 μm on a SUPERFROST S9441 (MATSUNAMI) glass slide using a cryostat. Tumor slices were washed with PBS (−) twice, and covered with a cover glass. The tumor sections were observed with an FV10i-LIV (Olympus).

Cell Cycle Assay. Cells were transfected with si-*PLK1* and control siRNA using *in vitro* optimized YSK-MEND 24 h after cells were seeded on a 6-well plate, and then incubated with YSK-MEND for 24 h, after which the cells were cultured in fresh medium for 24 h. Then, 1.0×10^6 cells were trypsinized, washed twice in PBS (−) containing 0.05% azide sodium and 0.5% fetal bovine serum, and fixed in cold 70% ethanol. Cells were stained with 50 μg/mL propidium iodide and 1.0 mg/mL Ribonuclease A at rt for 30 min at 37 °C. DNA content was determined using a FACSCalibur cytometer, and cell cycles of the cell populations in S, G1, G2, and M phases were determined using the ModFit software program (Becton Dickinson, Franklin Lakes, NJ).

Statistical Analysis. Comparisons between multiple treatments were made using one-way ANOVA, followed by the Bonferroni test. Pairwise comparisons between treatments were made using a Student's *t* test. A *p*-value of <0.05 was considered to be significant.

RESULTS

***In Vivo* Therapeutic Effect of a Single si-*PLK1* Treatment.** The polo-like kinase 1 (*PLK1*) represents a well-validated gene in several types of cancer, inhibitors for which are currently under development for clinical use.[11,12] We evaluated the knockdown ability and consequent therapeutic effect of YSK-MEND encapsulating anti-*PLK1* siRNA (si-*PLK1*) on a human renal cell carcinoma (RCC) model (OS-RC-2 cells). When mice bearing OS-RC-2 were treated once with 3.0 mg of si-*PLK1*/kg, *PLK1* mRNA expression was greatly reduced to the half level of the nontreatment (NT) group 24 h after only a single injection (around 50%, Figure 1). We previously reported that this suppression was likely mediated by siRNA induced RNA interference.[9] To evaluate the therapeutic efficacy of *PLK1* knockdown in OS-RC-2 cells, mice were continuously administered the siRNA-encapsulating YSK-MEND for 2 weeks and tumor volume was measured as a function of time. The si-*PLK1* treatment failed to inhibit the tumor growth compared to the nontreatment (NT) group (Figure 2A), although OS-RC-2 cells responded to si-*PLK1* knockdown *in vitro* (Figure S1 in the Supporting Information). No significant body weight change was observed in any of the groups (Figure 2B).

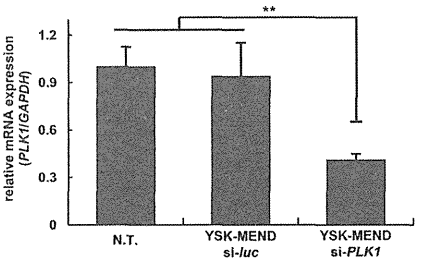

Figure 1. Knockdown effect of the anti-*PLK1* siRNA-loading YSK-MEND. Mice were treated with the YSK-MEND encapsulating the indicated control and anti-*PLK1* siRNAs at a dose of 3.0 mg/kg as the amount of siRNA. 24 h after the injection, tumor tissues were excised, and total RNAs were extracted from tumor homogenates. *PLK1* mRNA expression was determined by qRT-PCR, and the expression was normalized to the housekeeping gene, *GAPDH*. Values indicate mRNA expression relative to nontreatment (NT) ± SD.

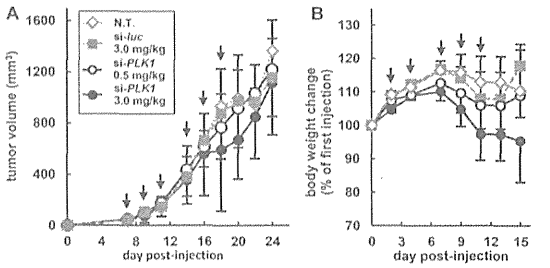

Figure 2. Therapeutic effect of anti-*PLK1* siRNA-loading YSK-MEND. (A) Tumor-bearing mice were intravenously injected with YSK-MEND encapsulating anti-luciferase siRNA (si-*luc*) or anti-*PLK1* (si-*PLK1*) at a dose of 0.5 or 3.0 mg/kg between day 7 and day 18. (B) The change in body weight was measured as an indicator of somatic toxicity. The body weight at the first injection day was regarded as 100%. Arrows indicate the injection of YSK-MEND. Values show mean tumor volumes (mm^3) ± SEM (n = 4–5).

An Efficient Combination Treatment with Doxorubicin (DOX) and si-*PLK1* Both *in Vivo* and *in Vivo*. To achieve an acceptable *in vivo* therapeutic effect, we attempted to combine a typical anticancer drug, doxorubicin (DOX), with si-*PLK1*. We first investigated the synergistic effect of such a combination on the cell-killing effect in human RCCs. *PLK1*-silenced cells were treated with DOX for 6 h, the DOX-containing medium was then removed, and the cells were recultured in fresh medium for 18 h. Cells that had been transfected with si-*PLK1* using the YSK-MEND optimized for *in vitro* transfection were significantly suppressed, as evidenced by a viability evaluation (Figure 3A). The DOX and si-*PLK1* combination significantly inhibited cell proliferation compared to the DOX only treatment (Figure 3B). The combination therapy induced approximately a 10-fold decrease in the IC_{50} of DOX concentration.

For the *in vivo* therapeutic study, DOX was formulated into liposomes (DOX-lip). The characteristics of the DOX-lip preparation were 105 ± 12 nm (in size) and −15 ± 3 mV (in zeta-potential). The si-*PLK1* MEND was intravenously injected into nude mice at a dose of 3.0 mg/kg. DOX-lip was administered at the same time at a dose of 1.5 mg/kg until the third si-*PLK1* injection. The combination of DOX and si-*PLK1* resulted in a moderate reduction in tumor growth without any evidence of acute toxicity (Figure 4A,B). On the other hand, si-*luc*, as a negative control, had no effect on the antitumor effect by DOX-lip (Figure 4C,D).

To examine why si-*PLK1* facilitated the anticancer effect by DOX, the extent of apoptosis in tumor tissue was determined by CLSM. When mice were injected with only DOX-lip or DOX-lip and control siRNA (si-*luc*), red signals indicating apoptosis were rarely observed (Figure 5). In the case of DOX-lip with si-*PLK1*, apoptosis was clearly observed in tumor tissue.

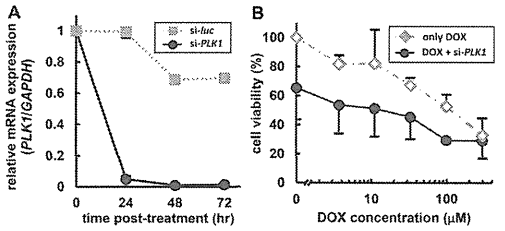

Figure 3. Combination effect on *in vitro* human renal cell carcinoma proliferation. (A) Cells were transfected with 20 nM siRNA using YSK-MEND optimized for *in vitro* transfection (YSK05/POPE/chol/PEG-DMG = 50/25/25/3). Cells were incubated with YSK-MEND for 24 h, and cultured in fresh medium until the indicated times. RNA was purified with TRIzol, and *PLK1* mRNA expression was determined by qRT-PCR. *PLK1* mRNA expression was normalized to the housekeeping gene, *GAPDH*. (B) Cells were incubated with YSK-MEND for 24 h, and then trypsinized and reseeded in 24-well plates. 24 h after reseeding, the cells were washed with PBS and incubated with DOX-containing medium for 6 h. DOX-containing medium was removed, and cells were washed with PBS and recovered 18 h after fresh medium incubation. Protein concentrations determined by the CA method were regarded as a viability. Values indicate mRNA expression relative to nontreatment (NT) ± SD.

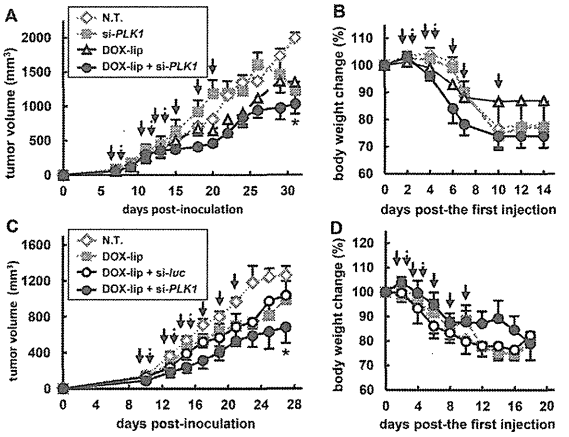

Figure 4. Therapeutic effect of anti-*PLK1* siRNA-loading YSK-MEND and doxorubicin-loading liposome. (A,C) Tumor-bearing mice were intravenously injected with the YSK-MEND encapsulating anti-luciferase siRNA (si-*luc*) or anti-*PLK1* (si-*PLK1*) at a dose of 0.5 or 3.0 mg/kg between day 9 and day 20. (B, D) The change in body weight was taken as an indicator of somatic toxicity. The body weight at the first injection day was regarded to be 100%. Arrows and dotted arrows indicate the injection of YSK-MEND and the injection of DOX-lip, respectively. Values show mean tumor volumes (mm^3) ± SEM (n = 4–5). *: $p < 0.05$ (one-way ANOVA followed by SNK test, vs NT).

PLK1 regulates a wide range of cell cycle related proteins. Cyclin B1 (*CCNB1*) is one of the target proteins by *PLK1* gene.[11] Cyclin dependent kinase 1 (*CDK1*) forms a complex with *CCNB1*, and this CDK1/CCNB1 complex plays a pivotal role in the G2/M phase transition.[13] We hypothesized that the si-*PLK1* treatment affected the expression of *CCNB1*, and consequently enhanced the sensitivity of OS-RC-2 cells to DOX. To validate this hypothesis, we next focused on cell cycle related events. When OS-RC-2 cells were treated with si-*PLK1*, *CCNB1* expression was dramatically decreased both *in vitro* (Figure 6A) and *in vivo* (Figure 6B). In addition, previous

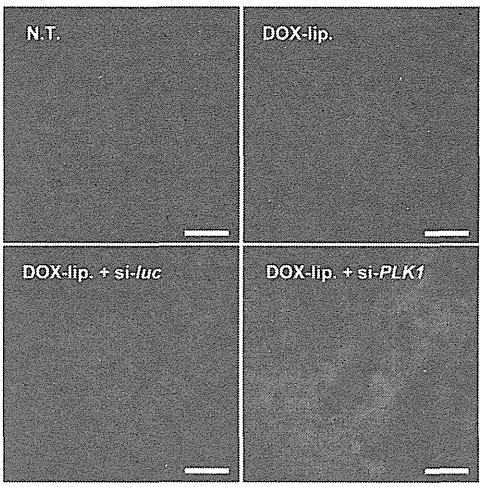

Figure 5. Apoptosis induced by DOX-lip and formulated siRNA. DOX-lip and/or siRNA-encapsulating YSK-MEND were injected into OS-RC-2 bearing mice three times daily at a dose of 1.5 and 3.0 mg/kg, respectively. APO-TRACE was administered via the tail vein 24 h after the final injection followed by excision of tumors. Tumor frozen sections were observed by confocal laser scanning microscopy. Red signals indicate apoptosis. Scale bars are 40 μm.

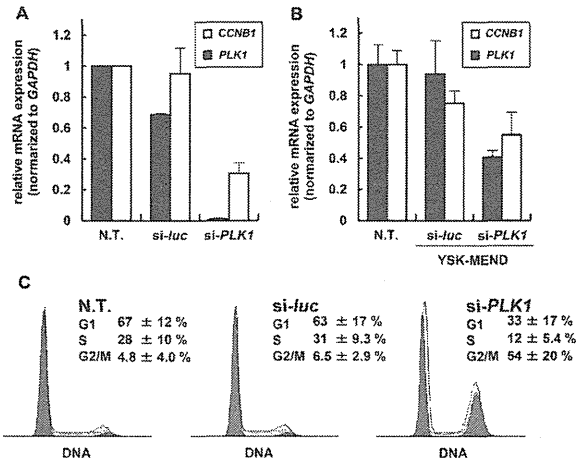

Figure 6. Effect of si-*PLK1* on the cell cycle related events. (A) OS-RC-2 cells were transfected with siRNAs using *in vitro* optimized YSK-MEND at 20 nM for 24 h. The cells were recovered and purified by TRIzol, and then *PLK1* and *CCNB1* mRNA expression were determined by qRT-PCR. (B) OS-RC-2 bearing mice were systemically injected with siRNA-encapsulating YSK-MENDs at 3.0 mg/kg, and 24 h after tumors were excised from mice. RNA was isolated from collected tumors, and then mRNA amount was measured by qRT-PCR. Black columns and white columns indicate *PLK1* and *CCNB1* expression, respectively. Values indicate mRNA expression relative to nontreatment (NT) ± SD (n = 3). (C) Cell cycle distributions were determined by flow cytometry analysis 48 h after transfection. The DNA of cells was stained with propidium iodide, and cells were analyzed by FACSCalibur. The cell population in various phases of cell cycle was decided using Mod Fit. The histogram is a typical shape, and the value in the figure is the mean of three independent experiment results.

studies have shown that *PLK1* inhibition caused a cell cycle defect, including G2 arrest. In the case of OS-RC-2 cells, the si-

PLK1 treatment increased the population of G2/M cells (4.8 to 54%, Figure 6C). No obvious change was observed when OS-RC-2 cells were treated with control siRNA (6.5%).

To further support this hypothesis, a small molecule inhibitor of CCNB1/CDK1 complex was cotreated with DOX. We therefore investigated the sensitivity of cells to DOX with/without the *CCNB1* inhibitor. The *CCNB1* inhibitor induced a 10-fold increase in sensitivity to DOX (Figure 7).

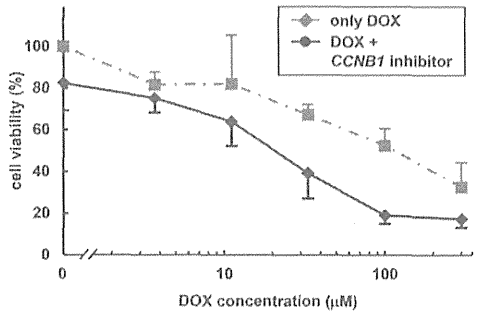

Figure 7. Effect of the CDK1/CCNB1 inhibitor on cell viability. Cells were cultured in DOX-containing medium with or without *CCNB1* inhibitor for 8 h, and then cultured in fresh medium for 16 h. The viability of cells was determined by calculating the protein concentration using the BCA method. Values indicate the relative viability to nontreatment (NT) ± SD (n = 3).

DISCUSSION

In this study, we examined the effectiveness of a therapy involving the use of a combination of DOX and *PLK1* for the treatment of RCCs, which are inherently resistant to DOX. First, we investigated the therapeutic potential of a single siRNA treatment using an anticancer siRNA-encapsulating YSK-MEND. No regression was observed when OS-RC-2 bearing mice were injected with si-*PLK1*-encapsulating YSK-MEND, while the YSK-MEND injection significantly resulted in a reduced *PLK1* gene expression. This discrepancy might be explained by assuming the following hypothesis. First, the siRNA is distributed heterogenously in the YSK-MEND. We previously reported that the YSK-MEND can deliver siRNA to, at most, 70% of cancer cells in tumor tissue in terms of knockdown percentage.[9] This heterogeneous delivery might produce a cell population whose *PLK1* expression was not inhibited. Second, OS-RC-2 cells are relatively resistant to DOX exposure. In fact, OS-RC-2 cells were much more resistant to *PLK1* knockdown than HeLa cells in terms of viability (Figure S2 in the Supporting Information), although other groups reported that *PLK1* expression was strongly interrelated with the overall survival of RCC patients, and that the *PLK1* inhibitor was applicable for the treatment of RCCs.[14] Bu et al. previously reported that, in a cell line reactive to *PLK1* knockdown, the sub G1 phase cell population appeared 48 or 72 h after treatment with an siRNA against *PLK1* transfection using four cell lines of esophageal cancers. On the other hand, OS-RC-2 did not develop a sub G1 phase after transfection. Although this difference would cause a weak cell-killing effect of OS-RC-2 cells by si-*PLK1*, the exact mechanism of the nonresponsiveness to si-*PLK1* is unclear at this time.

To produce a sufficient curative effect, we combined si-*PLK1* with a traditional anticancer drug, DOX. Previous reports suggested that PLK1 inhibition by a small molecule inhibitor, BI 2536, improved the cytotoxicity of certain types of anticancer drugs, such as paclitaxel, 5-fluorouracil, cisplatin, DOX, and radiotherapy.[15−18] Actually, si-*PLK1* formulated into the YSK-MEND resulted in OS-RC-2 cells being more sensitive to DOX both *in vitro* and *in vivo*, accompanied by an increased apoptosis in tumor tissue. In addition, the subpopulation in the G2/M phase was increased and the subpopulation in the G1 phase was decreased. A previous study revealed that cells in the G2/M phase were 3-fold more sensitive than cells in the G1 phase in the case of synchronized murine leukemia-derived cells.[19] The enhanced chemosensitivity toward DOX might be partly due to controlling the cell cycle of OS-RC-2 cells by *PLK1* silencing, although DOX itself is a concentration-dependent anticancer drug.[20]

The change in cell cycle phase could be caused by *CCNB1* suppression through *PLK1* silencing. Actually, *CCNB1* downregulation was observed when OS-RC-2 cells were treated with si-*PLK1* both *in vitro* and *in vivo*. *CCNB1* plays a key role in the transition to the G2/M phase in concert with *CDK1* through phosphorylating some proteins including *CDC25*, as mentioned above. Some groups have suggested that the reduction in *PLK1* by siRNA induced *CCNB1* suppression,[21] and this decrease was mediated by *FOXM1*.[22] In breast cancers, *CCNB1* siRNA facilitated cytotoxicity by DOX.[23] Other groups reported that *PLK1* suppression increased the protein level of CCNB1 by the degradation of CCNB1 via a decrease in ubiquitin dependent proteolysis. This point is currently controversial. At least, in RCCs, *FOXM1* depletion by siRNA appeared to cause a reduction in *CCNB1*.[24] The inhibition of the CCNB1/CDK1 complex decreased the IC_{50} value for the viability of OS-RC-2 by DOX exposure by approximately 3-fold. This is consistent with results reported in a previous study, in which siRNA against *CCNB1* was reported to exert a DOX cytotoxicity of murine melanomas both *in vivo* and *in vitro*.[25] In summary, the YSK-MEND encapsulating si-*PLK1* appears to enhance the effect of DOX on the viability of OS-RC-2 cells via *CCNB1* downregulation.

The results for body weight also indicated that the combination therapy of si-*PLK1* and DOX-lip would be well tolerated and safe. A liposomal carrier might do harm in the liver because the liver is the main clearance organ in the body regardless of the presence or absence of PEG,[26] and intravenously injected siRNA stimulates the immune system via the toll-like receptor and RIG-I.[27] Both carrier and cargo might cause adverse events. We previously reported that YSK-MEND encapsulating si-*PLK1* did not induce toxicity or produce inflammatory cytokines in the liver or other organs.[9] Moreover, several reports suggested that *PLK1* inhibition did not induce apoptosis in normal cell lines.[28,29] Actually, no change in the body weight of mice treated with si-*PLK1* and DOX-lip was observed. These facts indicate that combining si-*PLK1* with DOX-lip should be a safe system, and therefore has considerable potential for use in the treatment of RCCs.

CONCLUSIONS

Monotherapy using only siRNA against *PLK1* (si-*PLK1*) failed to induce a sufficient tumor cell killing effect. When *PLK1* expression was silenced by siRNA, cells became more sensitive to doxorubicin (DOX). The growth of renal cell carcinomas is difficult to suppress when typical anticancer drugs are used. The combination therapy using si-*PLK1* and DOX appears to be a useful strategy for developing a new treatment for renal cell carcinomas. Moreover, the YSK-MEND system could propose

an attractive methodology which enables us to analyze cancer biology in the *in situ* circumstance.

■ ASSOCIATED CONTENT

Supporting Information

Figures depicting the effect of *PLK1* knockdown on the viability of OS-RC-2 cells and comparison between HeLa cells (DOX sensitive) and OS-RC-2 cells (DOX resistant) in the response to DOX treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Review Article JOURNAL OF CLINICAL AND EXPERIMENTAL HEPATOLOGY

Designing Immune Therapy for Chronic Hepatitis B

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Presently-available antiviral drugs may not be a satisfactory option for treatment of patients with chronic hepatitis B (CHB). In spite of presence of several antiviral drugs, sustained off-treatment clinical responses are not common in CHB patients treated with antiviral drugs. In addition, antiviral drug treatment may have limited effects on blocking the progression of HBV-related complications. However, substantial long-term risk of viral resistance and drug toxicity are related with maintenance antiviral therapy in CHB patients with presently-available antiviral agents. The infinite treatments with antiviral drugs for CHB patients are also costly and may be unbearable by most patients of developing and resource-constrained countries. In this situation, there is pressing need to develop new and innovative therapeutic approaches for patients with chronic hepatitis B virus (HBV) infection. Immune therapy has emerged as an alternate therapeutic approach for CHB patients because studies have shown that host immunity is either impaired or derailed or distorted or diminished in CHB patients compared to patients with acute resolved hepatitis B who contain the HBV replication and control liver damages. Both non antigen-specific immune modulators and HBV antigen-specific agents have been used in CHB patients during last three decades. However, similar to antiviral therapy, the ongoing regimens of immune therapeutic approaches have also been unable to show real promises for treating CHB patients. The concept of immune therapy for treating CHB patients seems to be rationale and scientific, however, concerns remain about suitable designs of immune therapy for CHB patients. (J Clin Exp Hepatol 2014;4:241–246)

THE EXTENT OF PROBLEM WITH HEPATITIS B VIRUS AND PRESENT THERAPEUTIC STRATEGIES FOR TREATING CHRONIC HEPATITIS B PATIENTS

At present, HBV accounts for an estimated 350 million chronic HBV-infected persons that indicate that these persons would continue to harbor HBV for rest of their life. About 20% of them may eventually develop serious complications like liver cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC).[1] With considerable information about the viral life cycle, epidemiology, immunology, pathogenesis, and complications of HBV, further progression of HBV has been drastically reduced by implementing various public health measures. In addition, protective vaccines against HBV are available for last 3 decades and millions of healthy persons are protected from future HBV infection by vaccination. Even then, several million people of the world are newly infected with HBV each year in different parts of the world, especially in developing countries. This exposes the complexity of HBV control program. One of the major causes of new HBV infection is related to persistence of millions of chronic HBV-infected persons for whom effective therapeutic measures have not been developed. About 0.5–0.7 million people die every year due to HBV-related liver diseases.[2] Unfortunately, this frustrating picture is prevailing in spite of the availability of several antiviral drugs against HBV that have been developed during the last twenty or thirty years. Most of these drugs are capable of reducing HBV replication, down-regulating alanine aminotransferase (ALT), negatively regulating hepatitis B e antigen (HBeAg), inducing seroconversion of antibody to HBeAg (anti-HBe), and containment of liver damage in some, but not in all, patients with CHB patients.[3–8] Even, long-term beneficial effects of interferon in the context development of HCC have been shown by Lin et al.[9] Recently, Marcellin et al have documented reversion from liver cirrhosis in considerable numbers of patients of chronic hepatitis B treated with tenofovir for 5 years.[10] These studies about utility of interferon and nucleoside analogs for treatment and chronic hepatitis B and regression

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Abbreviations: CHB: chronic hepatitis B; HBV: hepatitis B virus; HBeAg: hepatitis B e antigen; DC: dendritic cells; RCT: randomized clinical trials; CTLs: cytotoxic T lymphocytes
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of liver cirrhosis inspire considerable optimisms, however, report contrary to these are also prevailing. A systemic review of the National Institutes of Health (NIH) Consensus Development Conference that assessed all randomized clinical trials (RCT) on antiviral drugs in CHB patients from 1989 to 2008 have shown that antiviral drug treatments may not block HBV-related complications or improve ultimate clinical outcomes. These drugs can modulate intermediate parameters in majority patients with CHB.[11,12] Taken together, the real therapeutic implications of antiviral drugs in CHB patients is yet to be explored in different geographical region and in different population groups. Further study about surrogacy of surrogate markers of HBV during therapy need to be re-assessed.[13]

NEW AND INNOVATIVE THERAPY FOR CHRONIC HEPATITIS B PATIENTS

HBV is a non cytopathic virus and the virus cannot cause liver damages and HBV-related complications by themselves. The replication of HBV and extent of liver damages in HBV-infected subjects are regulated mainly, if not solely, by the nature and magnitude of immune responses of the host to the HBV.[14-17] Studies have suggested that the HBV-specific immune responses are weak and narrowly focused in patients with CHB who are unable to contain HBV replication and liver damages compared to those with resolved acute hepatitis B that successfully resolve HBV infection, control HBV replication and contain liver damages.[14] Also, it has been observed that sustained control of HBV replication and containment of liver damage is usually associated with the restoration of host immunity of CHB patients.[18,19] Also, it has been found that host immunity may also be related to liver damages and allow HBV replication in CHB patients.[20] Thus, the nature of host immunity, not the magnitude of host immunity, seems to be a decisive factor for containment of HBV and control of liver damages of CHB patients and also for designing the nature of innovative therapy.

PREVAILING STRATEGIES OF IMMUNE THERAPY FOR CHRONIC HEPATITIS B PATIENTS AND ITS LIMITATIONS

Although these facts indicate that restoration of host immunity may have therapeutic implications in CHB patients, non antigen-specific polyclonal immune modulators could not stand the test of time as therapeutic agents in CHB patients.[21-31] Regarding interleukin-2 (IL-2) therapy in chronic hepatitis B, it was found that neither a biologically active but non-toxic dose of 300,000 U, nor a toxic dose of 1.0 million U of IL-2 resulted in sustained clearance of HBeAg[21] in these patients. Artillo et al also found no notable therapeutic efficacy of IL-2 in patients with chronic hepatitis B.[22] On the other hand, considerable reservation remains about the safety of IL-12 in chronic hepatitis B because 3 of 46 patients were withdrawn from therapy prematurely due to adverse events.[23] Also, follow up data using granulocyte-macrophage colony stimulating factor in chronic hepatitis B is lacking although this agent induced altered cytokine profile in these patients.[24] Ruiz-Moreno et al have shown that levamisole and interferon are neither safe nor efficacious in their cohort.[25] Even a combination of thymus humoral factor and interferon could induce HBV DNA negativity in only one third patients.[26] Woltman et al could not find any notable therapeutic effect of alpha galactosylceramide in patients with chronic hepatitis B.[27] Although Hirayama et al indicated that propagermanium may be an alternative therapeutic approach for chronic hepatitis B, no follow up data of their trial is available to substantiate their claim.[28] Although Iino et al[30] showed a promise with thymosin alpha-1 in Japanese patients with chronic hepatitis B, Yang et al did not find any additional benefit of this drug compared to interferon alpha monotherapy.[31] Taken together, most of these agents induced up-regulation of host immunity but failed to attain sustained control of HBV replication and liver damages of CHB patients. Moreover, it is really difficult to assess the real impact of these drugs as there have been no follow up study after end of treatment. Most of these studies were conducted as pilot studies or clinical trials. Phase III clinical trial with proper follow up data is mostly unavailable about non antigen-specific immune modulators. Thus, little has been exposed regarding sustained effects of non antigen-specific immune modulators in the context of HBV replication, liver damages and progression to HBV-related complications. In addition, adverse effects were documented in CHB patients treated by non antigen-specific immune modulators. Also, mechanisms of action of non antigen-specific immune modulators have not been elucidated. At present, it is elusive if non antigen-specific immune modulators can be a new and innovative therapeutic regimen for CHB patients.

HEPATITIS B VIRUS ANTIGEN-SPECIFIC IMMUNE THERAPY IN CHRONIC HEPATITIS B

With the postulation that restoration of HBV-specific immunity may be reflected in therapeutic effects in CHB patients, Pol et al first used HBsAg-based vaccine as a therapeutic vaccine in CHB patients.[32] They showed that HBsAg-based vaccine therapy induced reduction of HBV DNA, HBeAg seronegativity and anti-HBe seroconversion in some CHB patients. However, nothing has been reported about long-term follow of these patients.

Subsequently, investigators conducted clinical trials to assess the therapeutic efficacy of HBsAg-based vaccine therapy in CHB.[33–48] Senturk et al used a vaccine containing pre-S2 protein and showed clinical response was achieved in some patients but they did not show any mechanism related to the action of vaccine therapy in their cohort.[33] Most of these studies were accomplished as pilot studies and considerable heterogeneity have been prevailing among studies. Almost, nothing has been reported about sustainability of therapeutic effects by HBsAg-based vaccines in CHB patients. The major limitations of these studies lie in the fact that the evaluation of the therapeutic efficacy of HBsAg-based vaccine therapy was done from the point of view of individual investigators. The effect of the vaccine on HBV replication was assessed by some, whereas, others checked HBeAg negativity or anti-HBe seroconversion. The effects of HBsAg-based vaccination on ALT levels were evaluated by others. The role of HBsAg-based vaccine on HBV replication, ALT levels, HBeAg seroconversion, and liver damages have not been addressed in same run. When investigators found that HBsAg-based vaccine may not be an optimal option of therapy for CHB patients, attempts were made to improve the therapeutic potentials of HBsAg-based vaccine by altering the nature of antigen and adjuvants, dose of vaccine, and duration of therapy. An antigen–antibody complex vaccine containing HBsAg and antibody to HBsAg (anti-HBs) was used as a therapeutic vaccine.[35,36] HBsAg/anti-HBs complex used by Wen et al[35] and Xu et al[36] also did not show any data about long-term effects of these vaccines in chronic hepatitis B. Others used a HBsAg-based vaccine with antiviral agents or other immune modulators to increase the therapeutic potential of these vaccines.[37–40] Strong adjuvants and DNA-based vaccine expressing HBsAg was also used in CHB patients.[41,42] Some investigators loaded antigen-presenting dendritic cells (DC) with HBsAg to produce HBsAg-pulsed DCs and used HBsAg-pulsed DCs as therapeutic vaccines in CHB patients.[42–45] Combination therapy by Dahmen et al[37] and Horiike et al[38] also failed to exhibit sustained response in chronic hepatitis B. Other types of combination therapy, DNA-based therapy and cell-based therapy did not provide data of long-term safety and efficacy and thus this HBsAg-based vaccine therapy has not been recommended for clinical usage till now.[39–45] In this context, Dikici et al have reported that there is no clinical benefit of using HBsAg-based vaccine in chronic hepatitis B.[46] Finally, Vandepapeliere et al provided a credible evidence of non-efficacy of a combination of vaccine therapy and antiviral therapy in chronic hepatitis B.[48] Taken together neither non antigen-specific polyclonal immune modulators nor HBsAg-based vaccine could not show considerable promises as alternative therapy for therapy of CHB patients. However, more studies are warranted before a firm conclusion is drawn in this regard.

DESIGNING IMMUNE THERAPY FOR CHRONIC HEPATITIS B PATIENTS: SCIENTIFIC ASPECTS

All antiviral drugs have been prepared to reduce replication of replicating HBV and kinetics studies have shown that antiviral drugs are capable of doing that during on-treatment period. However, there is no committed antiviral drug that can block all forms of HBV, mainly covalently closed circular DNA (cccDNA). cccDNA can act as a template for replication of HBV DNA and thus any drug that cannot block cccDNA may not provide a real promise for treatment of CHB patients. However, circumstantial evidences have shown that cccDNA may be controlled by host immunity in many CHB patients. But, the immune modulatory capacities of antiviral drugs are not sufficient to block of cccDNA in majority of CHB patients. At present, there is no new drug on the list of antiviral drugs that can show potent immune modulatory capacities to block cccDNA in the liver of CHB patients (Figure 1).

Non-antigen-specific immune modulators also failed to show potent therapeutic effects in CHB patients. These drugs increased the magnitudes of host immunity in CHB patients but the nature of immunity that was induced by non antigen-specific immune modulators might not have protective capacity in CHB patients. Thus, the concept of 'protective immunity' and 'pathogenic immunity' in HBV infection has been surfaced. Although the nature of immunity induced by non antigen-specific immune modulators have not been dissected properly, it seems that host immunity that was upregulated by these modulators were not of 'protective' type for CHB patients (Figure 1).

The apparent failure of HBsAg-based vaccine to cure CHB patients or to have sustained therapeutic effects in spite of inducing HBsAg-specific immunity may be due to the fact that HBsAg-based immunity is not able to block HBV replication or contain liver damages in these patients. In fact, the role of HBsAg-specific immunity for prevention of HBV infection (prophylactic vaccine) and for treatment of CHB patients (therapeutic vaccine) should be separately analyzed. The fundamental difference between a prophylactic vaccine and a therapeutic vaccine should be kept in mind for formulating an amicable design of immune therapy for CHB patients (Figure 1).

In this context, it is natural to ask what type of immunity may have protective role in CHB patients. The concept of using HBcAg as a therapeutic vaccine received scientific support because patients with CHB who control HBV replication and contain progressive liver damage harbor significantly higher numbers of HBcAg-specific cytotoxic T lymphocytes (CTLs) in the liver compared to patients with CHB who are unable to control HBV replication and liver damage.[20,49]

Recently, HBcAg-based vaccine has been used in HBV TM, a murine model of the HBV carrier state, that express

1. Antiviral therapy
Scope and limitation:
A. Reduce replicating HBV but cannot control cccDNA and off-treatment control of HBV replication is not achieved
B. Contain liver damages transiently but sustained control and blocking of complications may not be achieved
C. Induce immunity, but the nature of restored immunity is elusive
D. Sustained maintenance of host immunity is not usually achieved

2. Non antigen-specific immune therapy
Scope and limitation
A. Upregulate host immunity but the nature is elusive
B. Sustained control of HBV replication and liver damages are not attained
C. Major concern remains about safety of these agents

3. HBsAg-based immune therapy
Scope and limitation
A. Induce HBsAg-specific immunity but that does not seem to be protective in nature
B. Sustained immunity is not available
C. HBcAg-specific immunity is not induced.

Figure 1 Scope and limitation of ongoing and innovative therapies for chronic hepatitis B.

HBV DNA and HBV-related antigens in the sera and liver. Immunization of HBV TM with the HBcAg-based vaccine induced wide-spread immunity in HBV TM. HBcAg-based vaccination in HBV TM caused (1) HBsAg-negativity, (2) production of anti-HBs, (3) HBsAg and HBcAg-specific T lymphocytes in lymphoid tissue, and (4) HBsAg- and HBcAg-specific CTL in the liver.[50,51] Although promising outcomes using the HBcAg-based vaccine have been shown in HBV TM, credible clinical trials with human consumable HBcAg have not been published till now. Even, we should that HBcAg is able to overcome tolerance to HBsAg in HBV TM.[51]

DESIGNING IMMUNE THERAPY FOR CHRONIC HEPATITIS B PATIENTS: CLINICAL ASPECTS

Circumstantial evidences have shown that both HBsAg- and HBcAg-specific immunity are essential for effective control of HBV replication and containment of liver damage in CHB patients. HBsAg-specific humoral immunity may be important for blocking HBV DNA or HBV-related antigens in the peripheral blood. On the other hand, HBcAg-specific cellular immune responses in the liver are important for controlling cccDNA in the liver and containment of liver damages.[20,49] These evidences indicate that an effective regimen of immunotherapy may be developed for CHB patients if adequate levels of immune responses to both HBsAg and HBcAg can be induced and maintained in CHB patients.

Although a therapeutic vaccine containing both HBsAg and HBcAg represents a tempting therapeutic approach for CHB patients, these vaccines have not been used in clinics mainly due to non-availability of human consumable HBsAg/HBcAg vaccine. However, epitopes of HBV-related antigens presented with DC showed considerable promise as a therapeutic vaccine in CHB patients.[45]

Recently, a human consumable HBsAg/HBcAg vaccine has been available and we have shown that this may be a potent therapeutic vaccine for chronic HBV-infected persons by assessing their safety and efficacy in HBV TM, a murine model of HBV carrier state.[50,51] Subsequently, a phase I/II clinical trial was performed in CHB patients to evaluate their safety, clinical efficacy and immune modulatory effects in CHB patients. The HBsAg/HBcAg-based immunization was safe for all patients administered via the nasal route or parenteral route. Administration of the HBsAg/HBcAg-based vaccine caused sustained negativity of HBV DNA in 9 of 18 CHB patients (50%). Also, persistent normalization of ALT was recorded in all patients in the study.[52] Currently, a phase III clinical trial is in progress using the HBsAg/HBcAg-based combined vaccine administered via the nasal and subcutaneous route in patients with CHB. The clinical outcome of these patients will be compared with patients receiving pegylated IFN for 48 weeks [Registered; ClinicalTrials.gov; NCT01374308].[53]

INFERENCE

There is a pressing need to develop new and novel therapeutic approaches for CHB patients because the limitations of commercially-available antiviral drugs in CHB patients have become evident. Moreover, antiviral drugs may induce more long-term confusions in developing countries where drug compliance rate is low and the health care delivery system do not allow proper follow up of these patients. It is

really a burning issue about how drug-induced mutant of CHB patients would be addressed by already overburdened health care delivery system of developing countries that harbor the major burden of CHB patients.

Non antigen-specific immune modulators and HBsAg-based immune therapy could not stand the test of time in clinics because studies with these agents during last three decades could not yield any credible phase III clinical trial so that the superiority of these agents over antiviral drugs can be substantiated.

Studies regarding immunotherapy in CHB patients during the last three decades have shown the fundamental concepts of immunotherapy represent an evidence-based approach, but major alterations are needed for designing effective regimens of immune therapy for CHB. Most CHB patients are not immune compromised and they have specific defects of HBV-specific immunity. Based on the emerging data, it seems that induction and restoration of both HBsAg and HBcAg-specific immunity may be another approach of immune therapy for these patients. As the safety of HBsAg/HBcAg vaccines has been confirmed, opportunities have been exposed if better therapeutic effects can be accomplished by using different combinations (vaccine/vaccine combinations, vaccine/antiviral combinations, vaccine/immune modulators combinations) of these vaccines through different routes (parenteral and/or mucosal). However, data supporting clinical utility of HBsAg/HBcAg-based vaccine is at their infancy. Thus, studies should be conducted in both patients with CHB and using an animal model of the HBV carrier state to develop insights about mechanisms underlying therapeutic potentials of HBsAg/HBcAg-based therapeutic vaccines. Designing an effective therapeutic vaccine against CHB would not only be a milestone for treatment of HBV infection, but would also contribute to developing therapeutic vaccines and immune-based therapeutic approaches for other chronic infections as well as for cancer, autoimmunity and allergies.

The fundamental limitation of immunotherapy to treat CHB can only be properly addressed if the fundamentals immune system and their role in chronic disease can be explored properly. It should be remembered that the functional implications of 'naturally-developing HBV-specific immunity' in CHB may not be the same or comparable with 'inducible HBV-specific immunity' that develops due to the administration of HBV-based vaccines. When an HBV-infected host develops HBV-specific immune cells due to natural interactions among immunocytes of the hosts, the hepatic microenvironment possibly supports their functional capacities. However, in the case of inducible immunity, questions remain regarding their persistency and functional implications. The challenge of our time is to restore inducible immunity by antiviral drugs, non antigen-specific immunity, HBsAg-based vaccines and HBsAg/HBcAg-based vaccines for prolonged period for persistence containment of HBV replication and control of liver damages. The proper designing of therapy for CHB patients may be unmasked when data about these agents from different clinical trials will be compiled.

CONFLICTS OF INTEREST

All authors have none to declare.

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Research Article

Clinical Features of Adult Patients with Acute Hepatitis B Virus Infection Progressing to Chronic Infection

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Background. Information regarding the progression of acute hepatitis B virus (HBV) infection to chronic infection in adults is scarce. *Methods.* Twenty-five adult patients with acute HBV infection (14 men and 11 women, 18–84 years old), whose clinical features progressed to those of chronic infection (group A) or did not (group B), were studied retrospectively. *Results.* There were 3 and 22 patients in groups A and B, respectively. Two of the 3 patients of group A lacked the typical symptoms of acute hepatitis. No differences were found between groups with respect to age, sex, or HBV genotypes. However, total bilirubin and alanine aminotransaminase levels were significantly lower in group A. *Conclusions.* Three of the 25 adult patients with acute HBV infection progressed to chronic infection. Hepatitis was mild in these patients. Patients with mild acute hepatitis B or unapparent HBV infection may have a higher risk of progressing to chronic infection.

1. Introduction

Hepatitis B virus (HBV) is a DNA virus with approximately 3200 base pairs. Approximately 350–400 million people are chronically infected with HBV and more than 3 billion people have been exposed to HBV worldwide [1, 2]. HBV induces a variety of liver diseases, ranging from acute or fulminant hepatitis to liver cirrhosis and hepatocellular carcinoma. HBV is one of the most important causes of liver cirrhosis and hepatocellular carcinoma [3]. On the other hand, hepatitis is self-limited in most adult patients with acute infection. Meanwhile 1-2% of patients progress to fulminant hepatic failure, and some progress to chronic infection. The rate of progression from acute to chronic HBV infection is reported to be 90% in newborns and 5–10% in adults [4, 5].

HBV can be classified into at least 8 genotypes with a divergence of more than 8% of nucleotide sequences [6–8]. There are some differences in clinical features and routes of transmission between genotypes [9, 10]. The rate of chronicity of genotype A infections is reported to be higher than those of other genotypes [11–14]. The progression of acute hepatitis B to chronic hepatitis is not rare in Western countries, but it is rare in Japan. The differences of the rates of chronicity of acute HBV infection supposed to be attributable to the different distribution of HBV genotypes; genotypes B and C are the predominant genotypes while genotype A was rare in Japan and common in Western countries. However, previous studies are based on the follow-up studies of apparent acute hepatitis B. The present study aimed to clarify the progression to chronic infection in adult patients with acute HBV infection including subclinical or unapparent patients who progressed to chronic infection.

2. Materials and Methods

2.1. Subjects. Among the 28 patients diagnosed with acute HBV infection who visited our hospital in the northwestern area of Shikoku Island, Japan, between 1998 and 2012, 25

Table 1: Clinical data of the subjects according to HBV genotypes.

	Group A	Group B	P
Number of patients	3	22	N.S.
Male : female	1 : 2	13 : 9	N.S.
Age	28 (20–84)	35 (18–81)	N.S.
Apparent acute hepatitis	1 (33%)	22 (100%)	$P < 0.05$
Fulminant hepatitis	0	3 (13.6%)	N.S.
T. bil >3.0 mg/dL	0	20 (90.9%)	$P < 0.01$
T. bil (mg/dL), max	1.00 ± 0.36	8.43 ± 5.14	$P < 0.05$
ALT >500 IU/L	0	22 (100%)	$P < 0.01$
ALT (IU/L), max	250 ± 211	2733 ± 1540	$P < 0.01$
HBeAg (positive)	3 (100%)	10 (45.5%)	N.S.
HBV-DNA >7 LC/mL	3 (100%)	1 (4.5%)	$P < 0.01$
Genotype A : B : C : D	0 : 0 : 2 : 1	3 : 1 : 14 : 1 (undetermined 3)	N.S.

N.S.: not significant; T. bil: total bilirubin; ALT: alanine aminotransferase; LC: log copies; HBeAg: hepatitis B e antigen; HBV: hepatitis B virus.

survived without liver transplantation and were included in the present study.

2.2. Methods. HBsAg was assayed by chemiluminescence immunoassay (CLIA, Architect HBsAg QT, Abbott Japan, Tokyo, Japan) or reverse passive hemagglutination assay (Mycell II, Institute of Immunology, Tokyo, Japan). Anti-HBs was tested by CLIA (Architect AUSAB, Abbott Japan) or hemagglutination assay (Mycell II anti-HBs, Institute of Immunology). Hepatitis B e antigen (HBeAg), anti-HBe, and IgM-type antihepatitis B core (anti-HBc) were assayed by CLIA (Architect HBeAg, Architect HBeAb, Architect HBc-II, and Architect HBc-M, Abbott Japan, resp.). Anti-HBc was tested by CLIA (Architect HBc-II, Abbott Japan) or enzyme immunoassay (EIA, F-HBc, Sysmex, Kobe, Japan). HBV-DNA level was assayed using polymerase chain reaction (PCR, Amplicor HBV Monitor, Test, Roche Molecular Systems Inc., Pleasanton, USA) or real-time PCR, (AccuGene m-HBV Abbott Japan). HBV genotype was determined by serial invasive signal amplification reaction assay (Invader assay; BML Inc., Saitama, Japan) [15]. When the genotype could not be identified using this method, an EIA was performed (Immunis, HBV genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan) [16]. Total bilirubin (T. bil) and alanine aminotransaminase (ALT) levels were examined every 1-2 weeks in the early stage of the disease. HBsAg was assayed every 1 or 2 months until negative results were yielded. The presence of human immunodeficiency virus (HIV) was assayed by the HIV antigen-antibody detection assay using a CLIA method.

The criteria of acute HBV infection were as follows: (1) positive for anti-HBc with a low titer (<10 by CLIA or <90% in 200-fold diluted serum by EIA) and positive for IgM-type anti-HBc or (2) positive for HBsAg in a previously HBsAg-negative patient. The criterion of progression to chronic infection was persistence of HBsAg from the onset of the disease for more than 6 months. Patients who lacked the typical symptoms or signs of acute hepatitis were deemed as having an unapparent infection.

The clinical features of subjects who progressed and did not progress to chronic infection were determined and analyzed. This study was retrospective in nature.

2.3. Statistical Analysis. Statistical analyses were performed using the χ^2 test, unpaired t-test, and Mann-Whitney U-test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Comparison of Clinical Features. Among the 25 patients, 3 progressed to chronic infection while the other 22 did not. All 25 subjects were HIV negative. The clinical data of groups A and B are summarized in Table 1. No differences were found between groups with respect to age or sex. All subjects in group B and 1 subject in group A exhibited typical symptoms of acute hepatitis and were easily diagnosed with acute hepatitis at the early stage of the disease. Meanwhile, 2 subjects in group A lacked the typical symptoms of acute hepatitis ($P < 0.01$). Zero and 20 subjects in group A and group B, respectively, had T. bil >3 mg/dL ($P < 0.01$). Zero and 22 subjects in group A and group B had ALT >500 IU/L, respectively ($P < 0.01$). In addition, the average levels of T. bil and ALT were lower in group A.

Three of 3 and 10 of 22 subjects in groups A and B, respectively, were HBeAg-positive. HBV-DNA level was >7.0 log copies (LC)/mL in 3 of 3 but only 1 of 22 subjects in groups A and B, respectively ($P < 0.01$). Two and 1 subject in group A had HBV genotypes C and D, respectively. There was no difference in HBV genotypes between groups A and B.

3.2. Patients Progressing to Chronic Infection. Patient 1 was an 84-year-old man who had chronic obstructive pulmonary disease and hypertension and was medicated by his home doctor. He had no history of liver disease and was negative for HBsAg in 2007. He had no family history of hepatitis B. He consulted his home doctor because of general fatigue in November 2011. He was not taking any medicine that

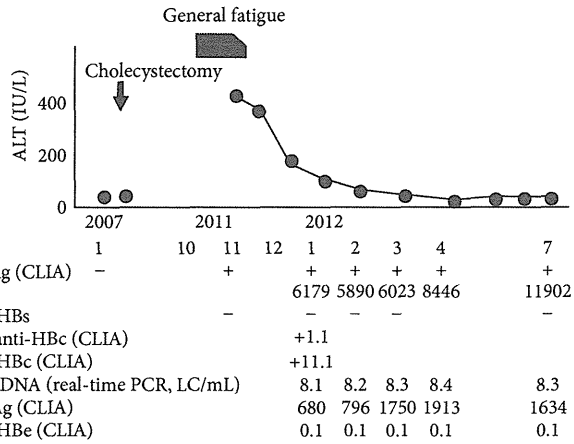

FIGURE 1: Clinical course of a patient infected with HBV genotype C who progressed to chronic infection (patient 1).

may have suppressed the immune function. He was HBsAg-positive and presented with elevated ALT level (466 IU/L). He consulted our hospital in January 2012. He was positive for HBeAg and HBV-DNA. The cutoff indexes of IgM-type anti-HBc and anti-HBc (CLIA) were 1.1 and 11.1, respectively. His general fatigue improved within a few weeks. ALT levels improved gradually, reaching the normal range at 8 months after onset, and remained within the normal range thereafter. However, he was consistently positive for HBsAg, HBeAg, and HBV-DNA (Figure 1). He was infected with HBV genotype C.

Patient 2 was a 28-year-old woman. She had been a sexual worker since 2007. She complained about mild fatigue and consulted our hospital in 2008. Her ALT level was 44 IU/L and she was positive for HBsAg, HBeAg, and HBV-DNA. Her ALT level was within the normal range, and HBsAg and HBeAg were positive thereafter. HBV-DNA was consistently >8 LC/mL. She had been diagnosed with chronic hepatitis with mild activity or asymptomatic HBV carrier status (i.e., the immune-tolerant phase); therefore, IgM-type anti-HBc and anti-HBc were not tested in 2008. However, in 1999, it was proven later that she had been negative for HBsAg when she gave birth to her daughter. Although the daughter did not receive HBV vaccine after birth, she was negative for HBsAg and anti-HBc in 2010. The patient was suspected to have had acute HBV infection with mild hepatitis, which was considered an unapparent infection. She was infected with HBV genotype C.

Patient 3 was a 20-year-old woman. She was negative for HBsAg and HBV-DNA when she donated blood in 1998. She was HBsAg-positive and had an elevated ALT level when she donated again in 1999. She had a history of sexual contact with a man a few months before the donation in 1999. She and her sexual partner were found to be infected with HBV genotype D. She was HBsAg- and HBeAg-positive. IgM-type anti-HBc was positive (cutoff index value 5.4) and anti-HBc was also positive with a high titer (98% in 200-fold diluted serum). Liver biopsy prompted a histological diagnosis of chronic hepatitis with stage 1 and grade 2. She was treated with interferon for 1 month in 2000. She became HBsAg- and HBeAg-negative after the treatment. This patient was also suspected to have had unapparent acute HBV infection.

All of these 3 patients were not drug abusers and were not alcohol abusers. They did not take any medicine that may suppress immune functions. The details of the clinical courses and complete nucleotide sequences of HBV in patients 2 and 3 have been reported previously as case reports [17–19]. In patient 1, precore (nt 1986) and core promoter (nt 1762 and nt 1764) sequence of HBV-DNA were analyzed by polymerase chain reaction-enzyme-linked minisequence assay (PCR-ELMA) and PCR-scintillation proximity assay (PCR-SPA), respectively. He was infected with HBV without mutations in these positions.

4. Discussion

It must be noted that the present study was not aimed at knowing the rate of chronicity in acutely HBV-infected patients, because it is extremely difficult to collect the unapparent cases of acute HBV infection. The purpose of this study is to know the clinical features of patients with acute HBV infection who progressed to chronic infection.

All 3 patients in group A in the present study had been negative for HBsAg for at least 1 year before testing positive. Data regarding anti-HBc and HBV-DNA negativity before the onset of hepatitis were available in patient 3 but not in patient 1 or patient 2. Therefore, the possibility of HBV reactivation from HBsAg-negative carrier or resolved hepatitis status in these 2 patients cannot be excluded completely. However, these patients did not take any medicine that may suppress immune function, were not drug abusers or alcohol abusers, and were not in immune-suppressed state. Therefore, the risk of HBV reactivation is presumed to be very low.

Details of the clinical course and complete HBV genome sequences of patient 2 and patient 3 in the present study had been reported as case reports. Patient 2 was infected with genotype C HBV, with no mutations in core promoter (nt 1762, nt 1764) and precore (nt 1896). The case report

indicated the possibility that acute infection of HBV genotype C infection in adult may progress to chronic infection, which had been reported to be rare in genotype C. Patient 3 was infected with genotype D HBV, and this HBV also had no mutations in core promoter (nt 1762, nt 1764) and precore (nt 1896). It has been shown in these case reports that the sequences of both strains were not very peculiar compared with many other strains of the same genotype in this district and specific mutations that related to chronicity of acute HBV infection had not been found.

Factors related to the severity of acute HBV infection and its clinical outcomes have been reported previously [20–22]. However, the factors related to the chronicity of HBV in acutely infected patients have not been fully elucidated. Both host and viral factors are suspected to affect the progression to chronic infection in acute HBV-infected patients [23–25]. The rate of chronicity in immunocompromised patients such as patients coinfected with HIV is reported to be high [24, 25]. In the present study, 3 of 25 adult patients with acute HBV infection progressed to chronic infection. These 3 patients were not compromised host. There were no differences between groups A and B with respect to age and sex, but T. bil and ALT levels were significantly lower in group A than those in group B. None of the 3 patients in group A had jaundice; their ALT levels were less than 500 IU/L, whereas HBV-DNA levels were high. Two of the 3 patients in group A lacked the typical symptoms of acute hepatitis. It is suspected that the progression from acute to chronic infection appears to represent a failure of immune clearance of virus-infected cells and it is marked by persistently high levels of HBV-DNA and HBeAg in serum. It has been described in a few literatures that the accompanying acute hepatitis is typically mild and subclinical with only modest serum ALT elevations and no jaundice in patients with acute hepatitis B who progressed to choric infection [5, 26]. The present data are concordant with these previous reports. Though the data of HBV sequences in the present study are not enough, all 3 patients of group A were infected with HBV without mutations in core promoter (nt 1762, nt 1764) and precore (nt 1896). It is well known that these mutations relate to severe hepatitis. It is needed to investigate further the relation of wild type HBV and chronicity of acute HBV infection in the future.

Among the 3 patients of group A, 2 and 1 were infected with genotypes C and D, respectively. There was no difference in HBV genotypes between groups A and B. These data indicate that acute infection with genotype C or D in adult can possibly progress to chronic infection in acutely infected adult patients, especially in those with mild acute hepatitis.

HBV genotype is a factor known to be related to the chronicity of acute HBV infection. The chronicity of genotype A is reported to be high, whereas that of genotype C is low; the rates of the chronicity of genotypes A and C infections are reported to be 3–23% and 0-1%, respectively [5–8]. Another report indicates that the chronicity of genotype D is supposed to be lower than that of genotype A [27]. The present results are not similar to the results of these reports in this respect. Many of these previous reports analyzed the follow-up data of patients presenting with clinical features of acute hepatitis. However, there is no report on the follow-up data of subclinical patients with acute HBV infection. Thus, the present study may indicate that the rate of chronicity in unapparent or subclinical cases of acute HBV infection with genotypes C and D might not be very low. However, it is difficult to study unapparent or subclinical cases of acute HBV infection. In the present study, 2 cases were unapparent infection, which were diagnosed as acute infection only because of accurate patient history. This highlights the importance of precisely taking the histories of patients and their families, even if they are supposedly chronically infected with HBV. The rate of chronicity of subclinical cases of acute HBV infection should be studied in greater detail in the near future.

In conclusion, adult patients with mild acute hepatitis B or subclinical HBV infection may have a higher possibility of progressing to chronic infection regardless of HBV genotypes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Circulating AIM as an Indicator of Liver Damage and Hepatocellular Carcinoma in Humans

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Abstract

Background: Hepatocellular carcinoma (HCC), the fifth most common cancer type and the third highest cause of cancer death worldwide, develops in different types of liver injuries, and is mostly associated with cirrhosis. However, non-alcoholic fatty liver disease often causes HCC with less fibrosis, and the number of patients with this disease is rapidly increasing. The high mortality rate and the pathological complexity of liver diseases and HCC require blood biomarkers that accurately reflect the state of liver damage and presence of HCC.

Methods and Findings: Here we demonstrate that a circulating protein, apoptosis inhibitor of macrophage (AIM) may meet this requirement. A large-scale analysis of healthy individuals across a wide age range revealed a mean blood AIM of 4.99±1.8 μg/ml in men and 6.06±2.1 μg/ml in women. AIM levels were significantly augmented in the younger generation (20s–40s), particularly in women. Interestingly, AIM levels were markedly higher in patients with advanced liver damage, regardless of disease type, and correlated significantly with multiple parameters representing liver function. In mice, AIM levels increased in response to carbon tetrachloride, confirming that the high AIM observed in humans is the result of liver damage. In addition, carbon tetrachloride caused comparable states of liver damage in AIM-deficient and wild-type mice, indicating no influence of AIM levels on liver injury progression. Intriguingly, certain combinations of AIM indexes normalized to liver marker score significantly distinguished HCC patients from non-HCC patients and thus could be applicable for HCC diagnosis.

Conclusion: AIM potently reveals both liver damage and HCC. Thus, our results may provide the basis for novel diagnostic strategies for this widespread and fatal disease.

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Introduction

Chronic liver injury is one of the most common and fatal diseases in modern society. It has multiple causes including hepatitis virus infection mostly due to hepatitis C virus (HCV) and to a lesser extent hepatitis B virus (HBV), alcohol injury, autoimmunity, and genetic disorders such as hemochromatosis [1–3]. In addition, the non-alcoholic fatty liver disease (NAFLD), which is associated with obesity, has been observed in a rapidly

growing number of patients due to recent and drastic changes in lifestyle. NAFLD comprises a wide variety of disease criteria ranging from benign simple steatosis to progressive inflammation and fibrosis, called non-alcoholic steatohepatitis (NASH) [4,5]. Such chronic liver diseases exhibit continuous inflammation and fibrosis and are a prominent risk for the development of hepatocellular carcinoma (HCC) [6–8]. In contrast to patients with HCV infection, who display a high susceptibility to HCC, only a limited proportion of NAFLD patients progress to carcinoma [9–11]. Intriguingly, recent evidence has revealed that although HCC develops largely on the basis of severe liver fibrosis/cirrhosis, it often occurs without cirrhosis in NAFLD/NASH patients exhibiting mild inflammation and fibrosis [12–18]. However, the mechanism of how each pathological background induces HCC remains to be elucidated. With such increasing risks and complicated pathogenesis, biomarkers that reflect the state of liver damage and the presence of HCC are important, particularly for the early diagnosis of HCC development. Ideally, markers that indicate an individual's susceptibility to HCC may be desirable from the prognostic and preventive views of HCC.

The circulating protein, apoptosis inhibitor of macrophage (AIM), also called CD5L, was initially identified as an apoptosis inhibitor that supports macrophage survival [19]. AIM is produced solely by tissue macrophages under transcriptional regulation by nuclear receptor liver X receptor alpha (LXRα) [20–22], and as a secreted molecule, AIM is detected in both human and mouse blood [19,23]. Interestingly, AIM associates with the immunoglobulin (Ig)M pentamer in the blood, and this association protects AIM from renal excretion, thereby maintaining circulating AIM at a relatively high concentration (approximately 2–5 μg/ml) in mice [23,24]. However, AIM's precise levels in healthy individuals and patients with various diseases remain controversial [25–28].

We recently identified that AIM is incorporated into adipocytes via CD36-mediated endocytosis where it inactivates cytoplasmic fatty acid synthase (FASN) through direct binding. This response reduces the production of lipid droplet-coating proteins such as fat-specific protein 27 (FSP27) and perilipin, thereby decreasing triacylglycerol deposition within adipocytes [29,30]. Consistent with this effect, adipocyte hypertrophy was found to be more advanced with a greater mass of visceral adipose tissue in AIM-deficient ($AIM^{-/-}$) mice than in wild-type ($AIM^{+/+}$) mice fed a high-fat diet (HFD) [29]. We also found that AIM prevents lipid storage in the liver, as in adipocytes [31]. Because a consensus has rapidly emerged that hepatocytic lipid metabolism impacts the pathogenesis of not only NAFLD but also other liver injuries, as well as HCC development, we decided to address the possible relationship in circulating AIM levels, the state of liver damage, and the presence of HCC in humans.

In this study, we first analyzed a large number of healthy individuals to determine the "normal level" of circulating AIM. We then assessed the correlation between circulating AIM levels and the state of liver damage using sera from patients with liver diseases. We also tested whether the difference in AIM levels correlated with the progression of liver damage using a mouse model. Furthermore, we investigated whether the AIM level can be applied for the diagnosis of HCC in humans.

Methods

Human subjects

Serum samples of healthy individuals were collected from volunteers who had annual medical examinations at Inoue Hospitals (Nagasaki, Japan). Serum samples of patients with liver diseases were obtained from Tokyo University Hospital, Ehime University Hospital and Jichi Medical University Hospital.

Ethic

For analysis of human subjects, informed consent in writing was obtained from each healthy volunteer and patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Committee of the University of Tokyo for Medical Experiments (Permission Numbers: #3358 & #2817). In addition, all animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo (Permit Number: P10-143). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Carbon tetrachloride (CCl4) administration

$AIM^{-/-}$ mice [19] had been backcrossed to C57BL/6 (B6) for 15 generations before used for experiments. Mice were intraperitoneally injected with CCl4 (Wako, Osaka, Japan) (1.6 g/kg body weight; dissolved in corn oil) twice a week for 3 or 12 wk. Mice were sacrificed 3 days after the last injection of CCl_4. All mice were maintained under a specific pathogen-free (SPF) condition.

Statistical analysis

Student's *t*-test was performed to compare values from two groups. Correlation coefficients and *p* values were calculated by Excel. Multiple linear regression analysis was performed by backward stepwise approach, with $t>1.5$ for entry and $t<1.5$ or inter-variables correlation coefficient>0.5 or probability $F>0.1$ for removal from the model. Multiple pairwise comparison among groups were performed by ANCOVA using JMP software (version 11).

ELISA assay

Human AIM was measured by an ELISA system using mouse anti-human AIM monoclonal antibodies (clones #6 and #7; established in our laboratory), which is now available from the Trans Genic Inc., Kumamoto, Japan. For ELISA of mouse AIM, we used two different rat anti-mouse AIM monoclonal antibodies (clones #36 and #35; established in our laboratory). Human IgM was measured by Human IgM ELISA Quantification Set (Bethyl Laboratories, Inc. Montgomery, USA).

Histology

Liver specimens were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) and replaced into 30% sucrose/PBS liquid. Samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Co.,Ltd., Tokyo), cut by 10 μm. For Sirius red staining, sections were washed in PBS for 5 min, counter stained with Mayer's Hematoxylin for 10 min, washed with running water for 2 min and subsequently soaked in hydrochloric acid alcohol (0.5% HCl in 70% EtOH) for 1 min. Sections were then stained with 0.03% Sirius red (Direct red 80, SIGMA-ALDRICH) in saturated picric acid solution for 15 min. HE staining was performed using Mayer's Hematoxylin (MUTO PURE CHEMICALS CO.,LTD., Tokyo) and Eosin (SIGMA-ALDRICH, St. Louis, USA).