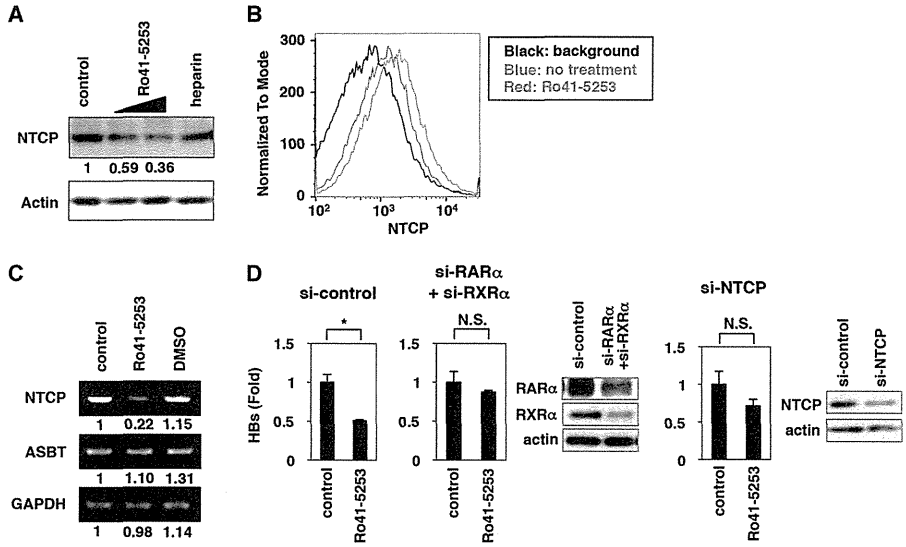

FIGURE 3. **Ro41-5253 reduced NTCP expression.** *A*, HepaRG cells were treated or untreated with 10 and 20 μM Ro41-5253 or 50 units/ml heparin for 12 h, and the levels of NTCP (*upper panel*) and actin (*lower panel*) were examined by Western blot analysis. The relative intensities for the bands of NTCP measured by densitometry are shown below the *upper panel*. *B*, flow cytometric determination of NTCP protein level on the cell surface of primary human hepatocytes treated with 20 μM Ro41-5253 (*red*) for 24 h or left untreated (*blue*). The *black line* indicates the background signal corresponding to the cells untreated with the primary antibody. *C*, RT-PCR determination of the mRNA levels for *NTCP* (*upper panel*), *ASBT* (*middle panel*), and *GAPDH* (*lower panel*) in cells treated with 20 μM Ro41-5253 or 0.1% DMSO for 12 h or left untreated. The relative intensities for the bands measured by densitometry are shown *below the panels*. *D*, HepaRG cells were treated with siRNA against RARa (*si-RARa*) plus that against RXRa (*si-RXRa*), that against NTCP (*si-NTCP*), and a randomized siRNA (*si-control*) for 3 days and then were re-treated with siRNAs for 3 days. The cells were pretreated with or without Ro41-5253 for 24 h and then infected with HBV for 16 h. HBs antigen produced from the infected cells were measured at 12 days postinfection. Statistical significance was determined using Student's *t* test (*, $p < 0.05$; *NS*, not significant).

tion was, at least in part, mediated by targeting NTCP. These data suggest that Ro41-5253 down-regulated NTCP, which probably contributed to the anti-HBV activity of Ro41-5253.

Retinoic Acid Receptor Regulated NTCP Promoter Activity—To determine the mechanism for Ro41-5253-induced down-regulation of NTCP, we used a reporter construct inserting nucleotides (nt) −1143 to +108 of the human *NTCP* (h*NTCP*) promoter upstream of the Gluc gene (Fig. 4*A*, *upper panel*). Ro41-5253 dose-dependently decreased the luciferase activity driven from this promoter, although the effect was modest and showed up to ~40% reduction (Fig. 4*A*, *left panel*). Ro41-5253 had little effect on the herpes simplex virus thymidine kinase promoter (Fig. 4*A*, *right panel*), suggesting that Ro41-5253 specifically repressed hNTCP promoter activity. As reported previously (38), Ro41-5253 specifically inhibited RAR-mediated transcription (Fig. 4, *B* and *C*). RARα, RARβ, and RARγ are members of the nuclear hormone receptor superfamily, which are ligand-activated transcription factors that regulate the transcription of specific downstream genes by binding to the RAR-responsive element (RARE) predominantly in the form of a heterodimer with RXR. We therefore asked whether RAR could regulate the h*NTCP* promoter. As shown in Fig. 4*D*, hNTCP promoter activity was stimulated by overexpression of either RARα, RARβ, or RARγ together with RXRα, and transcription augmented by RAR could be repressed by Ro41-5253 (Fig. 4*D*). Knockdown of endogenous RARα, RXRα, or both dramatically impaired the activity of the h*NTCP* promoter (Fig. 4*E*). These results suggest that RAR/RXR is involved in the transcriptional regulation of the h*NTCP* gene. Consistently, an RAR agonist, ATRA, induced *NTCP* mRNA expression (Fig. 4*F*).

Importantly, endogenous expression of RARα was more abundant in differentiated HepaRG cells, which are susceptible to HBV infection, than that in undifferentiated HepaRG and HepG2 cells, which are not susceptible (Fig. 4*G*) (29). This expression pattern was consistent with the expression of NTCP and with HBV susceptibility, suggesting the significance of RAR in regulating NTCP expression.

Promoter Analysis of hNTCP—We next examined whether RAR regulation of the h*NTCP* promoter is direct or indirect. From the analyses so far using the rat *Ntcp* (r*Ntcp*) promoter, one of the major regulators for rNtcp expression is farnesoid X receptor (FXR), which is a nuclear receptor recognizing bile acids (39). FXR, which is activated upon intracellular bile acids, indirectly regulates r*Ntcp* expression; FXR induces its downstream small heterodimer partner (Shp), another nuclear receptor, and Shp recruits to the r*Ntcp* promoter to repress the promoter activity (39). Then we examined whether RAR affected the expression of human SHP. As shown in Fig. 5*A*, although an FXR agonist GW4064 remarkably induced SHP expression as reported (39), RAR did not have a remarkable effect on the SHP level in HepaRG cells (Fig. 5*A*). To assess the direct involvement of RAR in h*NTCP* regulation, the ChIP assay showed that RAR was associated with the h*NTCP* promoter both in the presence and absence of ATRA (Fig. 5*B*), consistent with the characteristic that RAR/RXR binds to RARE regardless of ligand stimulation (40). The Genomatix software predicts that the h*NTCP* promoter possesses five putative RAREs in nt −1143 to +108 (Fig. 5*C*). Introduction of mutations in all of these five elements lost the promoter activation by RAR/RXR overexpression (Fig. 5*C*, *5-Mut*). Although the promoters mutated in the motif nt −491 to −479, −368 to −356, −274 to −258, or −179 to −167 were activated by ectopic expression of RAR/RXR and this activation was cancelled by Ro41-5253 treatment, the h*NTCP* promoter with

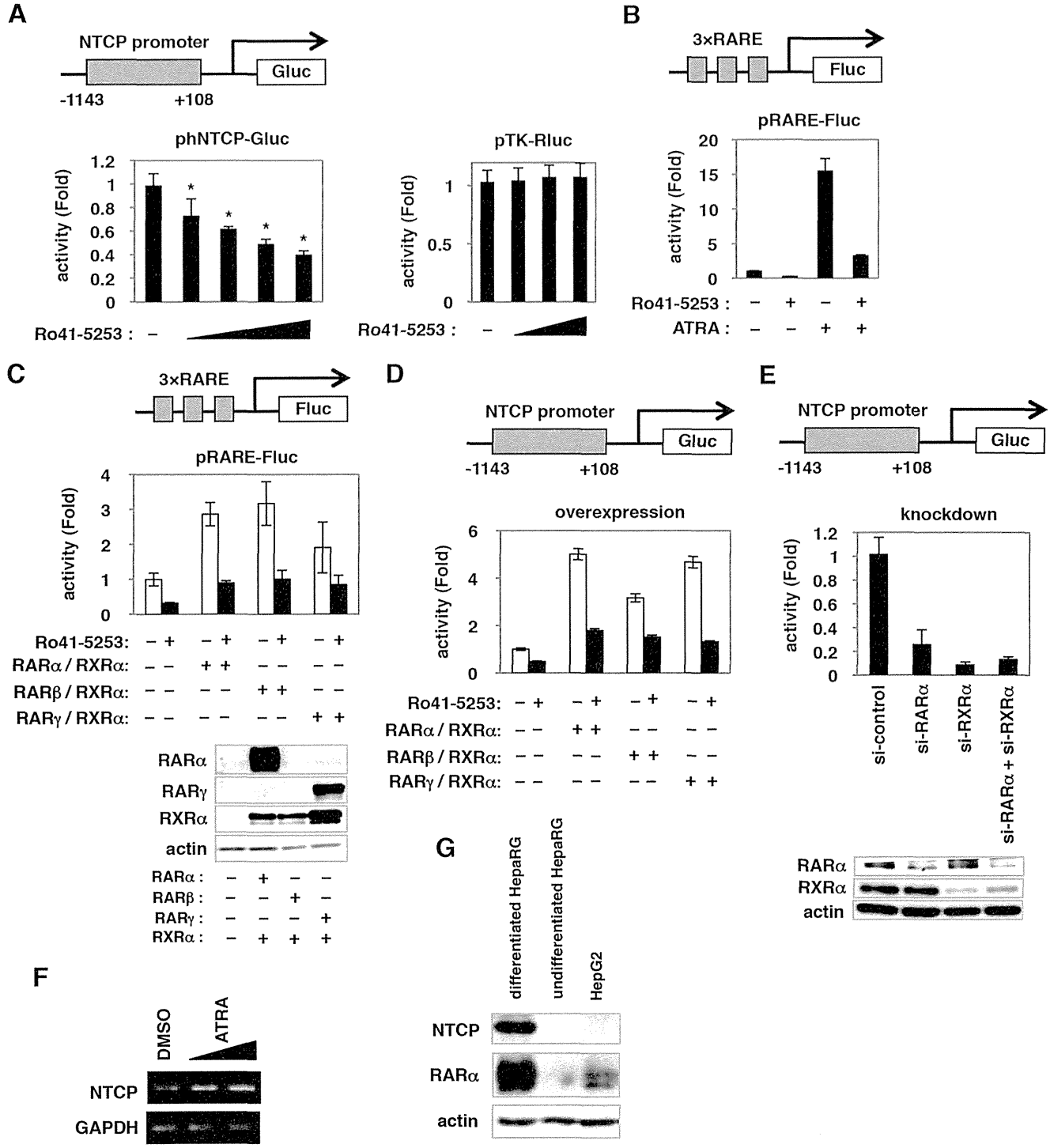

FIGURE 4. **RAR could regulate h*NTCP* promoter activity.** *A, left panel,* HuS-E/2 cells were transfected for 6 h with an hNTCP reporter construct with −1143/+108 of the h*NTCP* promoter region cloned upstream of the Gluc gene (*upper panel, phNTCP-Gluc*), together with an internal control plasmid expressing SEAP (pSEAP). Cells were treated or untreated with various concentrations of Ro41-5253 (5–40 μM) for 48 h. The Gluc and SEAP activities were determined, and the Gluc values normalized by SEAP are shown. *Right panel,* HuS-E/2 cells transfected with a reporter construct carrying the herpes simplex virus thymidine kinase promoter (*pTK-Rluc*) were examined for luciferase activity in the presence or absence of Ro41-5253 (10–40 μM). *B,* HuS-E/2 cells transfected with a Fluc-encoding reporter plasmid carrying three tandem repeats of RARE (*upper panel, pRARE-Fluc*), and Rluc-encoding reporter plasmid driven from herpes simplex virus thymidine kinase promoter (*pTK-Rluc*) were treated with or without 20 μM Ro41-5253 in the presence or absence of an RAR agonist, ATRA, 1 μM for 24 h. Relative values for Fluc normalized by Rluc are shown. *C,* HuS-E/2 cells transfected with pRARE-Fluc and pTK-Rluc with or without expression plasmids for RARs (RARα, RARβ, or RARγ) and RXRα were treated with (*black*) or without (*white*) Ro41-5253 for 48 h. Relative values for Fluc/Rluc are shown. *D,* HuS-E/2 cells were cotransfected with phNTCP-Gluc and pSEAP with or without the expression plasmids for RARs (RARα, RARβ, or RARγ) and RXRα, followed by 24 h of treatment or no treatment with 20 μM Ro41-5253. Relative Gluc/SEAP values are shown. *E,* phNTCP-Gluc and pSEAP were transfected into HuS-E/2 cells together with siRNAs against RARα (*si-RARα*), RXRα (*si-RXRα*), si-RARα plus si-RXRα, or randomized siRNA (*si-control*) for 48 h. Relative Gluc/SEAP values are indicated. Endogenous RARα, RXRα, and actin proteins were detected by Western blot analysis (*lower panels*). *F,* mRNA levels for *NTCP* and *GAPDH* were detected in differentiated HepaRG cells treated with or without ATRA (0.5 and 1 μM) for 24 h. *G,* protein levels for endogenous NTCP (*upper panel*), RARα (*middle panel*), and actin (*lower panel,* as an internal control) were determined by Western blot analysis of differentiated HepaRG, undifferentiated HepaRG, and HepG2 cells. Statistical significance was determined using Student's *t* test (*, $p < 0.05$).

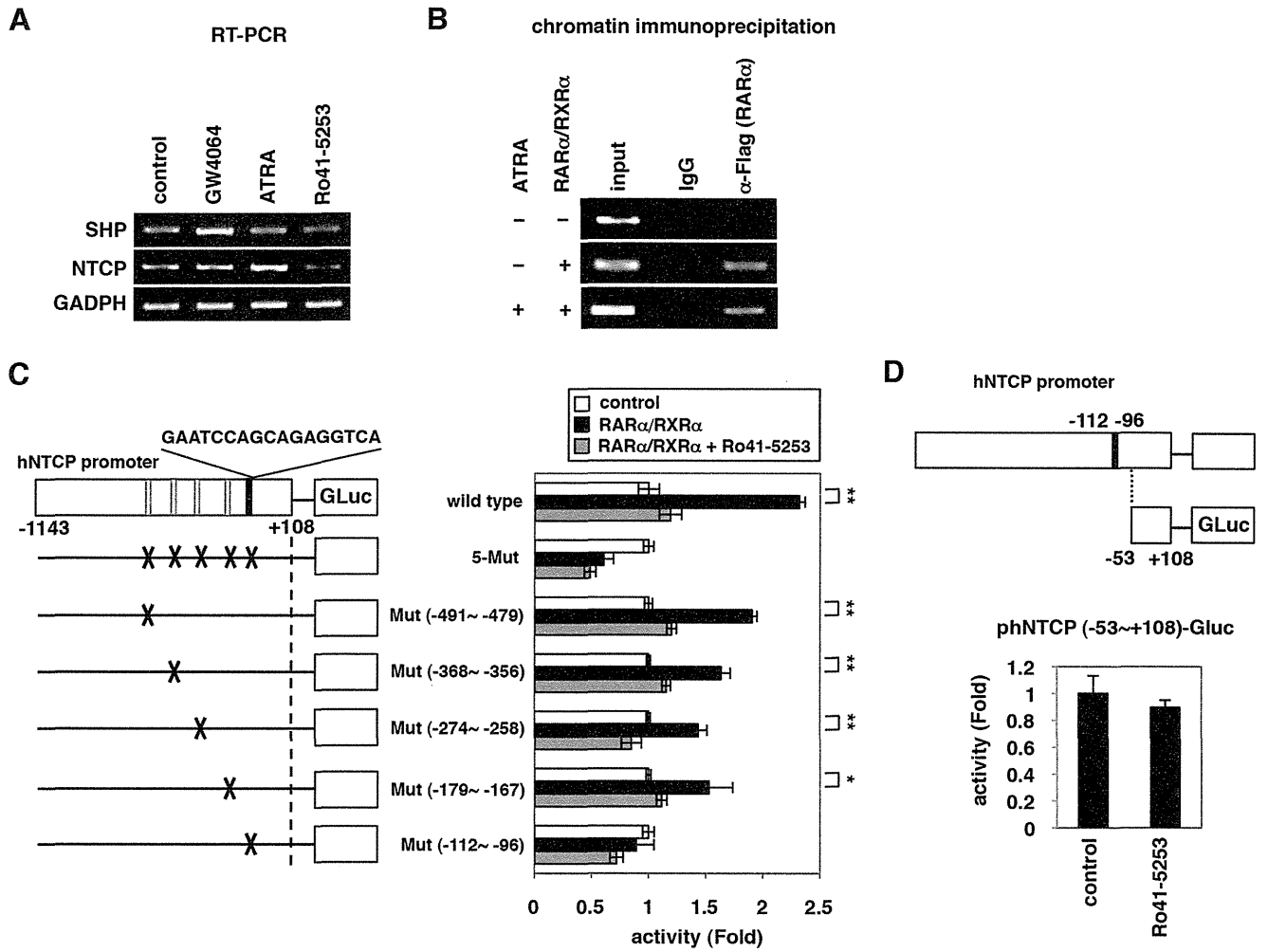

FIGURE 5. **RAR directly regulated the activity of hNTCP promoter.** *A,* HepaRG cells were treated with or without ATRA, Ro41-5253, or a positive control GW4064, which is an FXR agonist, for 24 h. mRNAs for SHP as well as *NTCP* and *GAPDH* were detected by RT-PCR. *B,* ChIP assay was performed as described under "Experimental Procedures" with Huh7-25 cells transfected with or without an expression plasmid for FLAG-tagged RARa plus that for RXRa in the presence or absence of ATRA stimulation. *C, left panel,* schematic representation of h*NTCP* promoter and the reporter constructs used in this study. h*NTCP* promoter has five putative RAREs (nt −491 to −479, −368 to −356, −274 to −258, −179 to −167 (*gray regions*), and −112 to −96 (*black regions,* GAATCCAGCAGAGGTCA)) in nt −1143 to +108 of h*NTCP*. The mutant constructs possessing mutations within each putative RAREs and in all of five elements (*5-Mut*) as well as the wild type construct are shown. *Right panel,* relative luciferase activities upon overexpression with or without RARa plus RXRa in the presence or absence of Ro41-5253. *D,* deletion reporter construct carrying the region nt −53 to +108 of the h*NTCP* upstream of the Gluc gene was used for the reporter assay in the presence or absence of Ro41-5253.

mutations in nt −112 to −96 had no significant response by RAR/RXR (Fig. 5*C*). Promoter activity of h*NTCP* that lacked the region nt −112 to −96 (nt −53 to +108) was not affected by Ro41-5253 (Fig. 5*D*). These data suggest that the nt −112 to −96 region is responsible for RAR-mediated transcriptional activation of h*NTCP*.

HBV Susceptibility was Decreased in RAR-inactivated Cells—We further investigated the impact of RAR antagonization on HBV infectivity. BMS195614, BMS493, and MM11253, which repressed RAR-mediated transcription (Fig. 6*A*), all decreased the susceptibility of HepaRG cells to HBV infection (Fig. 6*B*) without significant cytotoxicity (Fig. 6*C*). These data confirmed that HBV infection was restricted in RAR-inactivated cells. Among these, CD2665, a synthetic retinoid that is known to inhibit RAR-mediated transcription (Fig. 7*A*), had more potent anti-HBV activity than Ro41-5253 (Fig. 7*B*), which was accompanied by the inhibition of the h*NTCP* promoter (Fig. 7*C*) and down-regulation of NTCP protein (Fig. 7*D*).

CD2665 Showed a Pan-genotypic Anti-HBV Effect—We then examined the effect of CD2665 on the infection of primary human hepatocytes with different HBV genotypes. CD2665 significantly reduced the infection of HBV genotypes A, B, C, and D, as revealed by quantification of HBs and HBe antigens in the culture supernatant of infected cells (Fig. 8, *A–D*). Additionally, this RAR inhibitor decreased the infection of the ETV- and LMV-resistant HBV genotype C clone carrying mutations in L180M, S202G, and M204V (Fig. 8, *E* and *F*). Thus, CD2665 showed pan-genotypic anti-HBV effects and was also effective on an HBV isolate with resistance to nucleoside analogs.

We further investigated whether RAR inhibitors could prevent HBV spread. It was recently reported that HBV infection in freshly isolated primary human hepatocytes could spread during long term culture through production of infectious virions and reinfection of surrounding cells (41). As shown in Fig. 8*G*, the percentage of HBV-positive cells increased up to 30 days postinfection without compound treatment (Fig. 8*G*,

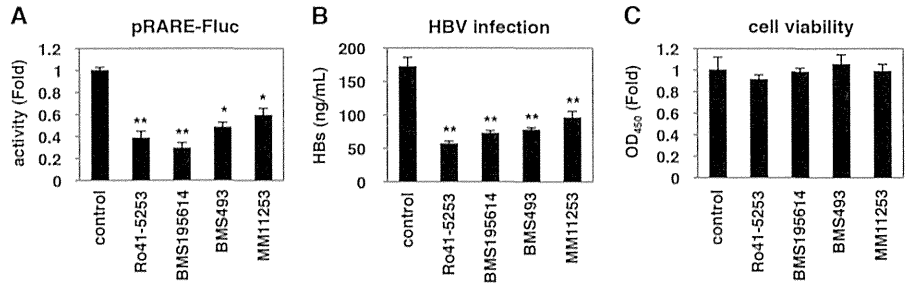

FIGURE 6. **HBV susceptibility was decreased in RAR-inactivated cells.** *A*, HuS-E/2 cells were transfected with the pRARE-Fluc and pTK-Rluc for 6 h followed by treatment with or without the indicated compounds at 20 μM for 48 h. Relative Fluc values normalized by Rluc are shown. *B* and *C*, HepaRG cells treated with or without the indicated compounds 20 μM were subjected to the HBV infection assay according to the scheme in Fig. 1*A*. HBs antigen in the culture supernatant was determined by ELISA (*B*). Cell viability was also quantified by MTT assay (*C*). Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

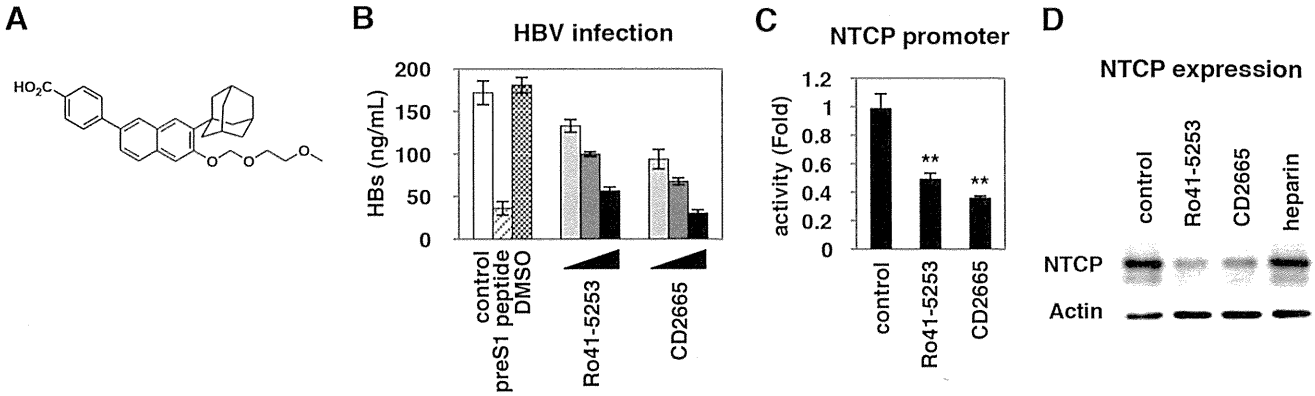

FIGURE 7. **CD2665 had a stronger anti-HBV activity than Ro41-5253.** *A*, chemical structure of CD2665. *B*, HepaRG cells treated with or without 1 μM preS1 peptide, 0.1% DMSO, or various concentrations of Ro41-5253 or CD2665 (5, 10, and 20 μM) were subjected to HBV infection according to the protocol shown in Fig. 1*A*. HBV infection was detected by quantifying the HBs secretion into the culture supernatant by ELISA. The efficiency of HBV infection was monitored by ELISA detection of secreted HBs. *C*, HuS-E/2 cells transfected with phNTCP-Gluc and pSEAP were treated with the indicated compounds at 20 μM for 24 h. Relative Gluc/SEAP values are shown. *D*, NTCP (*upper panel*) and actin proteins as an internal control (*lower panel*) were examined by Western blot analysis of HepaRG cells treated with or without the indicated compounds at 20 μM. Statistical significance was determined using Student's *t* test (**, $p < 0.01$).

panels a–d). However, such HBV spread was clearly interrupted by treatment with Ro41-5253 and CD2665 as well as preS1 peptide (Fig. 8*G*, *panels e–p*). The rise of HBs antigen in the culture supernatant along with the culture time up to 30 days was remarkably inhibited by continuous treatment with Ro41-5253 and CD2665 as well as preS1 peptide without serious cytotoxicity (Fig. 8*G*, *right graph*). Thus, continuous RAR inactivation could inhibit the spread of HBV by interrupting *de novo* infection.

DISCUSSION

In this study, we screened a chemical library using a HepaRG-based HBV infection system and found that pretreatment with Ro41-5253 decreased HBV infection by blocking viral entry. HBV entry follows multiple steps starting with low affinity viral attachment to the cell surface followed by specific binding to entry receptor(s), including NTCP. NTCP is reported to be essential for HBV entry (42). So far, we and other groups have reported that NTCP-binding agents, including cyclosporin A and its derivatives, as well as bile acids, including ursodeoxycholic acid and taurocholic acid, inhibited HBV entry by interrupting the interaction between NTCP and HBV large surface protein (19, 35). Ro41-5253 was distinct from these agents and was found to decrease host susceptibility to HBV infection by modulating the expression levels of NTCP. These results suggest that the regulatory circuit for NTCP expression is one of the determinants for susceptibility to HBV infection. We previously showed that the cell surface NTCP protein expression correlated with susceptibility to HBV infection (43). We therefore screened for compounds inhibiting h*NTCP* promoter activity to identify HBV entry inhibitors (data not shown) (44). Intriguingly, all of the compounds identified as repressors of the h*NTCP* promoter were inhibitors of RAR-mediated transcription. This strongly suggests that RAR plays a crucial role in regulating the activity of the h*NTCP* promoter (Fig. 9). We consistently found that RAR was abundantly expressed in differentiated HepaRG cells susceptible to HBV infection, in contrast to the low expression of RAR in undifferentiated HepaRG and HepG2 cells, which were not susceptible to HBV (Fig. 4*G*). RARE is also found in the HBV enhancer I region (45). RAR is likely to have multiple roles in regulating the HBV life cycle.

So far, only transcriptional regulation of rat Ntcp has been extensively analyzed (39, 46, 47). However, the transcription of h*NTCP* was shown to be differently regulated mainly because of sequence divergence in the promoter region (48), and transcriptional regulation of h*NTCP* remains poorly understood. Hepatocyte nuclear factor (HNF)1α and HNF4α, which positively regulated the rat *Ntcp* promoter, had little effect on h*NTCP* promoter activity (48). HNF3β bound to the promoter region and inhibited promoter activities of both h*NTCP* and rat

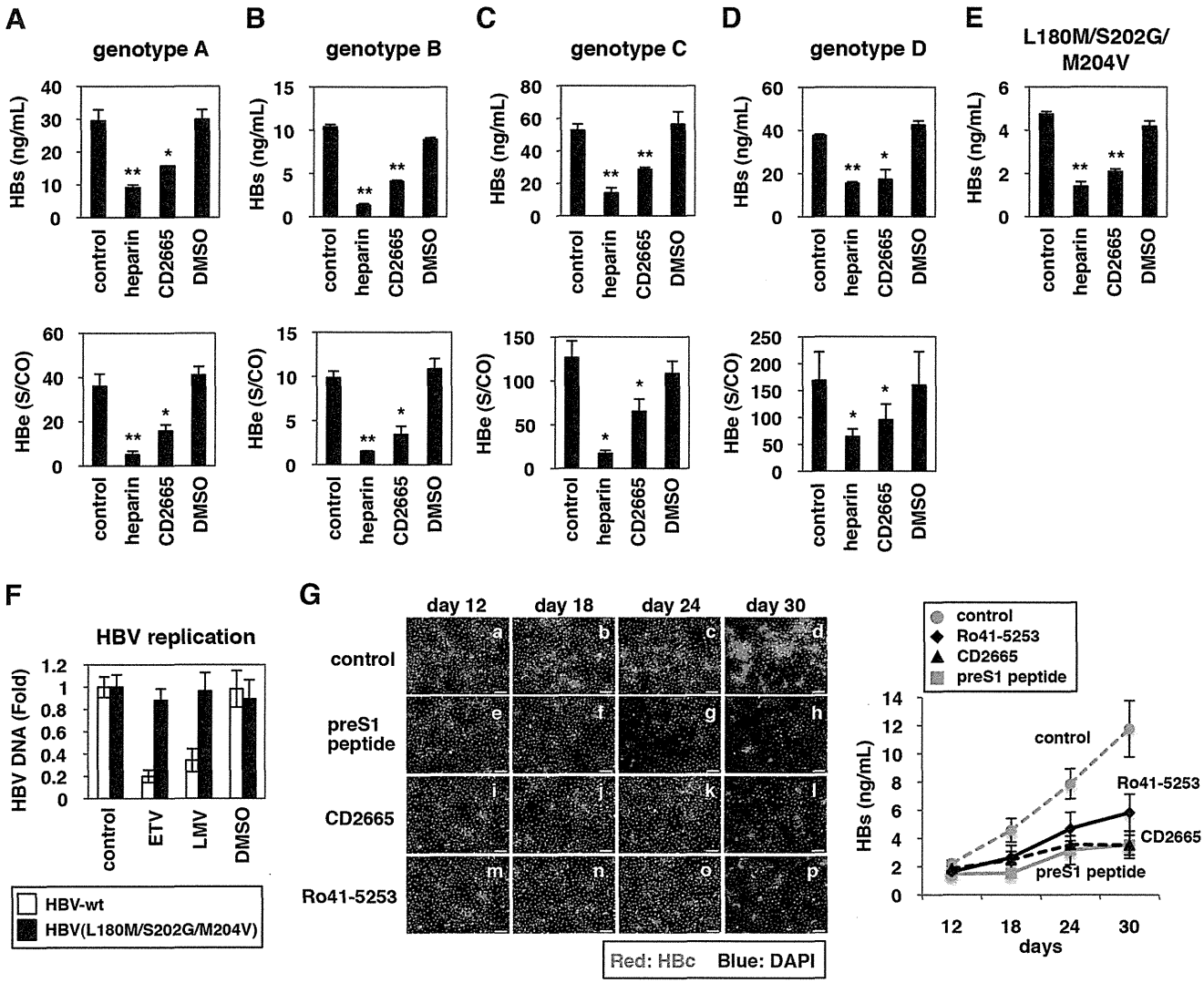

FIGURE 8. **CD2665 showed a pan-genotypic anti-HBV activity.** *A–E,* primary human hepatocytes were pretreated with or without compounds (50 units/ml heparin, 20 μM CD2665, or 0.1% DMSO) and inoculated with different genotypes of HBV according to the scheme show in Fig. 1*A*. HBs (*A–E*) and HBe (*A–D*) antigen secreted into the culture supernatant was quantified by ELISA. Genotypes A (*A*), B (*B*), C (*C*), D (*D*), and an HBV carrying mutations (L180M/S202G/M204V) (*E*) were used as inoculum. *F,* HBV(L180M/S202G/M204V) was resistant to nucleoside analogs. HepG2 cells transfected with the expression plasmid for HBV/C-AT (*white*) or HBV/C-AT(L180M/S202G/M204V) (*black*) were treated with or without 1 μM ETV, 1 μM LMV, or 0.1% DMSO for 72 h. The cells were lysed, and the nucleocapsid-associated HBV DNAs were recovered. Relative values for HBV DNAs are indicated. *G,* continuous RAR inactivation could inhibit HBV spread. Freshly isolated primary human hepatocytes were pretreated with or without indicated compounds (1 μM preS1 peptide, 10 μM Ro41-5253, or 10 μM CD2665) and inoculated with HBV at day 0. After removing free viruses, primary human hepatocytes were cultured in the medium supplemented with the indicated compounds for up to 30 days postinfection. At 12, 18, 24, and 30 days postinfection, HBc protein in the cells (*left panels, red*) and HBs antigen secreted into the culture supernatant (*right graph*) were detected by immunofluorescence and ELISA, respectively. *Red* and *blue* signals in the *left panels* show the detection of HBc protein and nucleus, respectively. Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

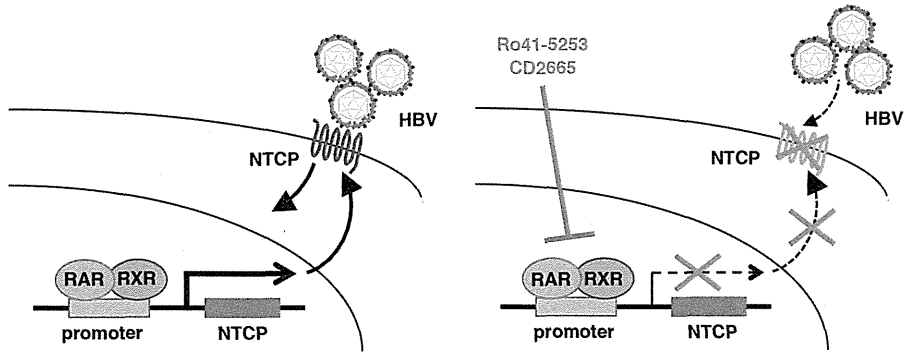

FIGURE 9. **Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection.** *Left panel,* RAR/RXR recruits to the promoter region of *NTCP* and regulates the transcription. The expression of NTCP in the plasma membrane supports HBV infection. *Right panel,* RAR antagonists, including Ro41-5253 and CD2665, repress the transcription of *NTCP* via RAR antagonization, which decreases the expression level of NTCP in the plasma membrane and abolishes the entry of HBV into host cells.

Ntcp. CCAAT/enhancer-binding protein also bound and regulated the h*NTCP* promoter (44, 48). A previous study, which was mainly based on reporter assays using a construct of the region from −188 to +83 of the h*NTCP* promoter, concluded that RAR did not affect h*NTCP* transcription (48). By using a reporter carrying a longer promoter region, our study is the first to implicate RARs in the regulation of h*NTCP* gene expression (Fig. 9). The turnover of NTCP protein was reported to be rapid, with a half-life of much less than 24 h (49). Consequently, reduction in the *NTCP* transcription by RAR inhibition could rapidly decrease the NTCP protein level and affect HBV susceptibility.

NTCP plays a major role in the hepatic influx of conjugated bile salts from portal circulation. Because NTCP knock-out mice are so far unavailable, it is not known whether loss of NTCP function can cause any physiological defect *in vivo*. However, no serious diseases are reported in individuals carrying single nucleotide polymorphisms that significantly decrease the transporter activity of NTCP (50, 51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} value for HBV infection was ~0.1 nM (52), although that for NTCP transporter function was 4 nM (28), suggesting that HBV infection could be inhibited without fully inactivating the NTCP transporter (53). HBV entry inhibitors are expected to be useful for preventing *de novo* infection upon post-exposure prophylaxis or vertical transmission where serious toxicity might be avoided with a short term treatment (54). For drug development studies against HIV, down-regulation of the HIV coreceptor CCR5 by ribozymes could inhibit HIV infection both *in vitro* and *in vivo* (55). Disruption of CCR5 by zinc finger nucleases could reduce permissiveness to HIV infection and was effective in decreasing viral load *in vivo* (56). Thus, interventions to regulate viral permissiveness could become a method for eliminating viral infection (55). Our findings suggest that the regulatory mechanisms of NTCP expression could serve as targets for the development of anti-HBV agents. High throughput screening with a reporter assay using an *NTCP* promoter-driven reporter, as exemplified by this study, will be useful for identifying more anti-HBV drugs.

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Microbiology:

Dysregulation of Retinoic Acid Receptor Diminishes Hepatocyte Permissiveness to Hepatitis B Virus Infection through Modulation of Sodium Taurocholate Cotransporting Polypeptide (NTCP) Expression

Senko Tsukuda, Koichi Watashi, Masashi Iwamoto, Ryosuke Suzuki, Hideki Aizaki, Maiko Okada, Masaya Sugiyama, Soichi Kojima, Yasuhito Tanaka, Masashi Mizokami, Jisu Li, Shuping Tong and Takaji Wakita

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Chronic Hepatitis B Prevalence among Children and Mothers: Results from a Nationwide, Population-Based Survey in Lao People's Democratic Republic

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Abstract

Background: Hepatitis B is regarded as a serious public health issue in Lao People's Democratic Republic (Lao PDR), a Southeast Asian country. However, disease epidemiology among the general population is not well known, and thus a nationwide cross-sectional survey for hepatitis B surface antigen (HBsAg) prevalence in children and their mothers was conducted.

Methods and findings: We applied three-stage cluster sampling using probability proportionate to size. After randomly selecting child (5 to 9 years old) and mother (15 to 45 years old) pairs from the selected villages, questionnaires and HBsAg rapid tests were conducted. Data from 965 child and mother pairs were analyzed. Multivariate logistic regression analyses were used to investigate the independent association of individual background characteristics for the odds of being HBsAg positive. In total, 17 children and 27 mothers were HBsAg positive. HBsAg prevalence was estimated to be 1.7% (95% confidence interval: 0.8%-2.6%) in children, and 2.9% (95% confidence interval: 1.7%–4.2%) in their mothers after taking sampling design and weight of each sample into account. Mother's infection status was positively associated with HBsAg positivity in children ($p<0.001$), whereas other potential risk factors, such as ethnicity, proximity to health centers, and history of surgery, were not. There were no significant associations between mother's HBsAg status and history of surgery, and other sociodemographic factors.

Conclusions: Despite the slow implementation of the hepatitis B vaccination program, HBsAg prevalence among children and their mothers was not high in Lao PDR compared to reports from neighboring countries. The reasons for the differences in prevalence among these countries are unclear. We recommend that prevalence surveys be conducted in populations born before and after the implementation of a hepatitis B vaccination program to better understand the epidemiology of hepatitis B.

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Introduction

More than two billion people have been infected with hepatitis B worldwide, and among these individuals, more than 350 million suffer from chronic hepatitis B virus (HBV) infection [1,2,3]. Infection with HBV results in 600,000 to 1.2 million deaths per year due to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2,4]. HBV is responsible for 60% to 80% of the world's hepatocellular carcinoma cases, one of the major three causes of death in Africa, Asia, and the Pacific Rim, and accordingly, has been categorized as a Group 1 carcinogenic agent to humans by the International Agency for Research on Cancer [5].

The prevalence of hepatitis B differs throughout the world. Southeast Asian countries have been estimated to have a chronic HBV infection rate of more than 8% before the introduction of hepatitis B vaccination [6]. The Western Pacific region of the World Health Organization (WHO), to which most of the Southeast Asian countries belong, is assumed to have a high prevalence of hepatitis B [7]. Specifically, the prevalence is estimated to be 9% to 12% among women of childbearing age [8] and 8% to 10% among children in pre-vaccine era [9]. The WHO

estimates that the region has 28% of the global population, while it accounts for almost half of all chronic hepatitis B infections worldwide [10].

Hepatitis B vaccination, especially within 24 hours after childbirth, is considered the most effective and efficient preventive measure against hepatitis B infection [3,11]. Based on these assumptions, the WHO set goals to lower the prevalence of chronic hepatitis B among children over 5 years of age to 2% by 2012 and 1% by 2017. To achieve these goals, the WHO plans to increase immunization coverage to 65% for the birth dose and 80% for the third dose of the hepatitis B vaccine [7].

Lao People's Democratic Republic (Lao PDR) is a Southeast Asian country, located in the center of the Indochina peninsula. The country is landlocked and surrounded by China, Vietnam, Cambodia, Thailand, and Myanmar. The neighboring countries report relatively high hepatitis B prevalence compared to other parts of the world. For example, a survey from two provinces in Cambodia reported a hepatitis B surface antigen (HBsAg) prevalence of 7.7% (95% CI: 6.2%–9.3%) among healthy volunteer adults [12]. Another population-based survey in a province in rural Vietnam found that 18.8% (95% CI: 15.7%–21.9%) of adults and 12.5% (95% CI: 9.7%–15.3%) of infants were HBsAg positive at the time of the survey [13]. Thus, Lao PDR has been regarded as one of the hyperendemic countries for hepatitis B for quite some time and is ranked as a priority country by the WHO [7,9] despite a lack of data on the prevalence in a representative population. Pre-vaccine era prevalence was estimated as 11.8% [4], 8–10% [9], or 8% or more [6] for Lao PDR and Indochina countries. In response to this situation, Lao PDR has implemented the hepatitis B vaccine into the routine immunization program since 2002 (at 6, 10, and 14 weeks after birth), as well as birth dosing since 2004. The birth dosing was initiated at referral hospitals in the capital city, and then gradually expanded into rural hospitals (2006), and eventually home deliveries (2010). However, since then, no direct investigation has been conducted, and thus a nationwide survey is warranted [7,9]. The routine immunization coverage is reported as 56% for BCG, 50% for the third DPT, 50% for the third hepatitis B, 40% for measles, and 46% for oral polio vaccine in 2007, when a proportion of target children were born [14].

The primary objective of the present study was to estimate the chronic HBV infection rates by measuring the seroprevalence of HBsAg among children aged 5 to 9 years, and their mothers aged 15 to 45 years.

Methods

Ethical considerations

The survey protocol was reviewed and approved by the Ethical Committee of the Ministry of Health, Lao PDR, and the institutional review board of the National Center for Global Health and Medicine, Japan (NCGM-G-001130-00). Access to selected households was granted by the Ministry of Health, and the provincial and district government authorities.

After obtaining approval to conduct the survey from local authorities, surveyors explained the purpose of the survey to village leaders, selected participants, and their caregivers, assured them that all information would be strictly confidential and that no names would be gathered, and that there would be no benefit or penalties for agreeing or refusing to participate. Written informed consent was obtained from each mother on behalf of her child for each pair. Written informed consent was obtained from legal representatives (next of kin, caregivers, or guardians) when mothers were illiterate. The respondents' names were not recorded on the questionnaire sheets.

Study population

The target population was children aged 5 to 9 years (date of birth: January 2, 2002 to January 1, 2007) and their mothers aged 15 to 45 years (date of birth: January 2, 1966 to January 1, 1997) living in the selected cluster at the time of the survey. The reasons for this selection criteria are: 1) the national and regional hepatitis control policy target is to reduce chronic hepatitis B prevalence among children aged 5 years or older [7]; 2) Lao PDR does not have reliable HBsAg prevalence data among healthy adults, and mothers of childbearing age are considered the major source of hepatitis B infection for children; and 3) our pilot survey revealed that between 20 and 25 mother and child pairs can be practically sampled from each village.

Calculation of sample size

The equation used to calculate the required sample size is as follows [15,16]:

$$n = Z^2 \times p(1-p)DEFF \times 2/(d^2 \times RR)$$

where n = sample size
Z = significance level for 95% confidence
p = expected prevalence
$DEFF$ = design effect
d = precision
RR = response rate

The sample size (n) of 961 was calculated on the basis of an expected HBsAg seroprevalence (p) of 5%, a 5% level of significance (Z), precision (d) of ±2.0%, design effect ($DEFF$) of 2.0, two strata, and response rate (RR) of 95%. For field practicability, we requested 24 survey teams to sample 21 child and mother pairs from each cluster, with the aim of gathering 1,008 pairs in total.

Survey design and sampling

The survey applied a stratified three-stage random cluster sampling design, a type of probability sampling recommended by the WHO [15,17]. The survey was carried out by 24 survey teams (two members per team). Team members were recruited from the same districts that were under investigation to implement the survey more smoothly. The survey teams consisted of epidemiology, surveillance, or laboratory staff. The survey teams were supervised by 11 national personnel (six from the National Immunization Program and five from the National Center for Laboratory and Epidemiology, Ministry of Health) as well as 13 provincial officers.

For stratified multistage cluster sampling, immunization coverage by district and population data were obtained from the National Immunization Program, the Ministry of Health, and the Department of Statistics, Lao PDR. For post-survey weight adjustment, the survey teams obtained the latest population data from village leaders or health volunteers.

All 143 districts in Lao PDR were stratified into two strata, one having high (more than 76%) and the other having low (76% or less) immunization coverage for the third diphtheria, pertussis, tetanus, and hepatitis B (DPT-HepB)vaccines as reported in 2010. For the first stage, we selected 12 districts from each stratum using probability proportionate to size (PPS) sampling based on the population census of 2005. For the second stage, we selected two villages from each selected district by PPS sampling, and 48

villages were randomly sampled in total. In the instances in which the selected village lacked a sufficient number of children or the survey team could not approach the selected village due to safety or security reasons, the nearest village on the way back to the district center was selected. For each selected village, surveyors obtained a list of households, including age and sex, primarily from the poverty reduction program data with the assistance of the village leader, women's union, and/or healthcare volunteer. From these lists, 21 mothers aged 15 to 45 years old with children aged 5 to 9 years were randomly selected using a paper-based lottery system. When a mother had multiple children aged 5 to 9 years old, the youngest child was chosen for the survey. Special attention was paid to ensure that the child's biological mother was surveyed, as adoption is common in rural Lao PDR.

The survey was carried out from January 25th to February 4th, 2012. Each survey team successfully approached their assigned villages, with the exception of one village, which could not be visited because of road difficulties. An alternative village was chosen according to the predetermined selection criteria. In total, 1,008 children and 1,008 mothers were sampled. The overall response rate for HBsAg was 100%; however, 43 pairs were excluded from the analysis due to age ineligibility. That is, one child was over 9 years of age and 33 were less than 5 years of age. Furthermore, three mothers were over 45 years of age and six were less than 15 years of age. This happened as 43 mothers confused calendar age with traditional age. In rural areas, newborns start at one year old and a year is added to their age for each passing of a Lunar New Year. The surveyors asked participants for their age in years and their date of birth, and checked that they matched. A total of 965 pairs were included for analysis.

Questionnaires

A brief face-to-face questionnaire was administered to the sampled mother. The questionnaire consisted of 25 questions in four domains of inquiry: sociodemographic background of the family (i.e., ethnicity, family head's occupation, and mother's education level), family history of liver diseases, including mother, demographic characteristics of the child (i.e., age, sex, and place of birth), and immunization records. Additionally, questions were asked regarding exposure to potential risk factors for acquiring hepatitis B infection (e.g., history of blood transfusion, surgical operation, and sharing of toothbrush). The questionnaire was developed in English, translated into Lao, back-translated into English, and then compared and revised by bilingual staff members. A small pilot test was conducted prior to the data collection.

Testing for HBsAg

We used a simple and rapid test (Alere Determine HBsAg test card; Alere Medical Co. Ltd., Chiba, Japan) rather than the traditional ELISA test, as it was better suited to use in the field [14]. The sensitivity and specificity of the test were reported as high in two Asian countries [18,19]. In Vietnam, the Determine HBsAg test validity was measured based on comparison with HBsAg EIA. Results were 100% in both sensitivity and specificity in 328 samples [18]. In China, the Determine HBsAg performance was evaluated in comparison with HBsAg EIA for 671 samples. The sensitivity was reported to be 98.9% and specificity 100% [19]. The Determine HBsAg examination kit is one of the most reliable point-of-care HBsAg tests, and is recommended by the WHO [15]. HBsAg testing was performed according to the manufacturer's instructions. Blood was collected from a finger prick using a safety lancet (BD Safety Lancet, Becton Dickinson, NJ, USA) and glass capillary tube, and the blood was applied onto the sample pad of the rapid test kit. After applying the chase buffer, surveyors assessed the results after at least 15 minutes, but no longer than 24 hours. When no control bar appeared after 15 minutes, the test results were considered invalid, and the test was repeated. Blood spots were collected onto filter paper for further testing. A 2-day training session was organized for surveyors and supervisors on the use of the rapid test and the completion of the questionnaire. To ensure the safety of the blood collection procedure, surveyors always used a new pair of latex gloves. Surveyors were instructed to place all capillary tubes and lancets into safety boxes immediately after use.

Data entry and statistical analysis

All of the completed questionnaires were brought to a centralized location and the data were entered into a Microsoft Excel 2007 spreadsheet. Data were double-entered and cross-checked. Logistic regression tests and odds ratios were used to examine the relationship between the independent variables and HBsAg results. Multivariate logistic regression was used to investigate the independent association of different household and individual characteristics with the odds of being HBsAg positive. All estimates and standard errors were calculated by taking the multistage clustered sampling design and the weight of each sample into account to give representative, unbiased results. A p value <0.05 was considered statistically significant.

In our regression analyses, we adjusted for potential confounders by using the following variables: third DPT-HepB immunization coverage at the location of current residence, mother's age, ethnic group, mother's education level, family head's occupation, and mother's HBsAg status. For multivariate logistic regression analyses, multicollinearity was tested by calculating the variance inflation factors for each independent variable, and a value of more than 10 was considered as having multicollinearity.

All statistical analyses were carried out using STATA version 12 (Stata Corp., College Station, TX). Means and proportions were calculated using STATA's 'svy' function, with each sample weighted according to estimated population size.

Results

Socioeconomic backgrounds

The baseline characteristics of the 965 mothers and their children are summarized in Table 1. The mean age of the mothers was 29.1 years (95% CI: 26.2–33.1), and the mean age of the children was 5.8 years (95% CI: 5.4–6.3). Of the sampled children, 474 (49.4%) were male and 486 (50.6%) were female (five were unknown).

HBsAg prevalence among the general population

Of the 965 pairs included in the study, 17 children and 27 mothers were positive for HBsAg. Six child and mother pairs were HBsAg positive. The estimated prevalence was 1.7% for children (95% CI: 0.8%–2.6%) and 2.9% for mothers (95% CI: 1.7%–4.2%) after taking the sampling design and weight of each sample into account. HBsAg prevalence did not change significantly between DPT-HepB3 high and low coverage districts in both children and mothers (Table 2).

Potential risk factors

To determine whether background characteristics affect HBsAg status, we conducted multivariate logistic regression analysis in children and their mothers. In children, the mother's HBsAg status was positively associated with hepatitis B infection (Table 3),

Table 1. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old) in Lao PDR by selected background characteristics.

		n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
Mothers' age(n = 965)	15–19	4	0.41	0	0.00		0	0.00	
	20–24	85	8.80	1	1.18	0.00–3.52	3	3.53	0.00–7.53
	25–29	294	30.47	7	2.38	0.63–4.13	8	2.72	0.85–4.59
	30–34	275	28.50	6	2.18	0.44–3.92	9	3.27	1.16–5.39
	35–39	176	18.24	3	1.70	0.00–3.64	3	1.70	0.00–3.64
	40–45	131	13.58	0	0.00		4	3.05	0.07–6.04
Ethnicity (n = 963)	Low land Lao	651	67.60	9	1.38	0.48–2.28	19	2.92	1.62–4.22
	Mid land Lao	248	25.75	6	2.42	0.49–4.34	5	2.02	0.25–3.78
	High land Lao	64	6.65	2	3.13	0.00–7.51	3	4.69	0.00–10.01
[1]Transportation (n = 939)	on foot	298	31.74	1	0.34	0.00–1.00	6	2.01	0.41–3.62
	bicycle	14	1.49	0	0.00		0	0.00	
	motor bike	364	38.76	7	1.92	0.51–3.34	10	2.75	1.06–4.43
	car	183	19.49	5	2.73	0.35–5.12	6	3.28	0.67–5.88
	hand tractor	66	7.03	3	4.55	0.00–9.71	4	6.06	0.15–11.97
	other	14	1.49	0	0.00		0	0.00	
[2]Time (n = 901)	< 5 minutes	31	3.44	0	0.00		1	3.23	0.00–9.81
	5 to 15 minutes	274	30.41	3	1.09	0.15–2.33	6	2.19	0.45–3.93
	15 to 30 minutes	231	25.64	5	2.16	0.27–4.06	11	4.76	2.00–7.53
	30 to 60 minutes	209	23.20	5	2.39	0.30–4.48	4	1.91	0.04–3.79
	> 60 minutes	156	17.31	3	1.56	0.00–4.68	4	2.56	0.06–5.07
[3]Education (n = 962)	did not finish primary school	307	31.91	7	2.28	0.60–3.96	12	3.91	1.73–6.09
	primary school	374	38.88	5	1.34	0.17–2.51	10	2.67	1.03–4.32
	junior high	185	19.23	3	1.62	0.00–3.46	2	1.08	0.00–2.59
	high school	73	7.59	0	0.00		1	1.37	0.00–4.10
	college/univ	20	2.08	1	5.00	0.00–15.47	2	10.00	0.00–24.41
	other or unknown	3	0.31	1	33.33	0.00–100.00	0	0.00	
[4]Occupation (n = 963)	farmer	683	70.92	13	1.90	0.88–2.93	19	2.78	1.55–4.02
	fisherman	5	0.52	0	0.00		0	0.00	
	laborer	92	9.55	1	1.09	0.00–3.25	5	5.43	0.71–10.16
	public officer	88	9.14	1	1.14	0.00–3.40	3	6.25	1.70–10.80
	factory employee	8	0.83	0	0.00		0	0.00	
	general employee	16	1.66	1	6.25	0.00–19.57	0	0.00	
	merchant	63	6.54	1	1.59	0.00–4.76	0	0.00	
	others	8	0.83	0	0.00		0	0.00	
Mother's surgery (n = 962)	yes	95	9.88	2	2.11	0.00–5.05	3	3.16	0.00–6.74
	no	867	90.12	15	1.73	0.86–2.60	24	2.77	1.67–3.86
Child's sex (n = 960)	male	474	49.38	9	1.89	0.67–3.13			
	female	486	50.63	7	1.44	0.38–2.50			
Place of delivery (n = 961)	province hospital	207	21.54	4	1.93	0.04–3.82	6	2.90	0.59–5.20
	district hospital	105	10.93	2	1.90	0.00–4.56	5	4.76	0.62–8.90
	health center	10	1.04	0	0.00		0	0.00	
	private clinic	11	1.14	0	0.00		1	9.09	0.00–29.35
	at home	569	59.21	8	1.41	0.44–2.38	14	2.46	1.18–3.74
	in the forest	56	5.83	3	5.36	0.00–11.44	1	1.79	0.00–5.36
	other health facility	3	0.32	0	0.00		0	0.00	
Child's surgery (n = 960)	yes	22	2.29	0	0.00				

Table 1. Cont.

	n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
no	938	97.71	16	1.71	0.88–2.54			

[1]Transportation to the nearest health facility, [2] Time to the nearest health facility, [3] Mothers' completed education, [4] Family head's occupation.
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whereas the other potential risk factors were not associated according to the adjusted odds ratio. We did not obtain information regarding the type of delivery, and we did not find significant differences in HBsAg prevalence associated with delivery settings. No independent factor was positively associated with HBsAg positivity in mothers, according to the adjusted odds ratio (Table 4).

Immunization status

Written immunization records were available for 213 out of 965 children (22.1%). One hundred ninety eight children were vaccinated with three doses of hepatitis B vaccine, and 34 children were immunized on the day of birth or the following day. Five out of 213 children with immunization records were HBsAg positive (2.35%; 95% CI: 0.30–4.40%), while 12 of 752 without immunization records were HBsAg positive (1.60%; 95% CI: 0.70–2.49%). The differences between the two groups were not significant ($p = 0.46$).

Discussion

HBsAg prevalence among the general population

The estimated HBsAg prevalence in the general population was much lower in both children and adults than that of previous reports from neighboring countries and Lao PDR. For example, HBsAg prevalence in adults in Cambodia, Thailand, and Vietnam was reported to be 7.7% (95% CI: 6.2%–9.3%) [12], 6 to 10% [15,20], and 18.8% (95% CI: 15.7%–21.9%) [13], respectively. Data on HBsAg prevalence amongst children was relatively scarce, and reported to be 3.5% (95% CI: 2.4%–4.8%) in Cambodia [21], and 18.4% (95% CI: 13.4%–23.4%) in Vietnam [13]. In Lao PDR, studies in blood donors, hospitalized patients, and Lao migrant workers tested in Thailand showed HBsAg prevalence of 8.73% (95% CI: 8.69%–8.77%) [22], 17.99% (95% CI: 17.81%–18.17%) [23], and 6.86% (95% CI: 6.80%–6.92%) [24] based on the given numerators and denominators in the articles, respectively.

Since the study objective was to estimate the nationwide HBsAg prevalence among the general population of Lao PDR, and thus the study design is a cross sectional survey, it is difficult to explain the reasons for the unexpectedly low prevalence. There are several potential explanations for this observation. The survey methodology used was very different from that used for blood donors, patients, and migrant workers. We used probability sampling and thus the results are representative of the whole population, whereas studies of blood donors, hospitalized patients, and migrant workers used non-probability sampling and therefore the results are restricted to these populations. The primary objective of our survey was to estimate HBsAg prevalence among the general population, so probability sampling was the most appropriate choice. Demographic conditions among the sampled population are determined by survey methodology, and therefore the results showed discrepancy. The WHO strongly recommends probability sampling for hepatitis B prevalence survey [7,15,17]. Although Lao PDR has the lowest population density of the Indochina peninsula countries [25], the precise effects on hepatitis B prevalence of the reduced frequency of human to human contact due to the country's relatively low population density and less developed infrastructure remain unclear.

The number of HBsAg positives varied from 0 to 4 per cluster. Since the sampling design of the survey aimed to estimate the prevalence in the whole country, it is difficult to determine whether these differences reflect the local endemic status.

Potential risk factors

Our survey revealed that no potential risk factors were significantly associated with the children's infection status, with the exception of the mothers' hepatitis B infection status. HBsAg prevalence surveys in other countries revealed that history of surgery [26,27], level of education [26], and ethnicity [28] were independently associated with hepatitis B infection. The reason why we could not find any potential risk factors positively associated with hepatitis B infection among children is not clear. However, it should be noted that the primary objective of the present study was to assess HBsAg prevalence, and not its risk factors. Additionally, some reports found that HIV positive individuals are positively associated with hepatitis B virus infection

Table 2. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old).

	Children's HBsAg (+)	%	95% CI	Standard error	Design effect	Mothers' HBsAg (+)	%	95% CI	Standard error	Design effect
High coverage districts (n = 486)	6	1.14	0.23–2.04	0.44	0.82	18	3.79	1.79–5.79	0.97	1.24
Low coverage districts (n = 479)	11	2.39	0.75–4.03	0.79	1.27	9	1.88	0.49–3.37	0.69	1.22
Total (n = 965)	17	1.72	0.81–2.63	0.44	1.10	27	2.93	1.65–4.20	0.61	1.28

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Table 3. Unadjusted and adjusted odds ratio for being HBsAg positive among children from five to nine years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	2.13	0.73–6.21	0.16	3.47	0.77–15.64	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	0.70	0.28–1.78	0.44	0.87	0.31–2.47	0.79
Ethnicity	Low land Lao	1(reference)					
	others	1.90	0.67–5.40	0.22	1.41	0.26–7.72	0.68
Education	none	1(reference)					
	finished primary school or upper	1.50	0.67–3.36	0.30	1.03	0.27–3.89	0.96
Occupation	white collar	1(reference)					
	blue collar	1.15	0.37–3.64	0.80	0.60	0.18–1.96	0.38
Sex	male	1(reference)					
	female	0.75	0.21–2.62	0.63	0.65	0.21–2.08	0.46
Birth place	health facility	1(reference)					
	non-health facility	0.98	0.39–2.49	0.97	0.79	0.28–2.21	0.64
Mothers' HBsAg	negative	1(reference)					
	positive	24.02	9.45–61.07	0.00	28.13	10.21–77.53	0.00

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[29,30]; however, we did not investigate HIV due to limited budget.

WHO's regional target

The interim target of the WHO is to reduce HBsAg prevalence to less than 2% in children aged at least 5 years old by 2012 [7,31]. The point prevalence is used for monitoring the control of hepatitis B. The Regional Office for the Western pacific recommended that the country conduct a national HBsAg prevalence survey to verify whether the country has reached the regional prevalence target [9]. Following these criteria, Lao PDR had already achieved its goal. However, it is unlikely that Lao PDR achieved the target through the immunization program alone because the country has the lowest immunization coverage of all countries in the region [7,9]. Considering the relatively lower HBsAg seroprevalence among the mothers compared to those reported in previous studies, it is likely that Lao PDR had a lower prevalence even before the introduction of the hepatitis B immunization program. Therefore, the final target of reducing HBsAg prevalence to less than 1% in children aged at least 5 years could be difficult to achieve if the country simply continues its current immunization policy.

A nationwide prevalence survey targeting the general population is ideally conducted before implementing the immunization

Table 4. Unadjusted and adjusted odds ratio for being HBsAg positive among mothers from 15 to 45 years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	0.50	0.20–1.28	0.14	0.47	0.19–1.16	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	1.03	0.43–2.51	0.94	0.94	0.39–2.25	0.88
Ethnicity	Low land Lao	1(reference)					
	others	0.80	0.30–2.17	0.65	0.68	0.25–1.85	0.44
Education	none	1(reference)					
	finished primary school or upper	1.68	0.70–4.01	0.23	2.04	0.89–4.68	0.09
Occupation	white collar	1(reference)					
	blue collar	1.71	0.53–5.55	0.35	1.93	0.68–5.50	0.21
History of surgery	no	1(reference)					
	yes	1.28	0.39–4.25	0.67	1.30	0.35–4.78	0.68

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strategy to evaluate hepatitis B epidemiology. However, we were able to understand the epidemiology to some degree, even after implementation of immunization policy, because adults usually represent the pre-vaccination era [15,17].

Strengths of the study

The present study is the first nationwide survey on the prevalence of hepatitis B in the general population both before and after the implementation of a hepatitis B immunization policy in Lao PDR and other Southeast Asian countries. We applied multistage stratified cluster sampling to better represent the general population. The design effect of prevalence was calculated between 0.8 and 1.3, which was acceptable as we set it around 2.0 before the survey.

The background characteristics of our sampled population were similar to those of another nationwide population-based study, the Lao PDR Reproductive Health Survey (LRHS) [32] conducted in 2005. For example, the locations of current residence (north, central, and south) were 33.3%, 41.7%, and 25.0% in our survey, and 38.6%, 38.9%, and 22.5% in the LRHS. The levels of mothers' completed education (none, primary school, secondary school or more) were 31.9%, 38.9%, and 29.2% in our survey, and 28.8%, 43.7%, and 27.5% in the LRHS. The LRHS applied the multistage stratified cluster sampling method and surveyed more than 13,000 women all over the country. A direct comparison of the populations sampled by the two different surveys is difficult to perform as the primary objectives were different. Despite this, our sampled population is considered to likely represent the general population in Lao PDR.

Limitations of the study

There are several limitations in our study that should be addressed. First, the population data is based on the census conducted in 2005. After 2005, the population distribution may have changed and some of the villages could have merged, thereby creating bias in the findings. Fortunately, we did not survey any villages that disappeared or merged.

Second, floating or marginal populations are likely to be missed from the residential lists, and these populations could be a source of HIV and hepatitis B virus infections [33]. In future seroprevalence surveys, these subpopulations should be accounted for by using specific approaches, such as oversampling.

Third, population immunity levels were difficult to measure or estimate. The possession of immunization certificates was low, because many participants had already finished their scheduled vaccinations before 12 months of age, and relevant documents were lost. In the present study, we did not have enough data from health centers due to time and budget limitations. Since we did not examine immunization markers, such as HBsAb, herd immunity levels are unknown.

Lastly, adult men were not included in the survey. Serological studies in the past indicated that men have higher HBsAg rates than women [8,21,28]. In Lao PDR, male blood donors presented with 9.7% HBsAg positive prevalence, while the prevalence in females was 6.2% [22]. When considering the disease burden of hepatitis B virus infections, it is better to include both sexes [26].

To the best of our knowledge, this is the first nationwide, population-based serological survey on chronic hepatitis B virus infections both before and after implementation of hepatitis B immunization in Southeast Asia, where disease burden is high. As such, our results provide valuable information on a hepatitis B immunization program and a useful baseline against which to compare future assessments in this region.

National immunization policy should be based on the disease epidemiology [3]. However, in Southeast Asia, understanding of the epidemiology of hepatitis B remains unsatisfactory. Even when a country implements a hepatitis B immunization program for children and the prevalence of disease reaches the target (i.e., less than 2% among children aged 5 years or older), we cannot conclude that the immunization program alone contributed to reduced disease prevalence without comparing it to the disease prevalence in the pre-vaccine generation, i.e., adults. Nationwide surveys assessing disease prevalence in the generations before and after the implementation of a vaccination program will provide valuable information for understanding hepatitis B epidemiology. Therefore, we recommend surveying hepatitis B seroprevalence in both generations.

Conclusions

We determined the nationwide HBsAg prevalence among children (1.7%; 95% CI: 0.8%–2.6%) and their mothers (2.9%; 95% CI: 1.6%–4.2%) in Lao PDR. This is the first report to estimate the nationwide prevalence of chronic hepatitis B in pre- and post-hepatitis B immunization generations in Southeast Asia, where hepatitis B infections are a substantial burden. The estimated prevalence was below that of previous studies, suggesting that our understanding of this disease's epidemiology is lacking and warrants further investigation. We recommend that the prevalence among the pre- and post-vaccine eras should be investigated when conducting hepatitis B seroprevalence surveys.

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Author Contributions

Conceived and designed the experiments: AX MH KI TW MS. Performed the experiments: KK TK PV CP KP DP BS VSO TS. Analyzed the data: KK TK MH. Contributed reagents/materials/analysis tools: KI TW MS. Wrote the paper: AX PV MH. Revised the manuscript: KK TK PV CP KP DP BS VSO KI TW MS. Arranged laboratory for diagnosis: PV KI TW MS.

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Chapter 6

The In Vivo Evaluation of the Therapeutic Potential of Human Adipose Tissue-Derived Mesenchymal Stem Cells for Acute Liver Disease

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Abstract

Mesenchymal stem cells (MSCs) have emerged as an attractive candidate for cell therapy applications. In the prior decade, many animal studies have demonstrated that MSCs are therapeutically beneficial for the treatment of liver disease. The carbon tetrachloride (CCl_4)-induced acute hepatitis model has been the most widely used model in these studies. Our group has utilized the CCl_4-induced mouse hepatitis model to study the therapeutic potential of human adipose tissue-derived MSCs (hADSCs). We have demonstrated that systemically administered hADSCs engrafted into the damaged liver and promoted tissue repair. This phenomenon likely reflected the paracrine effects of the administered hADSCs. In this chapter, we describe a method to evaluate the therapeutic efficacy of the systemic administration of hADSCs in the CCl_4-induced mouse model of acute hepatitis.

Key words Acute hepatitis, Carbon tetrachloride (CCl_4), Mesenchymal stem cell (MSC), Adipose-tissue derived MSC (ADSC), Intravenous transplantation

1 Introduction

Mesenchymal stem cell (MSC) transplantation has attracted a great deal of attention as a novel therapeutic option for liver diseases. At present, the only treatment for serious liver diseases is liver transplantation, and the application of this treatment has been limited by the shortage of donors. To compensate for this donor shortage, researchers have intensively studied hepatocyte transplantation as a potential treatment approach. However, the hepatocyte transplantation procedure is hampered by the low liver engraftment rate of transplanted hepatocytes and the low availability of transplantable hepatocytes [1]. Moreover, recent studies have not only examined approaches in which MSCs are used to repopulate a damaged liver, but also demonstrated that MSCs act via paracrine

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effects that significantly contribute to tissue repair in injured livers [2]. MSCs can be isolated from various adult connective tissues, including bone marrow and adipose tissues, the placenta, amniotic fluid, and umbilical cord blood [3, 4]. MSCs initially attracted research interest due to their ability to differentiate into cells of the mesodermal lineage. However, in recent years, greater attention has been devoted to exploring their capacity to secrete cytokines and growth factors [2, 5–7]. To date, numerous animal studies have demonstrated that MSCs are therapeutically beneficial for the treatment of liver diseases.

Several animal models for acute liver disease have been proposed, and these models have provided a great deal of insight with respect to evaluating the therapeutic efficacy of MSCs for these diseases. The most widely used model of acute liver disease is the carbon tetrachloride (CCl_4) treatment model [8–13]. In this model, hepatitis is induced by reactive metabolic trichloromethyl radicals ($\cdot CCl_3$) and peroxytrichloromethyl radicals ($\cdot OOCCl_3$), which are mainly metabolized from CCl_4 by cytochrome P450 2E1 (CYP2E1) [14]. Because CYP2E1 is preferentially localized in the pericentral zone of the liver acinus, the main sites of liver injury in the CCl_4-induced model are these pericentral regions. Similarly, acetaminophen (AAP) can also be used to generate an acute hepatitis model in rodents [15]. An overdose of AAP results in the generation of *N*-acetyl-*p*-benzoquinoneimine by CYP2E1 [16] and thereby produces hepatocyte necrosis. In contrast, concanavalin A (ConA) causes acute hepatitis through an excessive auto-immune reaction induced by the overproduction of various cytokines, such as tumor necrosis factor-α and interferon-γ [17]. It has been reported that the immunosuppressive effects of MSCs can improve ConA-induced acute hepatitis [18, 19]. The co-administration of lipopolysaccharide, a component of gram-negative cell walls, and D-galactosamine, another hepatotoxin, has also been used for the induction of acute hepatitis in mice [20].

Using the CCl_4-induced hepatitis model, we have demonstrated that human adipose tissue-derived MSCs (hADSCs) significantly contribute to tissue repair in acute hepatitis. Our research group has previously reported that hADSC-derived hepatocyte-like cells (hADSC-Heps) could be generated from hADSCs [9, 11] stimulated with growth factors that induce the differentiation of embryonic stem (ES) cells into hepatocyte-like cells [21]. Importantly, we confirmed that transplanted hADSC-Heps ameliorated liver injury in the CCl_4-induced mouse hepatitis model [9, 11]. Interestingly, however, we observed that in this model, undifferentiated hADSCs produced greater therapeutic effects than hADSC-Heps [10]. This finding has provided support for the notion that the therapeutic effects of hADSCs are mainly produced by the paracrine factors secreted by these cells rather than MSC

functions related to the repopulation of the liver mass. In this chapter, we describe a method to evaluate the therapeutic efficacy of the systemic administration of hADSCs in the CCl_4-induced acute hepatitis mouse model [10].

2 Materials

2.1 Animals

Six-week-old female BALB/c nude mice (CLEA Japan Inc., Tokyo, Japan) were used in this study (*see* **Note 1**).

2.2 Isolation and Culturing of hADSCs

1. 0.15 % type I collagenase in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS(−)) (*see* **Note 2**).
2. Sterilized surgical scissors.
3. Water bath equipped with a heating circulator.
4. Dulbecco's modified Eagle's medium (DMEM; high glucose, Invitrogen).
5. Fetal bovine serum (FBS).
6. 160 mM NH_4Cl.
7. 40 μm cell strainer (BD).
8. Hemocytometer.
9. MesenPRO RS™ Medium (Invitrogen).
10. Antibiotic-Antimycotic (Invitrogen).
11. GlutaMAX (Invitrogen).
12. CellBIND™ Surface 100 mm dish (Corning).

2.3 Routine Culturing of hADSCs

1. MesenPRO RS™ Medium (Invitrogen).
2. Antibiotic-Antimycotic (Invitrogen).
3. GlutaMAX (Invitrogen).
4. CellBIND™ Surface 100 mm dish (Corning).
5. Accutase.
6. PBS(−).

2.4 Systemic Administration of hADSCs in the CCl_4-Induced Mouse Model of Acute Liver Disease

1. Carbon tetrachloride (CCl_4).
2. Olive oil.
3. 26-G needle.
4. 1 mL syringe.
5. 27-G needle.
6. Mouse holder for intravenous injections.
7. 40 μm cell strainer.

2.5 Sampling of Serum and Liver Tissue

1. Isoflurane.
2. 24-G needle.
3. 1.5 mL tube.
4. PBS(−) containing 10 % formalin.

2.6 Histological Analyses of Mouse Liver Sections After Cell Transplantation

1. Hematoxylin.
2. Eosin.
3. Anti-human leukocyte antigen (HLA) class I antibody (clone W6/32; Sigma, 1:250).
4. Alexa Fluor 594 (Invitrogen).

3 Methods

3.1 Isolation and Culturing of hADSCs

This portion of the methods section is based on a protocol that was previously published by our laboratory [22].

1. Use surgical scissors to mince adipose tissue into pieces that are less than 3 mm in size. Collect these tissue pieces into a tube, add an equal volume of PBS(−), and mix vigorously at room temperature.
2. Let the mixture stand at room temperature until it separates into two phases.
3. Collect the upper phase, which contains stem cells, adipocytes, blood, and PBS(−), into a new tube, and wash this phase three times with fresh PBS(−). Discard the lower phase.
4. Add an equal volume of PBS(−) containing 0.15 % type I collagenase (thus achieving a final collagenase concentration of 0.075 %), and shake the resulting mixture for 30 min in a 37 °C water bath.
5. Add an equal volume of DMEM containing 10 % FBS, shake the resulting mixture well, and allow this mixture to incubate for 10 min. The mixture will separate into two phases during this incubation.
6. Discard the upper phase. Centrifuge the lower phase at 280 × *g* for 5 min at room temperature.
7. Resuspend the cellular pellet in 5 mL of 160 mM NH_4Cl over the course of 3 min. Filter the resulting mixture through a 40 μm cell strainer into a new tube containing 5 mL DMEM with 10 % FBS.
8. Centrifuge at 280 × *g* for 5 min at room temperature.
9. Dissolve the cell pellet in MesenPRO RS™ complete medium (*see* **Note 3**), and seed the cells onto CellBIND™ Surface 100 mm dishes at 1.0–5.0 × 10^4 cells/cm^2 (*see* **Note 4**).