Protein preparation. The N-terminal histidine or 3xFLAG-tagged NS3 protease, and the extracellular domain of human TβRI and TβRII were expressed in *Escherichia coli* by isopropyl-β-thiogalactopyranoside induction. The protein was purified by affinity chromatography in a HisTrap HP column (GE Healthcare, Waukesha, WI). Detailed procedures are in the Supplementary information.

Enzyme-linked immunosorbent assay (ELISA). TGF-β2 ELISA was performed using a TGF-β2 Emax® Immune Assay System ELISA kit (Promega, Madison, WI) according to the manufacturer's instructions.

Luciferase assay. The mink lung epithelial cell line CCL64, which stably expressed $(CAGA)_9$-MLP-luciferase and contained nine copies of a Smad-binding CAGA box element upstream of a minimal adenovirus major late promoter (2×10^4 cells/well)[23], was seeded into 96-well plates. The next day, the medium was replaced with fresh medium containing 0.1% bovine serum albumin, and the cells were cultured for an additional 24 hours. The cells were extracted with lysis buffer, and luciferase activity was measured by a Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Real-time RT-PCR. The isolation of total RNA and real-time RT-PCR were performed as described previously[24]. Briefly, total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. RNA (0.5 μg) was reverse transcribed to cDNA using the PrimeScript® RT Master Mix (Takara Bio Inc., Shiga, Japan). The mRNA expression levels were determined using real-time PCR. Real-time PCR was performed with the Thermal Cycler Dice® Real Time System, using the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) and normalized to *GAPDH* mRNA expression. The primer sequences used were as follows: human TGF-β1 forward: 5′-ACT ATT GCT TCA GCT CCA CGG A-3′, reverse: 5′-GGT CCT TGC GGA AGT CAA TGT A-3′; human collagen α1 (I) forward: 5′-ACG AAG ACA TCC CAC CAA TC-3′, reverse: 5′-AGA TCA CGT CAT CGC ACA AC-3′; human *GAPDH* forward: 5′-GGA GTC AAC GGA TTT GGT-3′, reverse: 5′-AAG ATG GTG ATG GGA TTT CCA-3′; and human TβRI forward: 5′-CTT AAT TCC TCG AGA TAG GC-3′, reverse: 5′-GTG AGA TGC AGA CGA AGC-3′.

Immunofluorescence staining. The cells were grown on eight-well chamber slides or glass bottom dishes and were incubated with HCV virion for 24 hours at 37°C. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 20 min at room temperature. After blocking with 3% BSA/10% normal goat serum/PBS for 30 min, the cells were incubated with primary antibodies for 2 hours, followed by incubation with secondary antibodies for 30 min at RT. For detecting NS3 and TβRI on the cell surface, the cells were fixed without permeabilization after incubation with the secondary antibodies. After being washed with PBS, the cells were mounted with Vectashield DAPI mounting medium (Vector Laboratories, Inc., Burlingame, CA) and observed under a Zeiss LSM 700 laser scanning confocal microscope. For quantitative fluorescence analyses, the intensity of phosphorylated Smad3 and the colocalization of NS3 and TβRI (Pearson's colocalization coefficient values) in each panel were calculated with ZEN software.

Proximity ligation assay (PLA). HCV-infected Huh-7.5.1 cells were fixed with 4% paraformaldehyde for 10 min at room temperature and subjected to in situ PLA using a Duolink in situ red starter kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. Briefly, cells were blocked and incubated with primary antibodies against NS3 and TβRI, followed by incubation with the PLA probes, which were secondary antibodies (anti-mouse and anti-rabbit) conjugated to oligonucleotides. DNA ligase was added to enable the formation of circular DNA strands when the PLA probes were in close proximity. This step was followed by incubation with oligonucleotides and polymerase for rolling circle amplification[25]. Texas red-labeled oligonucleotides, which hybridize to the amplified products, were used for visualization. The cells were observed under a Zeiss LSM 700 laser scanning confocal microscope.

Immunoprecipitation and immunoblotting. Anti-FLAG M2 affinity beads were pretreated with 5% bovine serum albumin in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl overnight. Isotype control IgG was bound to Protein G PLUS-Agarose (Santa Cruz) pretreated with 5% bovine serum albumin in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl. Cell lysates with 3xFLAG or 3x-FLAG-NS3 (2 mg protein) were incubated with 50 μl of the beads (10% slurry) at 4°C for 3 hours. The beads were then washed three times with the lysis buffer and incubated with lysates containing 6xHis-TβRI or 6xHis-TβRII (0.5 mg protein) at 4°C overnight. The bound proteins were eluted with the SDS-PAGE sample buffer after washing four times with the lysis buffer and then were subjected to SDS-PAGE (15% acrylamide) followed by transfer onto a PVDF membrane (Pall). The proteins were then visualized using anti-His tag HRP DirectT (MBL, 1/5000) or anti-FLAG BioM2 antibody (Sigma, 10 μg/ml) and horseradish peroxidase-conjugated anti-biotin antibody (Cell Signaling) using the ECL Western blotting detection reagent (GE Healthcare).

In silico docking simulation. The protein-protein docking simulation was implemented based on the geometric complementarity[26] between NS3 protease (PDB ID, 1NS3) and TβRI (PDB ID, 2PJY). Specifically, coordinates of the proteins were projected onto three-dimensional grids separated from each other at regular intervals. A surface score and an intramolecular score were assigned to each grid. This operation was conducted for both the receptor and the ligand. Next, convolution between the obtained grids was performed, the surfaces were explored exhaustively, and the complementarities of the binding states were calculated based on the scores. Amino acid residues appearing frequently in binding states with high complementarity scores can be estimated to be residues that are highly likely to appear in the interaction with an actual receptor. Accordingly, amino acid residues with an interatomic distance of 3.8 Å or less in the putative binding states were defined as contact residues and regarded as the putative contact residues of NS3 and TβRI.

Animal experiment. Chimeric mice with humanized livers were generated as previously described using urokinase-type plasminogen activator (uPA)-transgenic/SCID mice[27]. All mice were transplanted with frozen human hepatocytes obtained from a single donor. All animal experiments were approved by RIKEN Institutional Animal Use and Care Administrative Advisory Committees and were performed in accordance with RIKEN guidelines and regulations. Infection, extraction of serum samples, and euthanasia were performed under isoflurane anesthesia. Male chimeric mice (12- to 14-week old) were intravenously injected with 100 μl HCV J6/JFH-1 strain (1×10^8 copies/ml). Four weeks after HCV inoculation, anti-NS3 antibodies against predicted binding sites with the TβRI receptor were administered at doses of 0.5 mg/kg of BW or 5 mg/kg of BW twice a week for twelve weeks. Normal mouse IgG was administered at a dose of 5 mg/kg of BW as a control. When the animals were euthanized, the livers were either fixed with 4% paraformaldehyde for histological analysis or frozen immediately in liquid nitrogen for mRNA isolation.

Staining of liver tissue sections. The liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and tissue sections (6 μm in thickness) were prepared with a Leica sliding microtome (Leica Microsystems, Nussloch, Germany). The liver tissue sections were deparaffinized, rehydrated, and incubated for 5 min with a drop of Proteinase K (Dako Envision) in 2 mL of 0.05 M Tris-HCl buffer (pH 7.5) at room temperature. The liver tissue sections were stained with Mayer's hematoxylin solution (Muto Chemicals) and 1% eosin Y solution (Muto Chemicals). Sirius Red, which results in a red staining of all fibrillar collagen, was used to evaluate fibrosis. Briefly, the liver sections were stained with 0.05% Fast Green FCF (ChemBlink, Inc. CAS: 2353-45-9) and 0.05% Direct Red 80 (Polysciences, Inc. CAS: 2610-10-18) in saturated picric acid (Muto Chemicals) for 90 min at room temperature. The ratios of Sirius Red positive/total area (%) from 6 randomly selected fields were measured for each group using WinROOF software (Mitani Corp., Tokyo, Japan).

Statistics. Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's post-hoc test. A two-tailed Student's *t*-test was used to evaluate differences between the two groups. The Kruskal-Wallis test followed by Dunn's post-hoc test was used for multiple comparisons of Sirius Red positive areas.

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Author contributions

Sakata K., Hara M. and Yaguchi S. performed experiments. Sakata K., Matsuura T., Miyazawa K., Imoto M. and Kojima S. wrote the manuscript. Terada T., Matsumoto T., Shirouzu M., Yokoyama S., Yamaguchi T. and Suzuki T. contributed to the production and the purification of recombinant NS3 and its antibodies. Watanabe N., Aizaki H. and Wakita T. contributed to the production and the purification of HCV and discussion from the point of view of virology. Takaya D. performed docking simulation to predict binding sites. Sakata K. and Kojima S. planned the research. Kojima S. supervised the entire project.

Additional information

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Specific inhibition of hepatitis C virus entry into host hepatocytes by fungi-derived sulochrin and its derivatives

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ABSTRACT

Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. Although various classes of anti-HCV agents have been under clinical development, most of these agents target RNA replication in the HCV life cycle. To achieve a more effective multidrug treatment, the development of new, less expensive anti-HCV agents that target a different step in the HCV life cycle is needed. We prepared an in-house natural product library consisting of compounds derived from fungal strains isolated from seaweeds, mosses, and other plants. A cell-based functional screening of the library identified sulochrin as a compound that decreased HCV infectivity in a multi-round HCV infection assay. Sulochrin inhibited HCV infection in a dose-dependent manner without any apparent cytotoxicity up to 50 μM. HCV pseudoparticle and trans-complemented particle assays suggested that this compound inhibited the entry step in the HCV life cycle. Sulochrin showed anti-HCV activities to multiple HCV genotypes 1a, 1b, and 2a. Co-treatment of sulochrin with interferon or a protease inhibitor telaprevir synergistically augmented their anti-HCV effects. Derivative analysis revealed anti-HCV compounds with higher potencies (IC_{50} < 5 μM). This is the first report showing an antiviral activity of methoxybenzoate derivatives. Thus, sulochrin derivatives are anti-HCV lead compounds with a new mode of action.

1. Introduction

Hepatitis C virus (HCV) infection is a major causative agent of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma [1]. The standard anti-HCV therapy has been a co-treatment with pegylated-interferon (IFN)α and ribavirin, but this therapy is limited by less efficacy to certain HCV genotypes, poor tolerability, serious side effects, and high cost [2,3]. In addition to the newly approved protease inhibitors, telaprevir and boceprevir, a variety of anti-HCV candidates are under clinical development. Although these drugs improve the virological response rate, the emergence of drug-resistant virus is expected to be a significant problem. Moreover, these compounds are expensive due to their complex structure and the many steps required for their total synthesis. To overcome the drug-resistant virus and achieve a long-term antiviral effect, multidrug treatment is essential. Thus, the development of drugs targeting a different step in the HCV life cycle and presumably requiring low cost is urgently needed.

HCV propagates in hepatocytes through its viral life cycle including: attachment and entry (defined as the early step in this study); translation, polyprotein processing, and RNA replication (the middle step); and assembly, trafficking, budding, and release (the late step) (Supplementary Fig. S1). The middle step has been extensively analysed, especially after the establishment of the HCV replicon system [4]. The early step can be analysed with HCV pseudoparticle (HCVpp) [5,6], which is a murine leukemia virus- or human immunodeficiency virus-based pseudovirus carrying HCV E1 and E2 as envelope proteins. The HCV-producing cell culture system (HCVcc) is used for analyzing the whole life cycle [7–9]. In addition, the HCV trans-complemented particle (HCVtcp) system carrying an HCV subgenomic replicon RNA packaged in HCV E1 and E2-containing particles can evaluate the life cycle from the early to the middle step [10]. The majority of anti-HCV agents currently under clinical development, such as inhibitors of protease, polymerase, NS5A, and cellular cyclophilin, inhibit polyprotein processing and/or RNA replication. A desirable approach

Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCVpp, HCV pseudoparticle; HCVcc, HCV derived from cell culture; HCVtcp, HCV trans-complemented particle; MOI, multiplicity of infection; HBs, HBV envelope protein; CsA, cyclosporin A; VSV, vesicular stomatitis virus.

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to achieving efficient multidrug therapy is to identify new antiviral drugs targeting different steps in the viral life cycle. A combination of drugs with different targets can greatly decrease the emergence of drug-resistant virus.

Natural products generally contain more characteristics of high chemical diversity than combinatorial chemical collections, and therefore have a wider range of physiological activities [11,12]. They offer major opportunities for finding novel lead structures that are active in a biological assay. Moreover, biologically active natural products are generally small molecules with drug-like properties, and thus development costs of producing orally active agents tend to be lower than that derived from combinatorial chemistry [13]. In addition, there is a wide variety of natural compounds reported to possess antiviral activity [14,15]. In the present study, we have taken advantage of the potential of natural products by screening a natural product library derived from fungal extracts with a cell-based assay that supports the whole life cycle of HCV.

2. Materials and methods

2.1. Cell culture

Huh-7.5.1 [8] and HepaRG cells [16] were cultured as described previously.

2.2. Natural product library and reagents

Natural products were extracted essentially as previously described [17]. Culture broths of fungal strains isolated from seaweeds, mosses, and other plants were extracted with CH_2Cl_2. The crude extracts were separated by silica gel column chromatography to purify compounds. The chemical structure of each compound was determined by NMR and mass spectrometry analyses. Thus, we prepared an in-house natural product library consisting of approximately 300 isolated compounds.

Cyclosporin A was purchased from Sigma. Bafilomycin A1 and chlorpromazine were purchased from Wako. Heparin was obtained from Mochida Pharmaceutical. IFNα was purchased from Schering-Plough.

2.3. Compound screening

Huh-7.5.1 cells were treated with HCV J6/JFH1 at a multiplicity of infection (MOI) of 0.15 for 4 h. The cells were washed and then cultured with growth medium treated with 10 μM of each compound for 72 h. The infectivity of HCV in the medium was quantified. Cell viability at 72 h post-treatment was simultaneously measured. Compounds that decreased the cell viability to less than 50% of that without treatment were eliminated for further evaluations. Normalised infectivity was calculated as HCV infectivity divided by cell viability. Compounds reducing the normalised infectivity to less than 40% were selected as initial hits. The initial hits were further evaluated for data reproduction and dose-dependency.

2.4. HCVcc assay

HCVcc was recovered from the medium of Huh-7.5.1 cells transfected with HCV J6/JFH-1 RNA as described [7]. HCVcc was infected into Huh-7.5.1 cells at 0.15 MOI for 4 h. After washing out the inoculated virus, the cells were cultured with normal growth medium in the presence or absence of compounds for 72 h. The infectivity of HCV and the amount of HCV core protein in the medium were quantified by infectious focus formation assay and chemiluminescent enzyme immunoassay (Lumipulse II HCV core assay, ortho clinical diagnostics), respectively [7,18].

2.5. Immunoblot analysis

Immunoblot analysis was performed as described previously [19]. The anti-HCV core antibody (2H9) was used as a primary antibody with 1:1000 dilution [7].

2.6. MTT assay

The viability of cells was quantified by using a Cell Proliferation Kit II XTT (Roche Diagnostics) as described previously [20].

2.7. HCV replicon assay

Huh-7.5.1 cells were transfected with an HCV subgenome replicon RNA (SGR-JFH1/Luc) for 4 h and then incubated with or without compounds for 48 h [21]. The cells were lysed with 1xPLB (Promega), and the luciferase activity was determined with a luciferase assay system (Promega) according to the manufacturer's protocol [22].

2.8. HCVpp assay

HCVpp was recovered from the medium of 293T cells transfected with expression plasmids for HCV JFH-1 E1E2, MLV Gag-Pol, and luciferase, which were kindly provided from Dr. Francois-Loic Cosset at Universite de Lyon [5]. Vesicular stomatitis virus pseudoparticles (VSVpp) was similarly recovered with transfection by replacing HCV E1E2 with VSV G.

Huh-7.5.1 cells were preincubated with compounds for 3 h and were then infected with HCVpp in the presence of compounds for 4 h. After washing out virus and compounds, cells were incubated for an additional 72 h before recovering the cell lysates and quantifying the luciferase activity.

2.9. HCVtcp assay

The HCVtcp assay was essentially performed as described [10]. Briefly, Huh-7 cells were transfected with expression plasmids for the HCV subgenomic replicon carrying the luciferase gene and for HCV core-NS2 based on genotype 1a (RMT) (kindly provided by Dr. Michinori Kohara at Tokyo Metropolitan Institute of Medical Science), 1b (Con1), and 2a (JFH-1) [4,10,23] to recover HCVtcp. HCVtcp can reproduce RNA replication as well as HCV-mediated entry into the cells [10].

2.10. Synergy analysis

To determine whether the effect of the drug combination was synergistic, additive, or antagonistic, MacSynergy (kindly provided by Mark Prichard), a mathematical model based on the Bliss independence theory, was used to analyse the experimental data shown in Fig. 3A. In this model, a theoretical additive effect with any given concentrations can be calculated by $Z = X + Y(1-X)$, where X and Y represent the inhibition produced by each drug alone, and Z represents the effect produced by the combination of two compounds if they were additive. The theoretical additive effects were compared to the actual experimental effects at various concentrations of the two compounds and were plotted as a three-dimensional differential surface that would appear as a horizontal plane at 0 if the combination were additive. Any peak above this plane (positive values) indicates synergy, whereas any depression below the plane (negative values) indicates antagonism. The 95% confidence interval of the experimental dose–response was considered to reveal only effects that were statistically significant.

3. Results

3.1. Screening of natural products possessing anti-HCV activity

We extracted culture broths of fungal strains isolated from seaweeds, mosses, and other plants and purified compounds as described in the Section 2 [17]. The chemical structure of each compound was determined by NMR and mass spectrometry analyses. Thus, we prepared an in-house natural product library consisting of approximately 300 isolated compounds. As shown in the Section 2, compounds reducing the normalised HCV infectivity to less than 40% as compared with DMSO were selected as primary hits. The primary hits were then validated by examining the reproducibility, dose-dependency, and cell viability in the HCVcc system. Sulochrin [methyl 2-(2,6-dihydroxy-4-methylbenzoyl)-5-hydroxy-3-methoxybenzoate] (Fig. 1A) was one of the compounds showing the highest anti-HCV activity, and the following analyses focus mainly on this compound.

3.2. Sulochrin decreased HCV infectivity in HCV cell culture assay

To characterise the anti-HCV activity of the compounds, Huh-7.5.1 cells were infected with HCV J6/JFH1 at an MOI of 0.15 and then cultured for 72 h in the presence or absence of compounds.

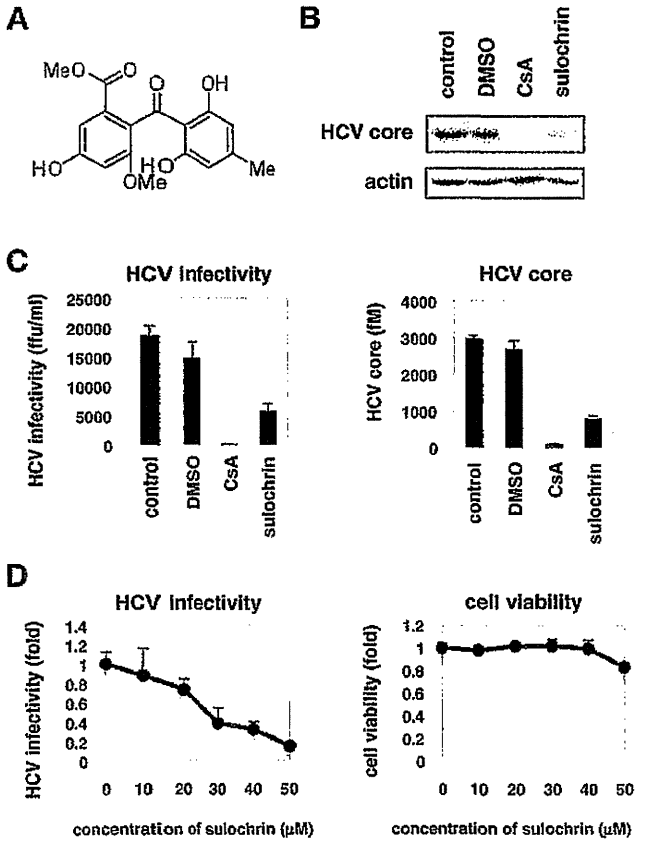


Fig. 1. Sulochrin decreased HCV production in a multi-round HCV infection assay. (A) Chemical structure of sulochrin. (B) Huh-7.5.1 cells were infected with HCV J6/JFH-1 at an MOI of 0.15 for 4 h and then incubated with or without 0.3% DMSO, 2 μM cyclosporin A (CsA), or 30 μM sulochrin for 72 h. The resultant medium was inoculated into naïve Huh-7.5.1 cells to detect intracellular HCV core and actin protein at 48 h postinoculation by immunoblot. (C) HCV infectivity (left) and HCV core protein (right) in the medium as prepared in (B) were quantified as shown in the Section 2. (D) HCV infectivity (left) determined as shown in (C) with varying concentrations (0–50 μM) of sulochrin. Cell viability was examined by MTT assay (right).

In this system, infectious HCV is secreted into the medium and then re-infects into uninfected cells to support the spread of HCV during a 72 h period (Section 2). Cell cultures were treated with sulochrin or cyclosporin A (CsA) as a positive control in this mul-

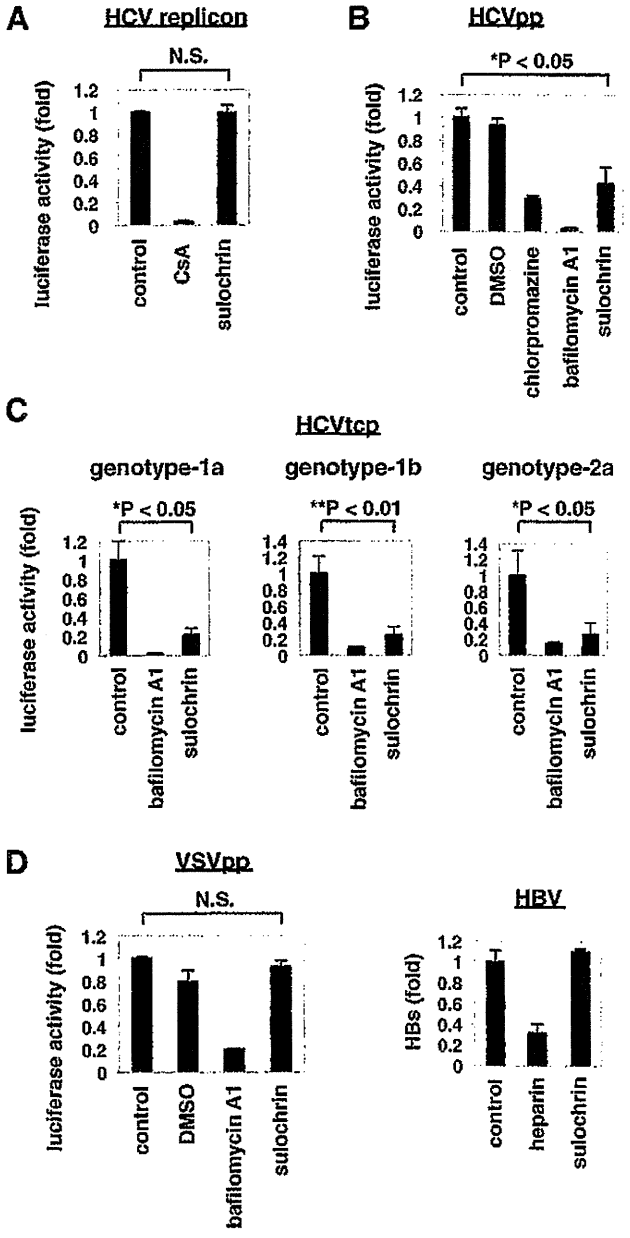


Fig. 2. Sulochrin blocked HCV entry. (A) Replicon assay. Huh-7.5.1 cells were transfected with an HCV subgenomic replicon RNA for 4 h followed by treatment with or without the indicated compounds for 48 h. Luciferase activity driven by the replication of the subgenomic replicon was quantified. (B and C) HCV pseudoparticle (HCVpp) and trans-complemented particle (HCVtcp) assay. Huh-7.5.1 cells were pretreated with the indicated compounds for 3 h and then infected with HCVpp (B) or HCVtcp (C) for 4 h. After washing out virus and compounds, cells were further incubated for 72 h and harvested for measuring luciferase activity driven by the infection of HCVpp or HCVtcp. HCVtcp assay was performed with HCV E1 and E2 derived from genotypes 1a (RMT), 1b (Con1), and 2a (JFH1). (D) Left, the pseudoparticle assay was performed as shown in (B) with VSV G instead of HCV E1 and E2. Right, HBV infection assay. HepaRG cells were pretreated with the indicated compounds for 3 h and then infected with HBV for 16 h. After washing out virus and compounds, cells were incubated for an additional 12 days. HBV infection was evaluated by measuring HBs secretion from the infected cells. Heparin was used as a positive control that inhibits HBV entry.

ti-round infection system. To examine the level of infectious HCV particles produced from the cells, the resultant medium was inoculated into naive Huh-7.5.1 cells to detect HCV core protein in the cells. As shown in Fig. 1B, intracellular production of HCV core but not that of actin was reduced in the cells inoculated with sulochrin- and CsA-treated medium (Fig. 1B). Quantitative analysis showed that sulochrin decreased HCV infectivity and HCV core protein in the medium to 1/3–1/4 of the untreated levels (Fig. 1C). Reduction of HCV infectivity by sulochrin was dose-dependent without serious cytotoxicity up to 50 μM (Fig. 1D).

3.3. Sulochrin blocked HCV entry

We investigated the step in the HCV life cycle that was inhibited by sulochrin. The middle step of the life cycle including translation and RNA replication was evaluated with the transient replication assay by using the HCV subgenomic replicon. Sulochrin had little effect on the replicon activity at doses up to 50 μM (Fig. 2A). In the HCVpp system, which reproduced the early step of HCV infection including entry, sulochrin significantly inhibited HCVpp infection (Fig. 2B). Sulochrin also inhibited the infection of HCVtcp, which reproduced both the viral entry and RNA replication, further supporting that this compound targeted the entry step (Fig. 2C). In contrast, VSV G-mediated viral entry efficiency was not altered by sulochrin treatment (Fig. 2D). Additionally, HBV entry was not inhibited by the presence of sulochrin (Fig. 2D). These data suggest that the inhibitory activity of sulochrin on viral entry is specific to HCV. The anti-HCV entry activity of sulochrin was conserved among different HCV genotypes, 1a (RMT), 1b (Con1), and 2a (JFH-1) [4,10,23] (Fig. 2C).

3.4. Synergistic effect of cotreatment of sulochrin with IFNα or telaprevir

We examined the anti-HCV activity of sulochrin co-administered with clinically available anti-HCV agents, IFNα and a prote-

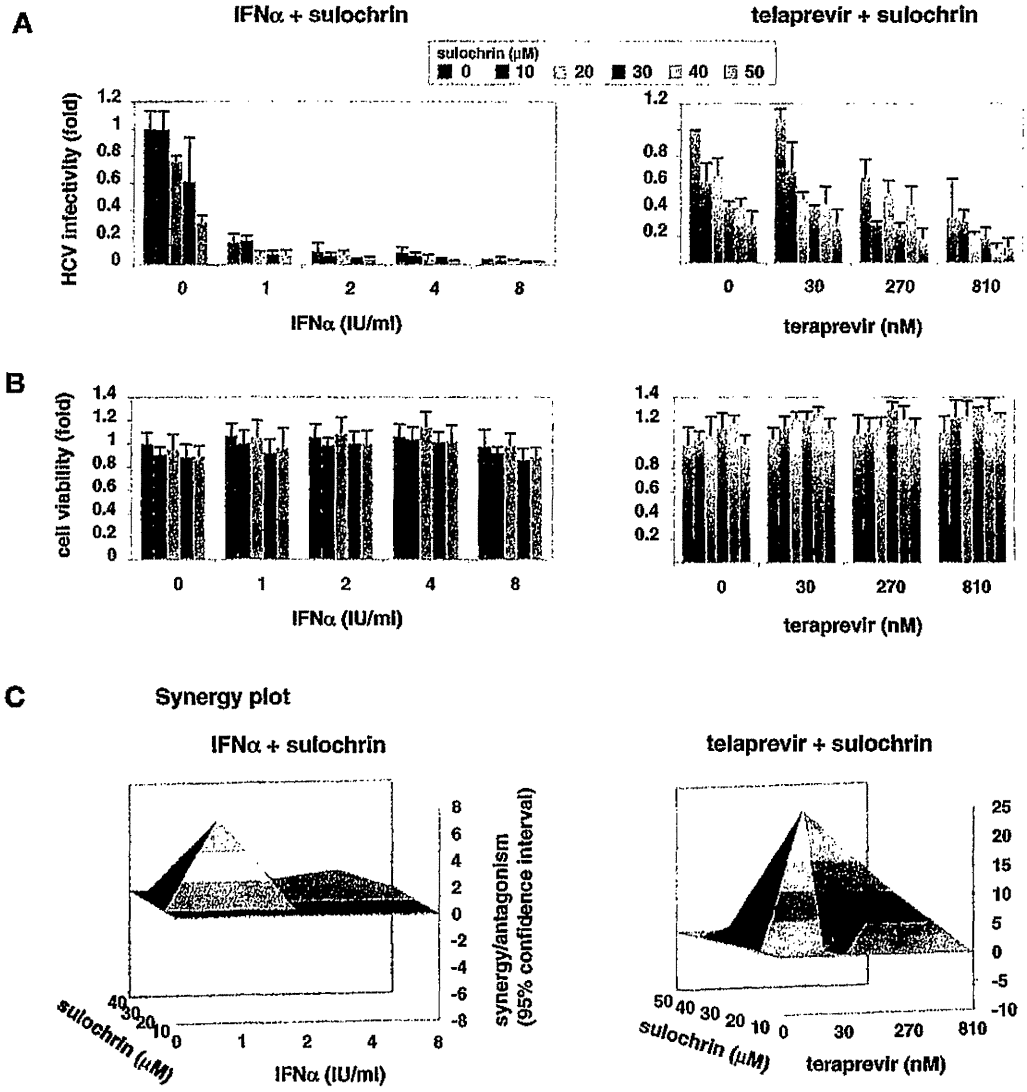


Fig. 3. Cotreatment of sulochrin with IFNα or telaprevir. (A, B) Huh-7.5.1 cells infected with HCV were treated with the indicated concentrations of sulochrin with IFNα (left) or telaprevir (right) to determine HCV infectivity in the medium (A) as shown in Fig. 1C. Cell viability was also quantified (B). (C) Synergy analysis. The results of the combinations shown in (A) were analysed with a mathematical model, MacSynergy, as described in the Section 2. The three-dimensional surface plot represents the difference between actual experimental effects and theoretical additive effects of the combination treatment (95% confidence interval). The theoretical additive effects are shown as the zero plane (dark gray) across the z-axis. A positive value in the z-axis as a peak above the plane indicates synergy, and a negative value with a valley below the plane indicates antagonism. Sulochrin in combination with IFNα (left) or telaprevir (right) produced synergistic antiviral effects that were greater than the theoretical additive effects.

ase inhibitor telaprevir. As shown in Fig. 3, addition of sulochrin with IFNα or telaprevir led to a further decrease in HCV infectivity (Fig. 3A) without significantly enhancing cytotoxicity (Fig. 3B) at any given concentrations. Thus, the combination of sulochrin and IFNα or telaprevir always resulted in a greater reduction in HCV infectivity as compared with that achieved by either agent alone. Synergy/antagonism analysis with the Bliss independence model showed that the experimental anti-HCV activity in combination with sulochrin and IFNα or telaprevir showed a peak above the zero plane in the *z*-axis, which shows the calculated theoretical additive effect (Fig. 3C). Any peak above the zero plane indicates more than an additive effect, namely, synergy (Section 2). The data clearly indicate that sulochrin had a synergistic anti-HCV effect with both IFNα and telaprevir.

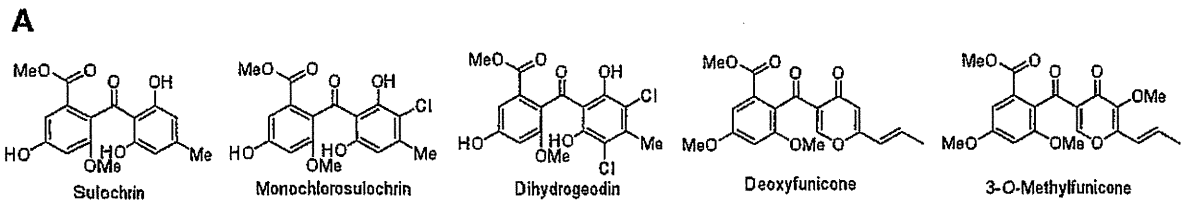
3.5. Derivative analysis of sulochrin

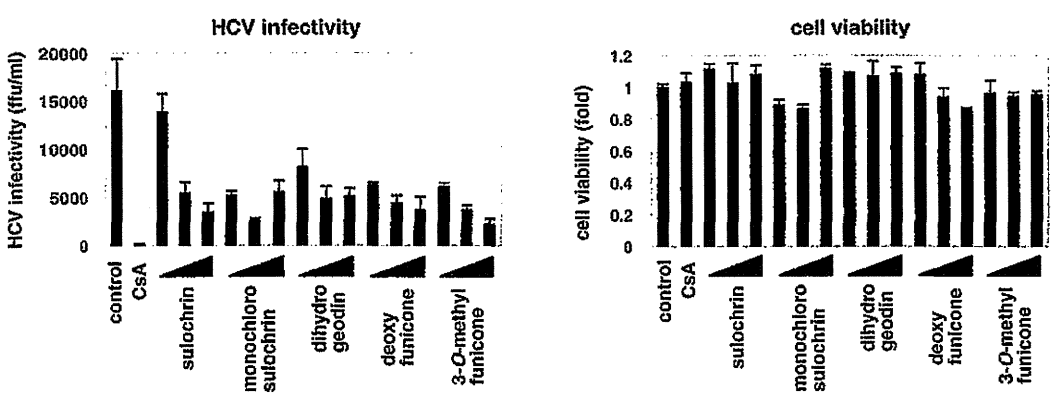
We examined the anti-HCV activity of a series of sulochrin derivatives (Fig. 4A) in the HCVcc system. Monochlorosulochrin and dihydrogeodin, mono- or dichloro-substituted derivatives of sulochrin, possessed even higher anti-HCV activity than sulochrin (Fig. 4B and C). Deoxyfunicone, of which one aromatic ring was replaced by a 4-pyrone ring, had approximately 5-fold greater HCV inhibitory activity as compared with sulochrin (Fig. 4B and C). An additional compound, 3-*O*-methylfunicone, also possessed anti-HCV activity (Fig. 4B and C). These data suggest that the 1,3-dihydroxy-5-methylbenzene moiety of sulochrin is important for anti-HCV activity. Furthermore, funicone derivatives as well as sulochrin derivatives are likely to be lead compounds for a new class of anti-HCV agents.

4. Discussion

In the present study, we prepared a natural product library consisting of approximately 300 isolated compounds derived from fungi extract [17]. Among these compounds, we focused on sulochrin, which reduced HCV infectivity in the HCVcc system. Sulochrin suppressed the viral entry efficiencies both in the HCVpp and the HCVtcp systems, suggesting that this compound blocked HCV envelope-mediated entry. HCV was reported to enter host cells through clathrin-dependent endocytosis after engagement to host receptors [24–27]. Sulochrin is not likely to be a general inhibitor of clathrin-dependent endocytosis, but rather is specific for HCV entry, as it did not affect the entry of other viruses such as VSVpp and HBV, which were reported to enter by clathrin-dependent manners [28,29].

Sulochrin inhibits eosinophil degranulation, activation, and chemotaxis [30,31]. It also inhibits VEGF-induced tube formation of human umbilical vein endothelial cells [32]. In addition, 3-*O*-methylfunicone, a sulochrin derivative possessing anti-HCV activity, has an anti-tumor activity [33]. It is unknown if these activities of the compounds are related to their anti-HCV activity. The establishment of drug-resistant virus and the identification of the target molecule are in progress to reveal the mechanism of action of sulochrin and its derivatives. However, the present study is the





C

IC_{50}s and CC_{50}s

compound	IC_{50} (μM)	CC_{50} (μM)
sulochrin	24.4 ± 2.0	> 50
monochlorosulochrin	4.3 ± 1.1	> 50
dihydrogeodin	10.3 ± 0.3	> 50
deoxyfunicone	4.6 ± 1.1	> 50
3-O-methylfunicone	6.2 ± 2.3	> 50

Fig. 4. Derivative analysis of sulochrin. (A) Chemical structures of sulochrin derivatives examined in this study, monochlorosulochrin, dihydrogeodin, deoxyfunicone, 3-*O*-methyfunicone, as well as sulochrin. (B) Anti-HCV effects of the sulochrin derivatives (10, 30, and 50 μM) were investigated as shown in Fig. 1C. (C) The IC_{50} and CC_{50} values of the sulochrin derivatives are shown.

first report to demonstrate the antiviral activity of these compounds. It is important to note that sulochrin inhibited the entry of HCV genotype 1a and b, which are the dominant genotypes in North America, Europe, and East Asia, indicating that this compound has potential clinical applications. Promising applications of entry inhibitors include the prevention of HCV recurrence in patients after liver transplantation. In patients with HCV-related end-stage liver diseases undergoing liver transplantation, re-infection of the graft is universal and characterised by accelerated progression of liver diseases. Entry inhibitors may be effective especially in these conditions under robust re-infection of HCV into hepatocytes. In the present study, we showed that co-treatment of sulochrin with IFNα and a protease inhibitor, teleprevir, synergistically augmented the anti-HCV effects of these approved drugs. These results suggest the possibility that co-treatment with sulochrin and probably its effective derivatives helps to inhibit the spread of HCV infection. We also identified the chemical structure and the derivatives of sulochrin as lead compounds for anti-HCV agents. Further derivatives analysis may identify more preferable anti-HCV agents.

In conclusion, our results demonstrate that sulochrin and its derivatives are potent and selective inhibitors of HCV infection in cell culture. Although further studies including an analysis of mode of action and pharmacological properties *in vivo* are required, this class of compounds should be pursued for its clinical potential in the treatment of HCV infection.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.100.

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Interleukin-1 and Tumor Necrosis Factor-α Trigger Restriction of Hepatitis B Virus Infection via a Cytidine Deaminase Activation-induced Cytidine Deaminase (AID)*

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Background: Cytokines and host factors triggering innate immunity against hepatitis B virus (HBV) are not well understood.
Results: IL-1 and TNFα induced cytidine deaminase AID, an anti-HBV host factor, and reduced HBV infection into hepatocytes.
Conclusion: IL-1/TNFα reduced host susceptibility to HBV infection through AID up-regulation.
Significance: Proinflammatory cytokines modulate HBV infection through a novel innate immune pathway involving AID.

Virus infection is restricted by intracellular immune responses in host cells, and this is typically modulated by stimulation of cytokines. The cytokines and host factors that determine the host cell restriction against hepatitis B virus (HBV) infection are not well understood. We screened 36 cytokines and chemokines to determine which were able to reduce the susceptibility of HepaRG cells to HBV infection. Here, we found that pretreatment with IL-1β and TNFα remarkably reduced the host cell susceptibility to HBV infection. This effect was mediated by activation of the NF-κB signaling pathway. A cytidine deaminase, activation-induced cytidine deaminase (AID), was up-regulated by both IL-1β and TNFα in a variety of hepatocyte cell lines and primary human hepatocytes. Another deaminase APOBEC3G was not induced by these proinflammatory cytokines. Knockdown of AID expression impaired the anti-HBV effect of IL-1β, and overexpression of AID antagonized HBV infection, suggesting that AID was one of the responsible factors for the anti-HBV activity of IL-1/TNFα. Although AID induced hypermutation of HBV DNA, this activity was dispensable for the anti-HBV activity. The antiviral effect of IL-1/TNFα was also observed on different HBV genotypes but not on hepatitis C virus. These results demonstrate that proinflammatory cytokines IL-1/TNFα trigger a novel antiviral mechanism involving AID to regulate host cell permissiveness to HBV infection.

The intracellular immune response can eliminate pathogens from a host, and host cells possess different mechanisms to counteract viral infection depending on the virus type. Human immunodeficiency virus (HIV) infection is restricted by cellular proteins designated as restriction factors, including APOBEC3G (A3G),[3] TRIM5α, tetherin/BST-2, and SAMHD1 (1, 2). All of these factors can be induced by stimulation with interferon (IFN). Hepatitis C virus (HCV) is eliminated by type I and III IFNs derived from dendritic cells or infected hepatocytes (3–6). In hepatocytes, this process involves a series of antiviral factors that are downstream genes of IFN, IFN-stimulated genes (ISGs). Influenza virus spread and virulence is inhibited by cytokines such as IFNs and TNFα. Responsive genes for these mechanisms include IFN-induced cellular Mx proteins that are dynamin-like GTPases (7, 8). However, these cytokine-induced antiviral immune responses are poorly understood in hepatitis B virus (HBV) infection.

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[3] The abbreviations used are: A3G, APOBEC3G; AID, activation-induced cytidine deaminase; HBV, hepatitis B virus; HCV, hepatitis C virus; ISG, IFN-stimulated gene; QNZ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; GEq, genome equivalent; PHH, primary human hepatocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ISRE, interferon sensitivity-responsive element; cccDNA, covalently closed circular DNA.

HBV infection is a worldwide health problem affecting more than 350 million people and is a major cause of the development of liver cirrhosis and hepatocellular carcinoma (9–11). During the course of infection, a number of cytokines and chemokines are up-regulated in HBV-infected patients, including IFNα/γ/λ, TNFα, IL-1, IL-6, IL-10, IL-12, IL-15, and IL-8 (12–15). Some of these cytokines are reported to suppress HBV replication (3, 16–21). In particular, type I, II, and III IFNs suppress the replication of HBV *in vitro* and *in vivo* (19, 20, 22–26). Although one of the downstream genes of IFN, *A3G*, has the potential to reduce HBV replication (27–34), it is still under discussion whether this protein is responsible for the anti-HBV activity of type I IFN, because it has been previously reported by Trono and co-workers (28, 35) that the induction of A3G does not explain the IFN-induced inhibition of HBV replication. Moreover, these studies were carried out using an HBV transgene that only reproduces a portion of the whole HBV life cycle, mainly focusing on intracellular HBV replication.

Here, we screened for cytokines and chemokines that affected HBV infection in HepaRG cells, a human hepatocyte cell line susceptible to HBV infection and reproducing the whole HBV life cycle (36, 37). IL-1 and TNFα decreased the host cell permissiveness to HBV infection, and this effect was at least partly mediated by the induction of activation-induced cytidine deaminase (AID). The anti-HBV activity of IL-1/TNFα was mechanistically different from that of IFNα. This study presents the activity of IL-1/TNFα to suppress HBV infection into hepatocytes independent of the effect on immune cells and the physiological role of AID in this machinery. Moreover, as far as we know, this is the first report to show the AID function to inhibit the infection of human pathogenic virus.

EXPERIMENTAL PROCEDURES

Reagents—All cytokines were purchased from PeproTech or R & D Systems. Heparin was obtained from Mochida Pharmaceutical. Lamivudine, PD98059, SP600125, SB203580, and Bay11-7082 were obtained from Sigma. Entecavir was obtained from Santa Cruz Biotechnology. BMS-345541 and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from Merck.

Cell Culture—HepaRG cells (Biopredic) were cultured with Williams' medium E (Invitrogen) supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 5 μg/ml insulin (Wako), 20 ng/ml EGF (PeproTech), 50 μM hydrocortisone (Sigma), and 2% DMSO (Sigma). HepG2, HepAD38 (kindly provided by Dr. Seeger at Fox Chase Cancer Center) (38), and HepG2.2.15 cells (a kind gift from Dr. Urban at Heidelberg University) (39) were cultured with DMEM/F-12 + GlutaMAX (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 50 μM hydrocortisone, and 5 μg/ml insulin in the presence (HepAD38 and HepG2.2.15) or absence (HepG2) of 400 μg/ml G418 (Nacalai Tesque). HepAD38 cells were cultured with 0.3 μg/ml tetracycline when terminating HBV induction. Huh-7.5.1 cells (kindly provided from Dr. Chisari at Scripps Research Institute) were cultured as described previously (40). Primary human hepatocytes (PHH) isolated from urokinase-type plasminogen activator transgenic/SCID mice inoculated with PHH (PhoenixBio) or purchased from Lonza were cultured with DMEM supplemented with 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 44 mM $NaHCO_3$ or with 1 mM pyruvate, nonessential amino acids, 20 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 0.25 μg/ml insulin (Wako), 5 ng/ml EGF, and 50 nM dexamethasone.

HBV Preparation and Infection—HBV used in this study was mainly derived from HepAD38 cells, which is classified as genotype D (38). Media from HepAD38 cells at days 7–31 post-induction of HBV by depletion of tetracycline were recovered every 3 days. Media were cleared through a 0.45-μm filter and precipitated with 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with medium at ~200-fold concentration. The HBV DNA was quantified by real time PCR. HBV genotype A and C in Fig. 7*B* was recovered from the media of HepG2 cells transfected with the plasmid pHBV/Aeus and pHBV/C-AT (41).

HepaRG cells were infected with HBV at 2000 (Fig. 7*B*) or 6000 (other figures) genome equivalent (GEq)/cell in the presence of 4% PEG8000 for 16 h as described previously (36). Urban and co-workers (42) reported that more than 10^3 GEq/cell amount of HBV derived from HepAD38 or HepG2.2.15 cells (*i.e.* $1.25–40 \times 10^4$ GEq/cell) as inoculum was required for efficient infection into HepaRG cells. The anti-HBV effect of IL-1/TNFα shown in this study was also observed when inoculated with HBV at 300 GEq/cell (data not shown).

Extraction of DNA and RNA—HBV DNA was extracted from the cells or from the medium using a DNA kit (Qiagen) according to the manufacturer's protocol. Total RNA was recovered with RNeasy mini kit (Qiagen) according to the manufacturer's protocol.

Real Time PCR and RT-PCR—HBV DNA was quantified by real time PCR analysis using the primer set 5′-ACTCACCAACCTCCTGTCCT-3′ and 5′-GACAAACGGGCAACATACCT-3′ and probe 5′-carboxyfluorescein (FAM)-TATCGCTGGATGTGTCTGCGGCGT-carboxytetramethylrhodamine (TAMRA)-3′ (43). The PCR was performed at 50 °C for 2 min, 94 °C for 10 min, and 50 cycles of 94 °C for 15 s and 60 °C for 1 min. Detection of cccDNA was achieved using 5′-CGTCTGTGCCTTCTCATCTGC-3′ and 5′-GCACAGCTTGGAGGCTTGAA-3′ as primers and 5′-CTGTAGGCATAAATTGGT (MGB)-3′ as a probe (44). This primer-probe set theoretically detected neither relaxed circular DNA nor HBV DNA integrated into host genome but can capture cccDNA as described previously (44). For quantification of cellular mRNA, cDNA was synthesized from extracted RNA using SuperScriptIII (Invitrogen), followed by PCR with TaqMan Gene Expression Master Mix (Applied Biosystems) and primer-probe set (TaqMan Gene Expression Assay, Applied Biosystems) or with Power SYBR Green PCR Master Mix (Applied Biosystems) and 5′-AAATGTCCGCTGGGCTAAGG-3′ and 5′-GGAGGAAGAGCAATTCCACGT-3′ as primers for *AID*.

RT-PCR was performed as described previously (45) using a one-step RNA PCR kit (Takara). Primers for amplifying each gene were as follows: 5′-CTCTGAGGTTTAGCATTTCA-3′ and 5′-CTCCAGGTCCAAAATGAATA-3′ for *cIAP*; 5′-GCA-

GATTTATCAACGGCTTT-3′ and 5′-CAGTTTTCCACCA-CAACAAA-3′ for *XIAP*; 5′-TAGCCAACATGTCCTCACA-GAC-3′ and 5′-TCTTCTACCACTGGTTTCATGC-3′ for *ISG56*; 5′-GCCTTTTCATCCAAATGGAATTC-3′ and 5′-GAAATCTGTTCTGGGCTCATG-3′ for *PKR*; and 5′-CCATG-GAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATG-ACC-3′ for *GAPDH*, respectively.

ELISA—HBs protein was quantified by ELISA using plates incubated at 4 °C overnight with a sheep anti-HBs antibody at 1:5000 dilution (Maxisorp nunc-immuno plate, Nunc catalog no. 439454) followed by coating with 0.2% BSA, 0.02% NaN_3, 1× PBS at 4 °C until use. Samples were incubated with the plates for 2 h and after washing with TBST four times, horse-radish peroxidase-labeled rabbit anti-HBs antibody was added for 2 h. The substrate solution (HCV core ELISA kit: Ortho) was reacted for 30 min before the A_{450} values were measured.

Indirect Immunofluorescence Analysis—Indirect immuno-fluorescence analysis was performed essentially as described previously (45). After fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100, an anti-HBc anti-body (DAKO, catalog no. B0586) was used as the primary antibody.

MTT Assay—The MTT assay was performed as described previously (46).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (47). The polyclonal antibody against AID was generated using a peptide derived from AID protein as an immunogen as described previously for preparation of the anti-AID antibody 1 (48). The specificity of the antibody was described previously (48, 49).

Lentiviral Vector-mediated Gene Transduction—Lentivirus carrying shRNAs was prepared with 293T cells transfected with expression plasmids for HIV-1 Gag-Pol, VSV G, and shRNAs (sh-control, sh-cyclophilin A, sh-AID(1), sh-AID(2); Mission shRNA) (Sigma) with Lipofectamine 2000 (Invitrogen). Recov-ered lentiviral vector was transduced into HepaRG cells fol-lowed by selection with 1.5 μg/ml puromycin. Lentivirus over-expressing AID, AID mutant, A3G, or the control lentivirus was recovered using expression plasmids for HIV-1 Gag-Pol, Rev, VSV G, and the corresponding expression vector as described previously (50).

Southern Blot Analysis—Southern blot was performed as described previously (41). After digestion of free nucleic acids with DNase I and RNase A, cell lysates were digested with pro-teinase K, and HBV DNA in the core particles was extracted with phenol/chloroform, followed by isopropyl alcohol precip-itation. Probe was prepared by cutting pHBV/D-IND60 (41) with SacII and BspHI to generate a full-length HBV DNA probe and labeled with AlkPhos direct labeling reagents (GE Health-care). Labeled bands were visualized with CDP-star detection reagent (GE Healthcare).

Quantification of Nucleocapsid-associated HBV RNA—After digestion of free nucleic acids with DNase I and RNase A, nucleocapsid was precipitated with PEG8000 (41). Total RNA was then extracted from the resuspended precipitates. HBV RNA was quantified by real time RT-PCR with 5′-TCC-CTCGCCTCGCAGACG-3′ and 5′-GTTTCCCACCTTAT-GAGTC-3′ as primers with Power SYBR Green PCR Master Mix (Applied Biosystems).

Co-immunoprecipitation Assay—Co-immunoprecipitation assay was essentially performed as described (45).

Differential DNA Denaturation PCR—Differential DNA denaturation PCR was performed as described previously (51).

Reporter Assay—DNA transfection was performed with pNF-κB-luc or pISRE-TA-luc (Stratagene) and pRL-TK (Pro-mega), which express firefly luciferase driven by NF-κB or ISRE and *Renilla* luciferase by herpes simplex virus thymidine kinase promoter, respectively, and Polyethylenimine Max (Poly-sciences Inc., catalog no. 24765). After compound or cytokine treatment, cells were lysed, and luciferase activities were measured as described previously (52). A reporter carrying HBV core promoter was constructed by inserting the DNA fragment (1413–1788 nucleotide number) of HBV DNA (D-IND60) into pGL4.28 vector (Promega) (41). In the reporter assay using this construct (Fig. 1*H*), HX531, a reti-noid X receptor antagonist was used as a positive control as retinoid X receptor was involved in the transcription from the core promoter (53).

RESULTS

IL-1 Reduced Host Cell Susceptibility to HBV Infection—To evaluate the effect of cytokines and chemokines on susceptibil-ity to HBV infection, we treated HepaRG cells (36) with cyto-kines for 3 h prior to and 16 h during HBV infection, followed by culture without stimuli for an additional 12 days (Fig. 1*A*, *lower scheme*). Heparin, a competitive inhibitor of HBV attachment (54), was used as a positive control and decreased secretion of the viral envelope surface protein (HBs) from HBV-infected cells (Fig. 1*A*, *upper graph, lane 38*), which suggests a successful HBV infection in this experiment. Examination of 36 cytokines and chemokines revealed that IL-1β drastically decreased pro-tein secretion from HBs (Fig. 1*A*, *upper graph, lane 8*). Although IFNs had a strong anti-HBV effect by a continuous treatment after HBV infection (Fig. 3*C*, *panel b*, and data not shown), they had only a limited effect in this screening where cytokines were only pretreated and cotreated with HBV (Fig. 1*A*, *lanes 2–7*). HBc protein expression (Fig. 1*B*) and HBV DNA (Fig. 1*C*) in the cells and medium (Fig. 1*D*) were significantly decreased by treatment with IL-1β without cytotoxicity (Fig. 1*G*). HBV cccDNA and HBV RNA was also decreased in infected cells treated with IL-1β (Fig. 1, *E* and *F*). IL-1β did not decrease HBV core promoter activity at least in HepG2 cells (Fig. 1*H*). These results suggest that IL-1β suppressed HBV infection to HepaRG cells. IL-1β did not decrease the expression of sodium taurocholate cotransporting polypeptide (*NTCP*), a recently reported HBV entry receptor (data not shown) (55). Similar results were obtained using primary human hepatocytes (Fig. 1*I*).

NF-κB Signaling Was Critical for Anti-HBV Activity—As shown in Fig. 2*A*, IL-1β suppressed HBV infection in a dose-dependent manner. This anti-HBV effect was reversed by cotreat-ment with a neutralizing antibody for the IL-1 receptor, IL-1RI (Fig. 2*B*), suggesting that receptor engagement was required for anti-HBV activity. IL-1Ra is a natural antagonist that associates with IL-1RI but does not trigger downstream signal transduc-

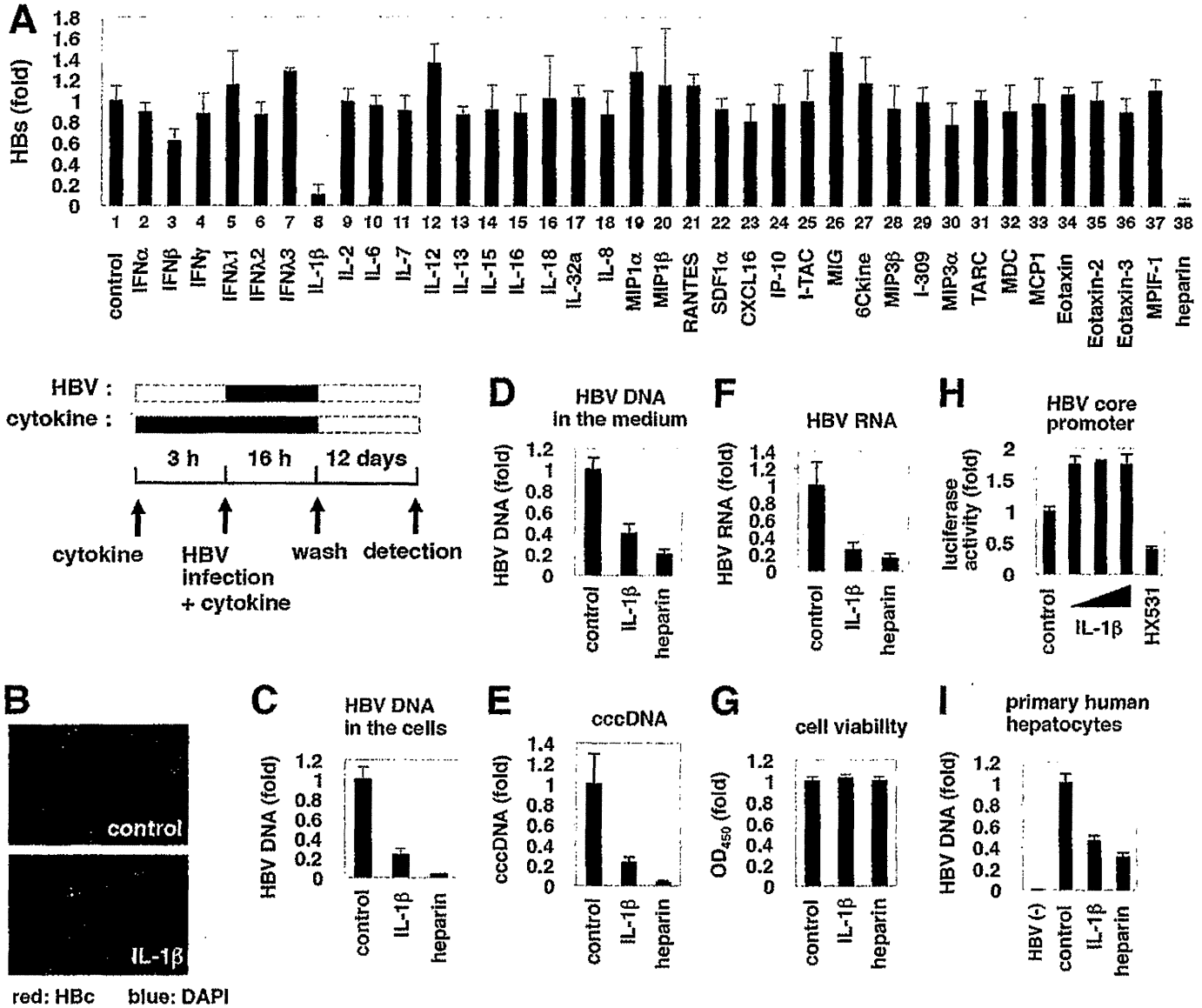

FIGURE 1. **Suppression of HBV infection by IL-1β.** *A, upper graph,* HepaRG cells were pretreated with cytokines at 100 ng/ml (except for IFNα and IFNβ at 100 IU/ml) or heparin at 25 units/ml as a positive control or were left untreated (*control*) for 3 h and then infected with HBV in the presence of each stimuli for 16 h. After washing, cells were cultured in normal growth medium for 12 days. HBs protein secreted into the medium was quantified by ELISA. *Lower scheme* indicates the treatment procedure for HepaRG cells. *Black* and *dashed line boxes* indicate the periods with and without treatment, respectively. *B–G* and *I*, HepaRG cells (*B–G*) or PHH (*I*) were treated as shown in *A* with or without 100 ng/ml IL-1β or 25 units/ml heparin as a positive control. HBc protein in the cells (*red*) was detected by indirect immunofluorescence analysis, and the nucleus was stained with DAPI (*blue*) at 12 days post-infection (*B*). HBV DNA (*C* and *I*), cccDNA (*E*), and HBV RNA (*F*) in the cells as well as HBV DNA in the medium (*D*) were detected. Cell viability was quantified by MTT assay (*G*). *HBV(−)* in *I* indicates uninfected cells. All of the data, except in *I*, are based on the average of three independent experiments. *I* shows the average results from one representative experiment, but the reproducibility of the data were confirmed in three independent experiments. *H*, reporter plasmid carrying the HBV core promoter was transfected with HepG2 cells and then treated with or without IL-1β (1, 10, and 100 ng/ml) and an retinoid X receptor antagonist HX531 as a positive control for 6 h. Luciferase activity was measured.

tion (56). Treatment with IL-1Ra did not decrease HBV infectivity (Fig. 2*C*), suggesting that signal transduction triggered by IL-1 was required for anti-HBV activity.

To identify the signal transduction pathway essential for anti-HBV activity, we treated HepaRG cells with PD98059, SP600125, SB203580, and Bay11-7082, which are inhibitors for MEK, JNK, p38, and NF-κB, respectively (57). As shown in Fig. 2*D*, only cotreatment with Bay11-7082 significantly removed the anti-HBV effect of IL-1β. Luciferase assay and RT-PCR analysis indicated that Bay11-7082, but not other inhibitors, blocked the transactivation of NF-κB (Fig. 2*E*, *upper panels*) and NF-κB downstream genes, *cIAP* and *XIAP* (Fig. 2*E*, *lower panels*). Additional NF-kB inhibitors, BMS-345541 and QNZ (Fig. 2*G*), also reversed the anti-HBV effect of IL-1β (Fig. 2*F*). These data suggest a critical role for NF-κB activation in the anti-HBV activity. Additionally, IL-1β did not augment the activity of interferon sensitivity-responsive element (ISRE) and mRNAs for ISGs, *ISG56*, and double-stranded RNA-dependent protein kinase (*PKR*) in HepaRG cells (Fig. 2*H*), suggesting that the anti-HBV activity is independent of ISG up-regulation. TNFα, another cytokine that activates NF-κB signaling (Fig. 2*E*, *lower panels*), also inhibited HBV infection (Fig. 2*I*). Thus, NF-κB activation in host hepatocytes was critical for the anti-HBV activity of proinflammatory cytokines.

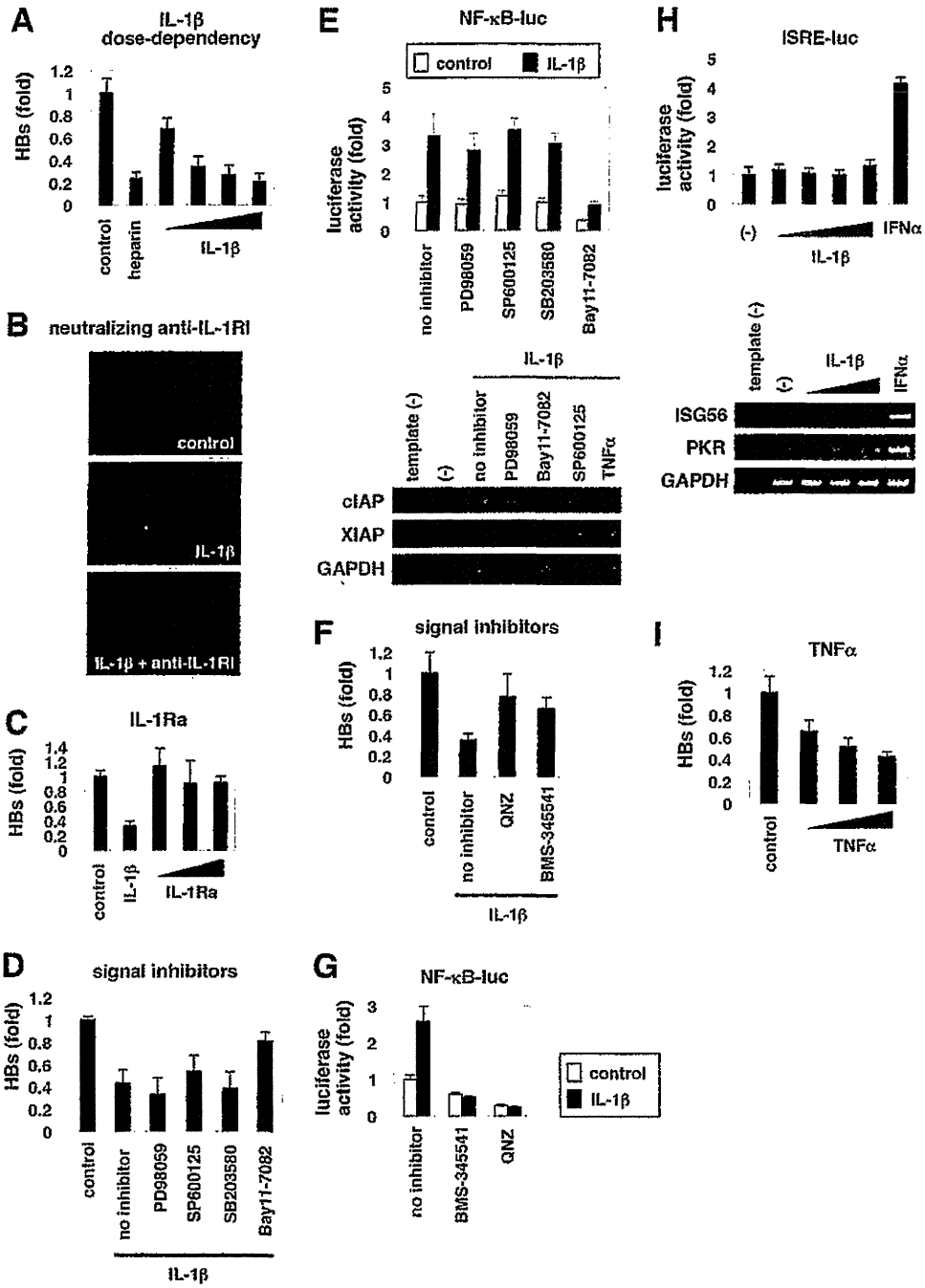

FIGURE 2. **NF-κB activation triggered by IL-1 and TNFα was critical for anti-HBV activity.** *A–D, F,* and *I,* HepaRG cells were left untreated (*control*) or treated with varying concentrations of IL-1β (1, 10, 30, and 100 ng/ml) or 25 units/ml heparin (*A*), with 30 ng/ml IL-1β together with or without a neutralizing anti-IL-1RI antibody at 20 μg/ml (*B*), with 10 ng/ml IL-1β or varying concentrations of IL-1Ra (10, 30, and 100 ng/ml) (*C*), with 3 ng/ml IL-1β together with or without PD98059, SP600125, SB203580, or Bay11-7082 (*D*), or QNZ or BMS-345541 (*F*), or with TNFα (10, 100, and 300 ng/ml) (*I*) according to the treatment schedule shown in Fig. 1*A*. HBV infection was monitored by HBs protein secretion into the medium in *A, C, D, F,* and *I* and with HBc protein in the cells in *B*. *E, G,* and *H,* NF-κB (*E* and *G*) and ISRE activity (*H*) were measured by reporter assay in the cells transfected with the reporter plasmid expressing luciferase driven from five tandem repeats of NF-κB elements (*E, upper graph,* and *G*) or ISRE (*H, upper graph*) or by RT-PCR in the cells (*E* and *H, lower panels*) upon signaling inhibitors used in *D* and *F* together with or without IL-1β (*E* and *G*), or upon IL-1β (10, 30, and 100 ng/ml) or IFNα 100 IU/ml as a positive control (*H*) for 6 h. The *white* and *black bars* in the *upper graph* of *E* and *G* show the data in the absence or presence of IL-1β, respectively. Bands for mRNA for *cIAP, XIAP,* and *GAPDH* (*E*) or *ISG56, PKR,* or *GAPDH* (*H*) are presented in the *lower panels*. All of the data are based on averages of three independent experiments.

Early Phase of HBV Infection as Well as HBV Replication Were Impaired by IL-1 Treatment—Although heparin, an attachment inhibitor, could block HBV infection only if added together with the HBV inoculum, pretreatment with IL-1β before HBV infection was sufficient to show anti-HBV activity (Fig. 3*A, panel b*). This activity was amplified by a prolonged treatment time of up to 12 h (Fig. 3*B*). Intriguingly, HBV cellular DNA was also reduced by IL-1β treatment following HBV infection (Fig. 3*C, panel b*). In contrast, IFNα was not effective by pretreatment (Figs. 3*C, panel a,* and 1*A*), although it did decrease HBV DNA by treatment after HBV infection (Fig. 3*C, panel b*), consistent with previous reports that IFNα can sup-

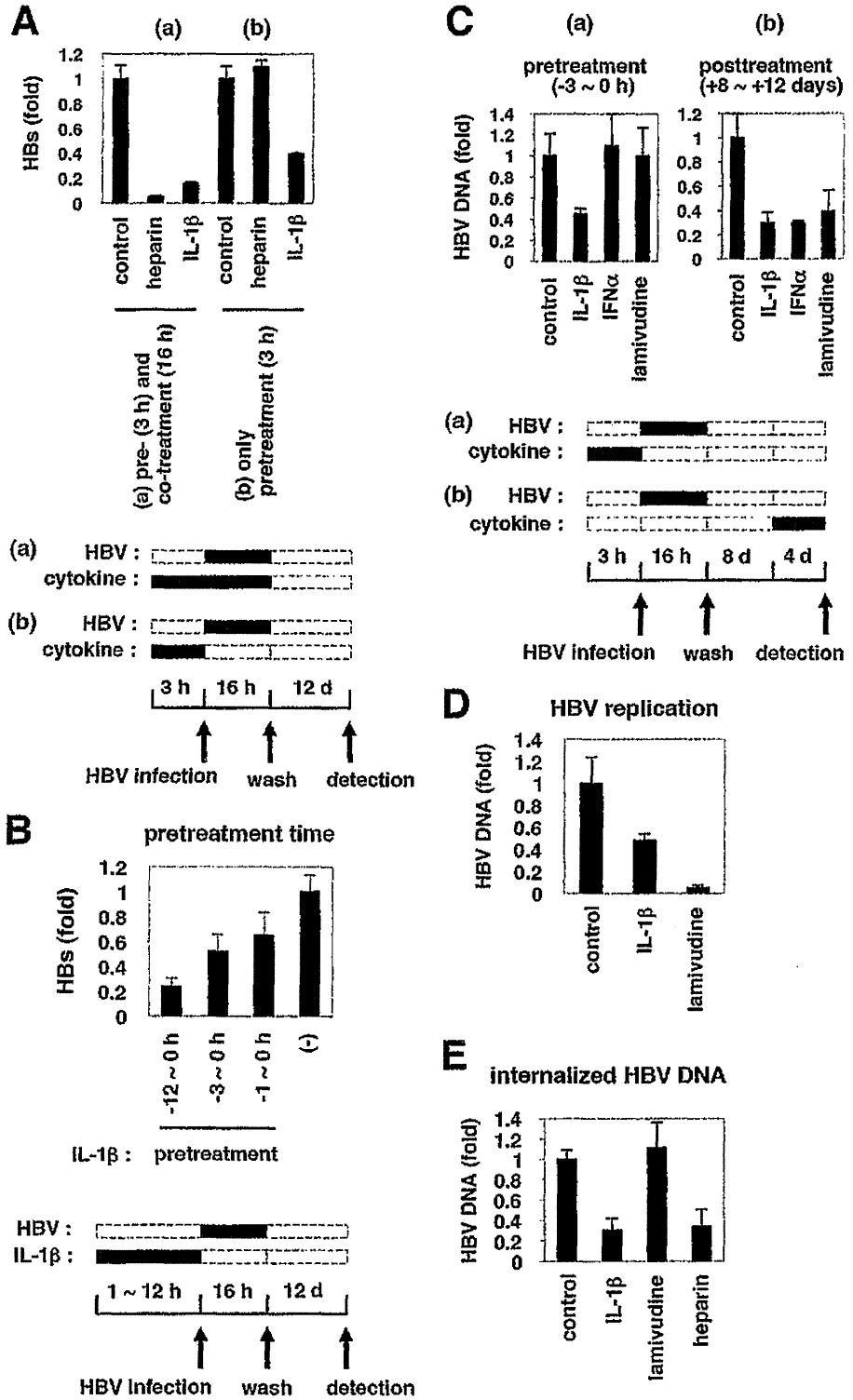

FIGURE 3. **Defining the steps of the HBV life cycle targeted by IL-1β.** *A*, HepaRG cells were pretreated with IL-1β or heparin for 3 h and then infected with HBV in the presence (*A, panel a*) or absence (*A, panel b*) of IL-1β or heparin for 16 h. HBV infection was monitored with HBs protein secretion from the infected cells. Only pretreatment with IL-1β and not heparin could inhibit HBV infectivity. *d*, day. *B*, HepaRG cells were pretreated with IL-1β or left untreated (−) for the indicated time (*h*) and infected with HBV without IL-1β. Anti-HBV activity was amplified by a prolonged treatment time. *C, panel a*, HepaRG cells were pretreated with 10 ng/ml IL-1β, 100 IU/ml IFNα, or 1 μM lamivudine for 3 h, followed by infection with HBV for 16 h in the absence of cytokines (*pretreatment*). *C, panel b*, HepaRG cells were infected with HBV for 16 h without pretreatment. After washing out the input virus, cells were cultured in normal medium for the first 8 days and then cultured with IL-1β, IFNα, or lamivudine for the following 4 days (*post-treatment*). HBV DNA in the cells was measured by real time PCR. IL-1β showed an anti-HBV activity in both pretreatment and post-treatment, although an anti-HBV effect of IFNα was seen only with post-treatment. *D*, HepAD38 cells were treated with 100 ng/ml IL-1β or 1 μM lamivudine, or left untreated for 6 days in the absence of tetracycline. HBV replication was evaluated by measurement of HBV DNA in the medium. *E*, HepaRG cells were pretreated with IL-1β, lamivudine, or heparin for 3 h or left untreated and infected with HBV for 16 h in the presence or absence of each compound. After trypsinization and extensive washing of the cells, cellular DNA was immediately recovered to detect HBV DNA. HBV DNA at 16 h post-infection was decreased by treatment with IL-1β but not lamivudine.

press HBV replication (19, 20, 26). Thus, the anti-HBV activity of IL-1β is likely to be mechanistically different from that of IFNα.

The HBV life cycle can be divided into at least two phases as follows: 1) the early phase of infection that includes attachment, entry, nuclear import, and cccDNA formation; and 2) the late phase representing HBV replication, including transcription, assembly, reverse transcription, DNA synthesis, and viral release (58). The early phase of HBV infection is not supported, but HBV DNAs persistently replicate in HepAD38 cells in the presence of tetracycline (38). IL-1β decreased the HBV DNA levels in HepAD38 cells (Fig. 3*D*), suggesting suppression of HBV replication. In addition, to examine the early phase preceding HBV replication, we infected HepaRG cells with HBV in the presence of IL-1β for 16 h and then immediately recovered cellular DNA in the trypsinized cells for quantification of HBV DNA (Fig. 3*E*). This procedure likely detected HBV DNA that had been internalized and evaded the host restriction before initiation of HBV replication because lamivudine showed no effect on the amount of DNA detected (Fig. 3*E*). In this experiment, IL-1β significantly decreased HBV DNA (Fig. 3*E*). cccDNA was also decreased by IL-1β, suggesting that the early phase of HBV infection before cccDNA formation was also interrupted by IL-1β.

IL-1 and TNFα Induced the Expression of AID—The innate immune pathway against HBV infection remains largely unknown. Recently, accumulating evidence suggested that several APOBEC family proteins, especially A3G, suppressed HBV replication when overexpressed (27–33). In contrast, there was no report available suggesting the anti-HBV function of other restriction factors against HIV, TRIM5α, tetherin/BST-2, and SAMHD1. We then investigated APOBEC family proteins as a candidate for an anti-HBV effector. The APOBEC family includes APOBEC1 (A1), A2, A3s, A4, and AID (59). Because some of these proteins are reported to be up-regulated in cytokine-stimulated hepatocytes (27, 28, 60, 61), we examined the expression of these genes in cells treated with IL-1β, TNFα, and IFNα as a control for 12 h. The mRNA levels of *A1*, *A2*, and *A3A* were below the detection threshold. *A3G* and *A3F* mRNA were significantly expressed in HepaRG cells, and their expression levels were remarkably increased by IFNα treatment (Fig. 4*A*), as observed in other reports (27, 28, 61). IL-1β and TNFα did not significantly up-regulate A3s, and only AID was up-regulated 6–10-fold by both cytokines (Fig. 4*A*). Induction of A3s by both IL-1β and TNFα was not observed at any time point examined until 12 h (data not shown). In contrast, induction of *AID* mRNA by IL-1β and TNFα was conserved in human hepatocyte cell lines, such as HepG2 and FLC4 cells, and in primary human hepatocytes (Fig. 4*B*). AID protein production was also increased in primary human hepatocytes by treatment with IL-1β and TNFα (Fig. 4*C*). This AID induction by IL-1β was suggested to be NF-κB-dependent, because the up-regulation of *AID* mRNA was canceled by addition of NF-κB inhibitors, Bay11-7082 or QNZ (Fig. 4*D*).

AID Played a Significant Role in the IL-1-mediated restriction of HBV—To examine the function of AID during HBV infection, we transduced AID ectopically into HepaRG cells using a lentiviral vector (Fig. 5*A*, *left panel*). The susceptibility of these AID-overexpressing cells to HBV was decreased by approximately one-third compared with the parental or empty vector-transduced HepaRG cells (Fig. 5*A*, *right panel*), suggesting that AID can restrict HBV infection. An AID mutant AID(M139V), with reported diminished activity to support class switching (48), also decreased the susceptibility to HBV infection, although the reduction in HBV susceptibility was moderate compared with the case of the wild type AID (Fig. 5*B*).

To examine the relevance of endogenous AID in the anti-HBV activity of IL-1, we transduced a lentiviral vector carrying a short hairpin RNA (shRNA) against AID (sh-AID) or a non-relevant protein cyclophilin A (Fig. 5*C*), and we observed the anti-HBV activity of IL-1β in these cells. IL-1β decreased HBV infection in the control and sh-cyclophilin A -transduced cells by ~3.0-fold as determined by HBs secretion (Fig. 5*D*, *lanes 1* and *2*, *black bars*). In contrast, anti-HBV activity of IL-1β was limited to only 1.6–1.7-fold in the cells transduced with sh-AIDs (Fig. 5*D*, *lanes 3* and *4*, *black bars*). Such relieved anti-HBV activity following AID knockdown was not observed in the case for heparin treatment (Fig. 5*D*, *lanes 1–4*, *gray bars*). Similar results were obtained by monitoring intracellular HBV DNA after infection (data not shown). Although the anti-HBV effect of IL-1β was not completely blunted, these data suggest that AID plays a significant role in mediating the anti-HBV effect of IL-1β.

Similar observations were obtained in HBV-replicating cells overexpressing AID (Fig. 5, *E* and *F*). Core particle-associated HBV DNA in HepG2 cells transfected with an HBV-encoding plasmid was decreased by overexpression with AID as well as with A3G (Fig. 5*E*, *lanes 1* and *3*). Intriguingly, HBV DNA in core particles was also decreased by expression of an AID mutant AID(H56Y), which contains a mutation in the cytidine deaminase motif and is derived from a class switch deficiency patient (Fig. 5*E*, *lane 2*) (48). Southern blot also showed that the HBV rcDNA level in HepG2.2.15 cells was reduced by transduction with AID and another mutant AID(M139V), with diminished activity to support class switching (Fig. 5*F*) (48). These data suggest that AID could suppress HBV replication, and this restriction activity can be still observed with reduced enzymatic activity. In addition, AID was shown to interact with HBV core protein by coimmunoprecipitation assay (Fig. 5*G*). Moreover, overexpression of AID reduced the levels for nucleocapsid-associated HBV RNA (Fig. 5*H*). These results further suggest an antiviral activity of AID against HBV replication.

AID Could Induce Hypermutation of HBV DNA—Major enzymatic activity for APOBEC family proteins is the introduction of hypermutation in target DNA/RNA, and hypermutation accounts for antiviral activity for A3G against HIV-1 to some extent (2). Several groups reported that APOBEC family proteins could induce hypermutation in HBV DNA (27, 30, 32, 34). Next we asked whether AID could induce hypermutations in HBV DNA. In differential DNA denaturation PCR analysis, a high content of A/T bases introduced by hypermutation decreased denaturation temperatures (51). As shown in Fig. 6*A*, ectopic expression of AID decreased the denaturation temperature of HBV DNA as shown by that of A3G. Sequence analyses of the HBV DNA X region amplified at 83 °C by differential DNA denaturation PCR indicated a massive accumulation of

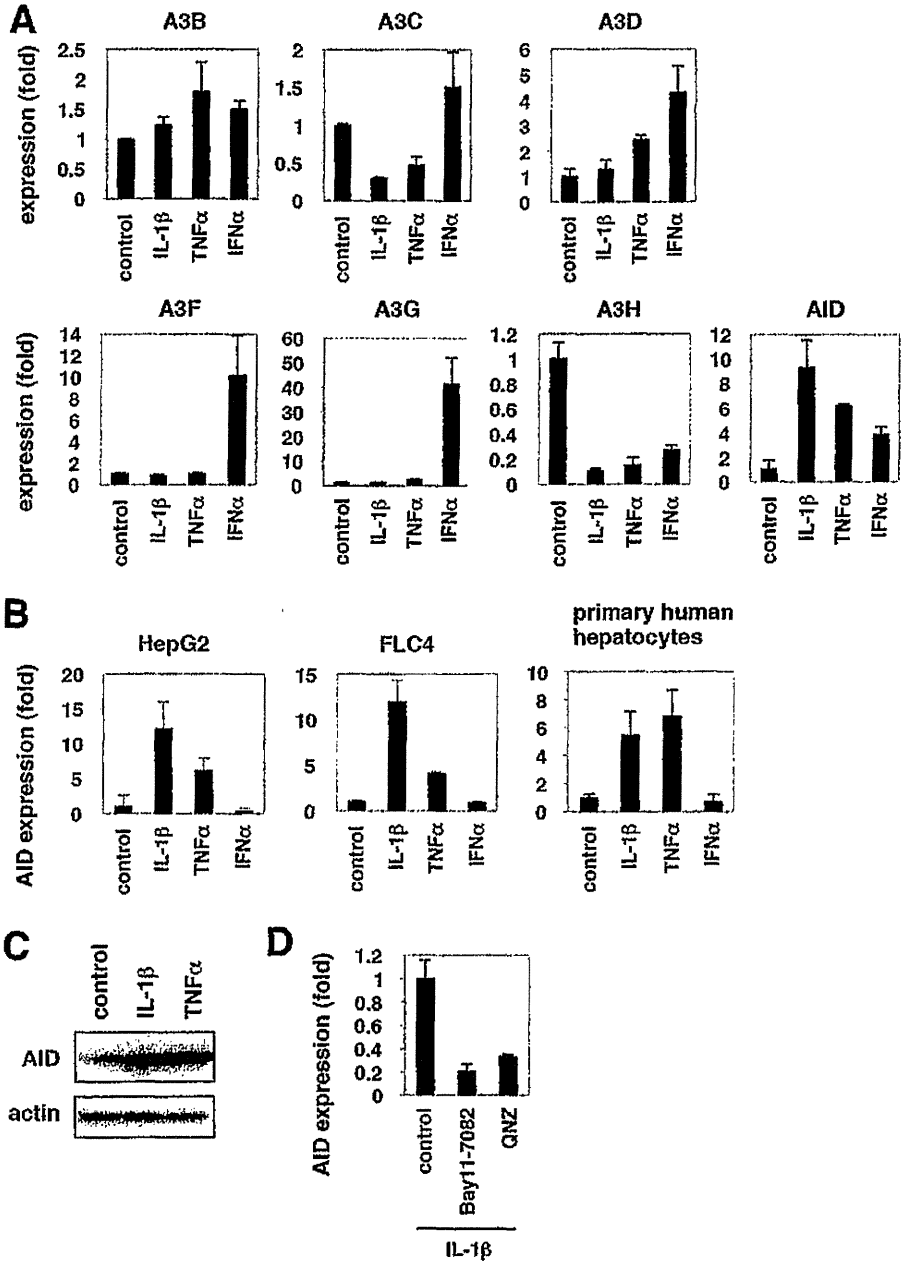

FIGURE 4. **AID expression was induced by IL-1β and TNFα.** *A*, mRNAs for *A3B*, *-C*, *-D*, *-F*, *-G*, *-H* and *AID* were quantified by real time RT-PCR analysis in HepaRG cells treated with 100 ng/ml IL-1β, 100 ng/ml TNFα, or 100 IU/ml IFNα for 12 h or left untreated. *Graphs* show the relative expression levels compared with the controls set at 1. *B*, *AID* mRNA was detected in HepG2, FLC4 cells, and PHH treated with IL-1β, TNFα, or IFNα or left untreated. Induction of AID by IL-1β and TNFα was observed in HepG2 and FLC4 cells and primary human hepatocytes. *C*, AID protein (*upper panel*) and actin levels as an internal control (*lower panel*) were examined by immunoblot of primary human hepatocytes treated with IL-1β or TNFα or left untreated. *D*, AID mRNA was detected in PHH treated with 100 ng/ml IL-1β in the presence or absence of NF-κB inhibitors, Bay11-7082, or QNZ for 12 h.

G-to-A mutations by AID (Fig. 6*B*). The frequency of G-to-A mutations was augmented by AID expression (Fig. 6*C*). In this experiment, AID(JP8Bdel), a hyper-active mutant of AID (62), further promoted the accumulation of the G-to-A and C-to-T mutations, although AID(H56Y) showed mutations in HBV DNA equivalent with mock GFP control sample (Fig. 6*C*). Thus, AID had the potential to introduce hypermutation in nucleocapsid-associated HBV DNA.

IL-1 Suppressed the Infection of Different HBV Genotypes but Not That of HCV—We examined whether the antiviral activity of IL-1β and TNFα could be generalized to other viruses or was specific to HBV. As shown in Fig. 7*A*, the production of infectious HCV and HCV core proteins in the medium was not significantly altered by treatment with these cytokines in HCV-infected cells, compared to when IFNα was used as a positive control (Fig. 7*A*). In contrast, IL-1 suppressed the infection of HBV genotype A and C into HepaRG cells (Fig. 7*B*) as well as genotype D (Fig. 1*C*). These data suggest that the antiviral activity of proinflammatory cytokines IL-1 and TNFα is specific to HBV.

DISCUSSION

In this study, cytokine screening revealed that IL-1 and TNFα decreased the host cell susceptibility to HBV infection.

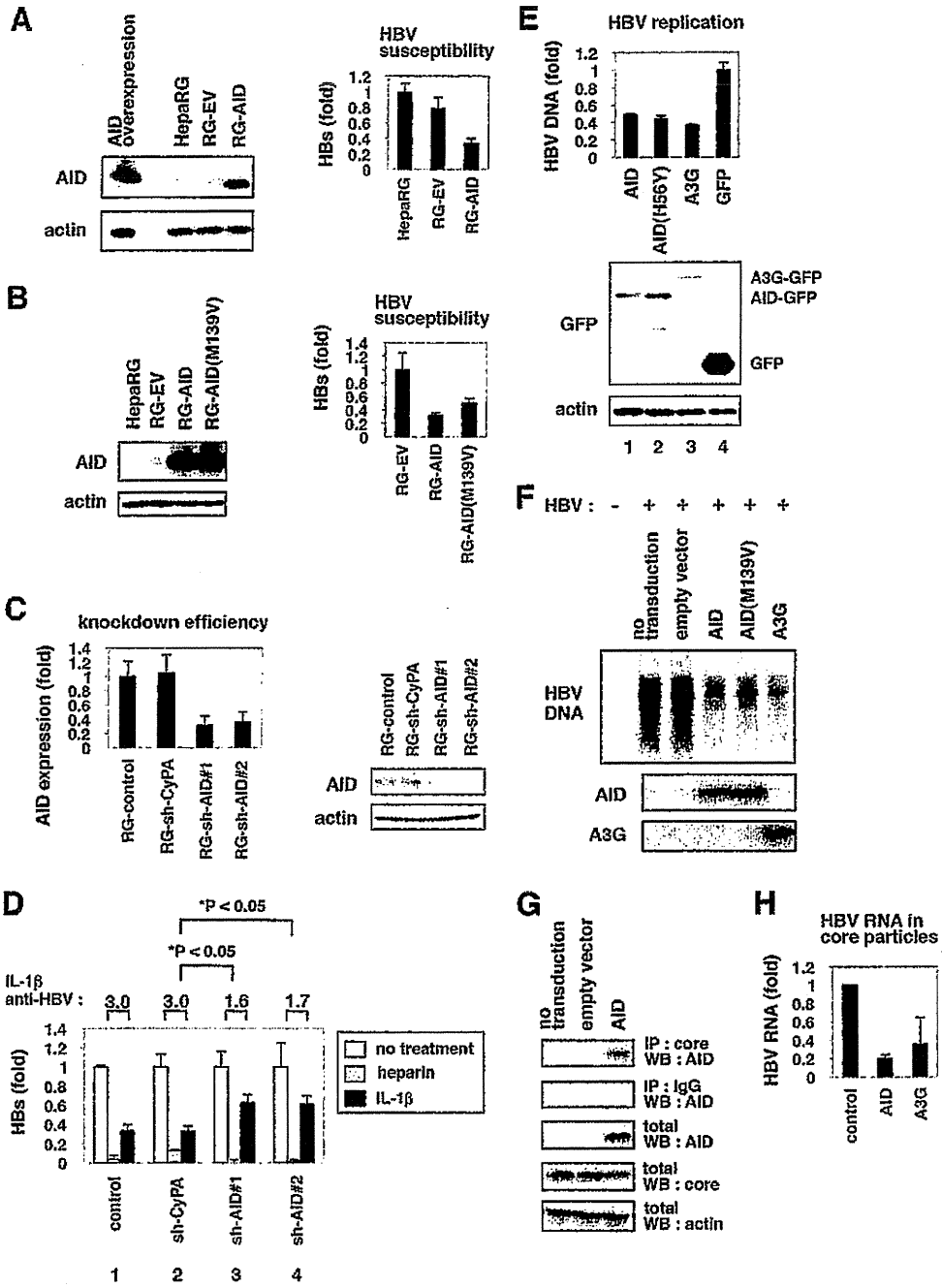

FIGURE 5. **AID played a significant role in IL-1-mediated anti-HBV activity.** *A* and *B*, *left panels*, HepaRG cells were transduced with a lentiviral vector carrying the expression plasmid for AID (*RG-AID*), AID(M139V) mutant (*RG-AID(M139V)*) (*B*), or the control vector (*RG-EV*). Protein expression for AID (*upper panel*) and actin (*lower panel*) in these cells, the parental HepaRG cells (*HepaRG*), and those transiently transfected with AID expression plasmid (*AID overexpression*) (*A*) was examined by immunoblot. *Right panels*, these cells were infected with HBV followed by detection of secreted HBs protein as Fig. 1*A*. AID-transduced cells were less susceptible to HBV infection. *C*, HepaRG cells were transduced with lentiviral vector carrying shRNAs for AID (*RG-shAID#1* and *RG-shAID#2*) or for cyclophilin A (*RG-shCyPA*) as a control. AID mRNA (*left panel*) and protein (*right panel*) were quantified by real time RT-PCR and immunoblot analysis. *D*, cells produced in C were infected with HBV in the absence or presence of IL-1β or heparin, and HBs was detected in the medium as in Fig. 1*A* to examine the anti-HBV effect of IL-1β and heparin. The fold reduction of HBV infection by IL-1β treatment is shown as *IL-1β anti-HBV* above the *graph*. The *white*, *gray*, and *black bars* indicate HBs value of the cells without treatment and with heparin and IL-1β treatment, respectively. The anti-HBV activity of IL-1β but not heparin was reduced in the AID-knockdown cells. *E*, AID and its mutant suppressed HBV replication. HepG2 cells were cotransfected with GFP-tagged AID, AID(H56Y), A3G, and GFP itself along with an HBV-encoding plasmid. Following 3 days, cytoplasmic nucleocapsid HBV DNA was quantified (*upper graph*), and the overexpressed proteins as well as actin were detected (*lower panels*). *F*, lentiviral vectors carrying AID, AID(M139V) mutant, A3G, or an empty vector (*empty vector*) were transduced or left untransduced (*no transduction*) into HepG2.2.15 cells. Nucleocapsid associated HBV DNA in these cells or in HepG2 cells (*HBV−*) was detected by Southern blot (*upper panel*). AID (*middle panel*) and A3G protein (*lower panel*) were also detected by immunoblot. *G*, HBV core interacted with AID. HepAD38 cells transduced without (*no transduction*) or with AID-expressing vector or the empty vector (*empty vector*) were lysed and treated with anti-core antibody (*1st panel*) or control normal IgG (*2nd panel*) for immunoprecipitation (*IP*). Total fraction without immunoprecipitation (*3rd* to *5th panels*) was also recovered to detect AID (*1st* to *3rd panels*), HBV core (*5th panel*), and actin (*5th panel*) by immunoblot. *WB*, Western blot. *H*, HBV RNA in core particles was extracted as shown under "Experimental Procedures" in HepG2 cells overexpressing HBV DNA together with or without AID or A3G.

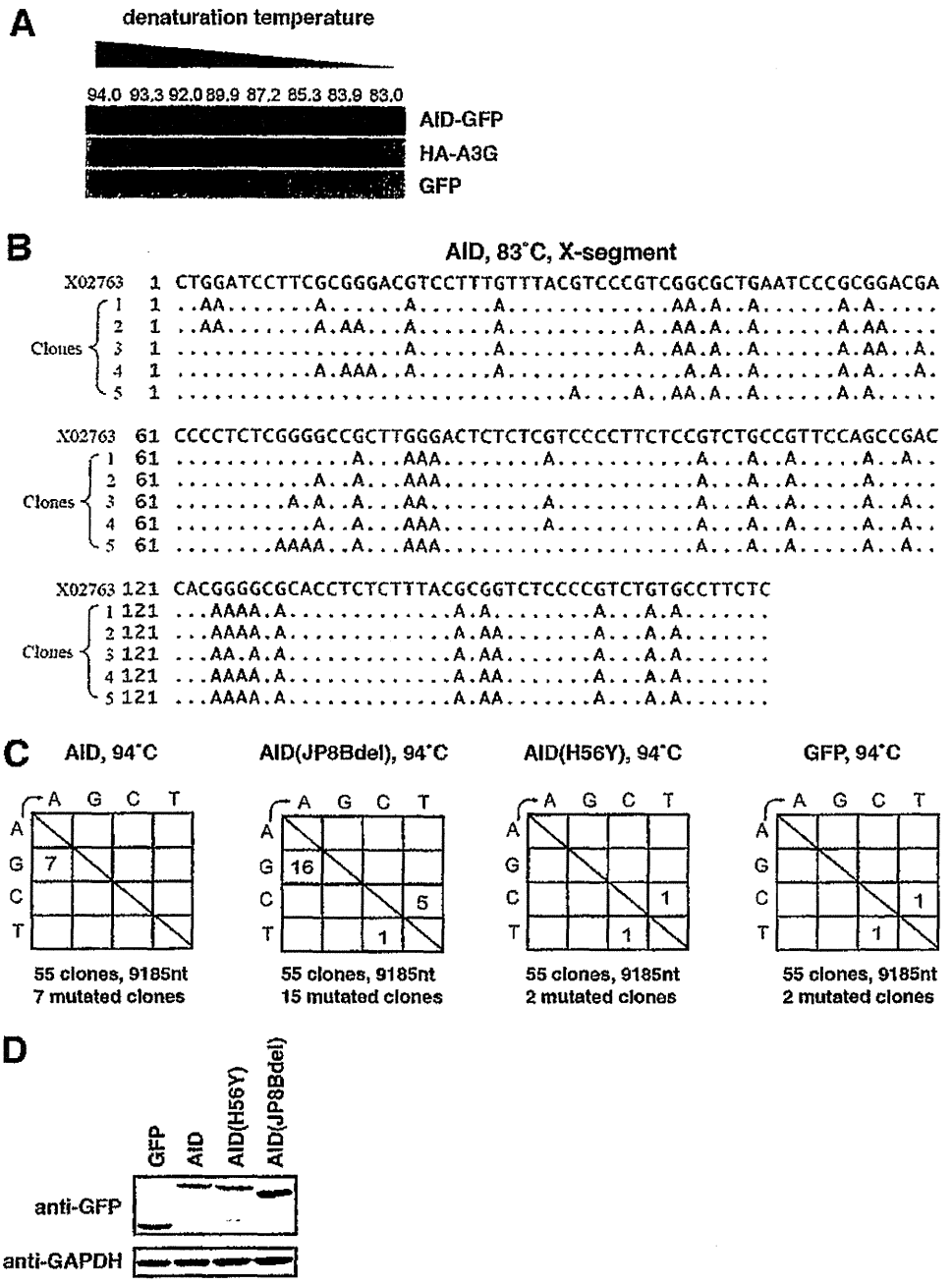

FIGURE 6. **AID could induce hypermutation of HBV DNA.** *A* and *B*, HepG2 cells were cotransfected with an expression vector for GFP-tagged AID, HA-tagged A3G, or GFP along with an HBV-encoding plasmid. 3 days after transfection, nucleocapsid-associated HBV DNA was extracted, and differential DNA denaturation PCR was performed to amplify the X gene segments. The *numbers above* the panels in *A* show denaturing temperatures. The X gene fragment amplified at 83 °C in the AID sample was cloned in to a T vector and sequenced in *B*. Alignment of independent five clones with reference sequence (X02763) is indicated. *C*, AID and its mutant (JP8Bdel) induced G-to-A and C-to-T hypermutations in HBV DNA. HepG2 cells were transfected with expression vectors of GFP-tagged AID, AID(H56Y), AID(JP8Bdel), or GFP itself together with HBV encoding plasmid. Three days after transfection, cells were harvested, and nucleocapsid-associated HBV DNA was extracted. X gene fragments were amplified at 94 °C and cloned in T vector. 55 clones were sequenced as described under "Experimental Procedures." The *numbers* indicate the clone numbers carrying the mutation. *D*, expression of GFP, GFP-tagged AID, AID(H56Y), and AID(JP8Bdel) is shown by immunoblot.

This antiviral mechanism is rather unique, given that the intracellular immune response against viruses is typically triggered by IFNs. So far, type I, II, and III IFNs are reported to suppress the replication step of the HBV life cycle (19, 20, 25, 26). In contrast, we suggest that IL-1 and TNFα inhibit the early phase of HBV infection as well as the replication. This is consistent with cumulative clinical evidence suggesting that these proinflammatory cytokines contribute to HBV elimination (63–65). IL-1 and TNFα are generally produced mainly in macrophages and also in other cell types, including T cells and endothelial cells (66). Although the main producer cells of these cytokines in hepatitis B patients are not defined, it has been reported that the secretion of IL-1 and TNFα in nonparenchymal cells were increased by HBV infection into hepatocytes (67). TNFα production in macrophages was augmented by addition of recombinant HBc (68). A number of clinical studies cumulatively

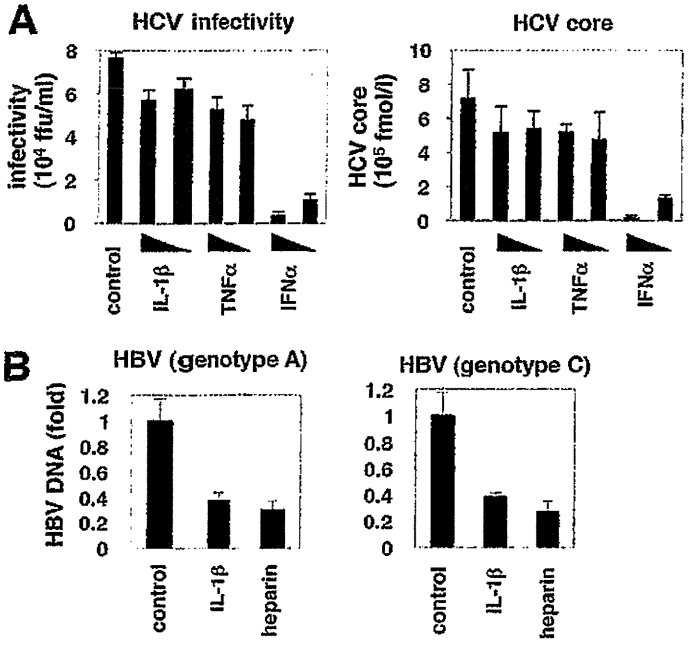

FIGURE 7. **Antiviral activity of AID was specific to HBV.** *A*, Huh-7.5.1 cells were pretreated with IL-1β, TNFα, or IFNα for 3 h or left untreated and then coincubated with HCV for 4 h. After washing HCV and cytokines and culturing the cells with normal medium for 72 h, the infectivity of HCV (*left panel*) as well as HCV core protein (*right panel*) in the medium was quantified. *B*, HepaRG cells were treated with IL-1β or heparin or left untreated for 3 h prior to and 16 h during infection of HBV genotype A (*left graph*) or C (*right graph*) as shown in Fig. 1*A*. HBV infection was monitored with cellular HBV DNA at 12 days after the infection as Fig. 1*C*.

show that serum levels of IL-1 and TNFα are increased in hepatitis B patients (12). Recently, it has been a significant clinical problem that HBV reactivates during the course of treatment with immunosuppressants such as anti-TNFα agents (64, 65). Taken together, it is proposed that acute or chronic HBV infection induces IL-1/TNFα from macrophages or other cells in the liver of infected patients, which can directly suppress HBV infection in hepatocytes, in addition to their immunomodulatory effects to the host immune cells. Although IL-1 level in HBV-infected patients varies between papers, Daniels *et al.* (63) reported that the peak IL-1β level in HBV-infected patients was 9–36 ng/ml under Toll-like receptor stimulation, at which concentration IL-1β showed significant anti-HBV effects in this study. In general, downstream genes of NF-κB include a number of antiviral factors such as *viperin*, *iNOS*, and *RANTES* (69). Although some of these genes may function cooperatively for IL-1- and TNFα-induced anti-HBV machinery, our data suggest that AID, at least in part, plays a role in the elimination of HBV that was potentiated by proinflammatory cytokines IL-1 and TNFα.

AID belongs to APOBEC family proteins that share enzyme activity to convert cytidine to uracil in mainly DNA, and occasionally RNA (51, 70, 71). Although AID was initially identified in B cells, chronic inflammation can trigger its expression in hepatocytes (60). The induction of AID was reportedly mediated by NF-κB (60), consistent with the results in this study. Although AID in B cells is essential for class switch recombination and somatic hypermutation of immunoglobulin genes (70, 72), the physiological role of AID in hepatocytes is unknown. Although expression of AID in hepatocytes is still lower than in B cells, AID is reportedly expressed in the liver both in cell culture and *in vivo* settings (34, 60). Our results raise the idea that AID plays a role in innate antiviral immunity. AID also has a role in virus-induced pathogenesis as it was reported to counteract oncogenesis induced by Abelson-murine leukemia virus (73). In addition, AID was reported to restrict L1 retrotransposition, which can predict the role of AID in innate immunity (74). This study is significant in that it revealed a biological function of AID in viral infection itself, linking it to the restriction of a pathogenic human virus. It will be interesting to analyze the role of AID in the infection process of other viruses in the future.

Although the mechanism for AID suppression of the HBV life cycle is the subject of future study, AID possibly targets the early phase of HBV infection, including entry as well as the replication stage, including assembly and reverse transcription (Fig. 3). It has been recently reported that chicken AID reduced cccDNA of duck HBV possibly through targeting cccDNA as well as nucleocapsid-associated HBV DNA (75). This study is likely to support the idea that AID may target cccDNA formed after HBV entry into hepatocytes, and also associates with nucleocapsid-associated HBV DNA during HBV replication, although it is not clear whether the innate immune machinery against HBV/duck HBV is conserved in human and chicken cells. A3G blocked HBV replication through the inhibition of reverse transcriptase (29), packaging of pregenomic RNA (33), and the destabilization of packaged pregenomic RNA (31) independently of its deaminase activity, and it also induced hypermutation of HBV DNA (27, 30, 32, 34). It was recently reported that AID was packaged into the HBV nucleocapsid (51). Moreover, AID induced C-to-T and G-to-A hypermutations in HBV DNA/RNA, although the anti-HBV activity has not been demonstrated so far (51). The hypermutation activity of AID was likely to be dispensable for its anti-HBV replication function (Figs. 5 and 6), as reported for APOBEC3G by several groups (29, 30, 33). Further analysis is required to elucidate the precise mechanisms for AID-mediated suppression of the HBV life cycle.

In conclusion, we have identified that host cell susceptibility to HBV infection is modulated by IL-1 and TNFα, and AID is involved in this machinery. This sheds new light on the link between proinflammatory cytokines and the development of the innate antiviral defense.

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