JOURNAL OF VIROLOGY, June 2007, p. 6128–6133 Vol. 81, No. 11
0022-538X/07/$08.00+0 doi:10.1128/JVI.02627-06

Human Serum Amyloid A Protein Inhibits Hepatitis C Virus Entry into Cells[▽]

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Received 28 November 2006/Accepted 21 February 2007

Serum amyloid A (SAA) is an acute-phase protein induced by a variety of inflammatory stimuli, including bacterial and viral infections. SAA was recently found to function as an opsonin for gram-negative bacteria. We report here that SAA inhibited hepatitis C virus (HCV) infection in cultured cells. SAA reduced HCV infectivity in a dose-dependent manner when added during HCV infection but not after virus entry. SAA bound HCV virions and specifically blocked HCV entry but did not affect virus attachment. These findings suggest that SAA functions as part of the host innate immune defense mechanisms against HCV infection in humans.

Serum amyloid A (SAA) is an acute-phase response protein whose plasma level is remarkably elevated in response to a variety of inflammatory stimuli, including infections, tissue damage, and cancer (18, 27). SAA is primarily produced by hepatocytes and is largely associated with high-density lipoprotein (HDL) upon secretion into the plasma. SAA plays an important role in HDL metabolism and cholesterol homeostasis (15). SAA is a ligand for the human scavenger receptor class B type I (SR-BI), an HDL receptor mediating the selective uptake of cholesterol ester, and thereby inhibits the interaction of HDL with SR-BI and decreases the uptake of cholesterol ester (2, 7). SAA also promotes cellular cholesterol efflux (14, 25, 28).

However, the physiological functions of SAA in host innate immunity are not well understood. The acute-phase response proteins, including mannose binding lectin, C-reactive protein (CRP), serum amyloid-P (SAP), and the long pentraxin 3 (PTX3) function as pattern recognition receptors to trigger the host antimicrobial defense mechanisms upon infection (10, 16). Both CRP and SAP were shown to bind different pathogens, including bacteria and viruses, and to activate the host complement system (16, 21, 26). A recent study demonstrated that the PTX3 is able to bind both human and murine cytomegalovirus in vitro and protects mice from primary murine cytomegalovirus infection and reactivation through the activation of the interferon regulatory factor 3 (6). Recently, SAA was shown to be an opsonin for gram-negative bacteria (11, 24). SAA is also a natural ligand for SR-BI (7), which has been reported to be a putative receptor or coreceptor for hepatitis C virus (HCV) infection (3, 4, 9, 13, 23). Thus, we believe that SAA likely inhibits HCV infection. In this study, we sought to determine the effect of human SAA protein on HCV infectivity in a human hepatoma cell line, Huh7.5 (5).

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▽ Published ahead of print on 28 February 2007.

SAA inhibits HCV infection. Several groups have recently reported robust cell culture systems for HCV propagation and infection (8, 12, 17, 29, 31). We have constructed stable human hepatoma cell lines that contain a chromosomally integrated JFH1 HCV cDNA and continuously produce infectious HCV (8). This provides a unique opportunity for us to determine viral and cellular factors that affect the HCV life cycle. To determine the effect of SAA on HCV infection, a recombinant human SAA protein (Biovision and Biodesign International) was mixed with HCV and then used to infect Huh7.5 cells at a multiplicity of infection (MOI) of about 0.1 to 0.5. After a 2-hour incubation, the mixture of SAA and HCV was removed and the HCV-infected cells were washed twice with phosphate-buffered saline (PBS). At 3 days postinfection (p.i.), the levels of HCV protein expression and RNA replication were determined by immunofluorescence assay (IFA), Western blotting, and RNase protection assay (RPA), respectively, as previously described (8). Strikingly, the HCV infectivity was remarkably suppressed by SAA in a dose-dependent manner, as determined initially by immunofluorescence staining (IFA) for HCV NS3 protein using an NS3-specific monoclonal antibody (8). The number of HCV-infected Huh7.5 cells was proportionally reduced by increasing amounts of SAA protein (Fig. 1). The HCV infectivity was completely suppressed by SAA at concentrations of 50 to 100 μg/ml. In sharp contrast, HCV infectivity was unaffected by two other acute-phase proteins, apolipoprotein A-I (ApoA-I) and CRP, at concentrations up to 100 μg/ml (Fig. 1A and B and data not shown). To further determine the efficacy of SAA for inhibiting HCV infectivity, the levels of HCV NS3 protein and positive-strand RNA were determined (Fig. 1B and C). Consistent with the IFA results, the levels of both HCV NS3 protein (Fig. 1B) and positive-strand RNA (Fig. 1C) were proportionally decreased by increasing amounts of SAA. The reduction of HCV protein expression and RNA replication correlated closely with increasing concentrations of SAA (Fig. 1D). The 50% effective concentration of SAA for inhibition of HCV infectivity was approximately 10 μg/ml when SAA from Biovision was used (Fig. 1D). It should be noted that the inhibitory activity of SAA

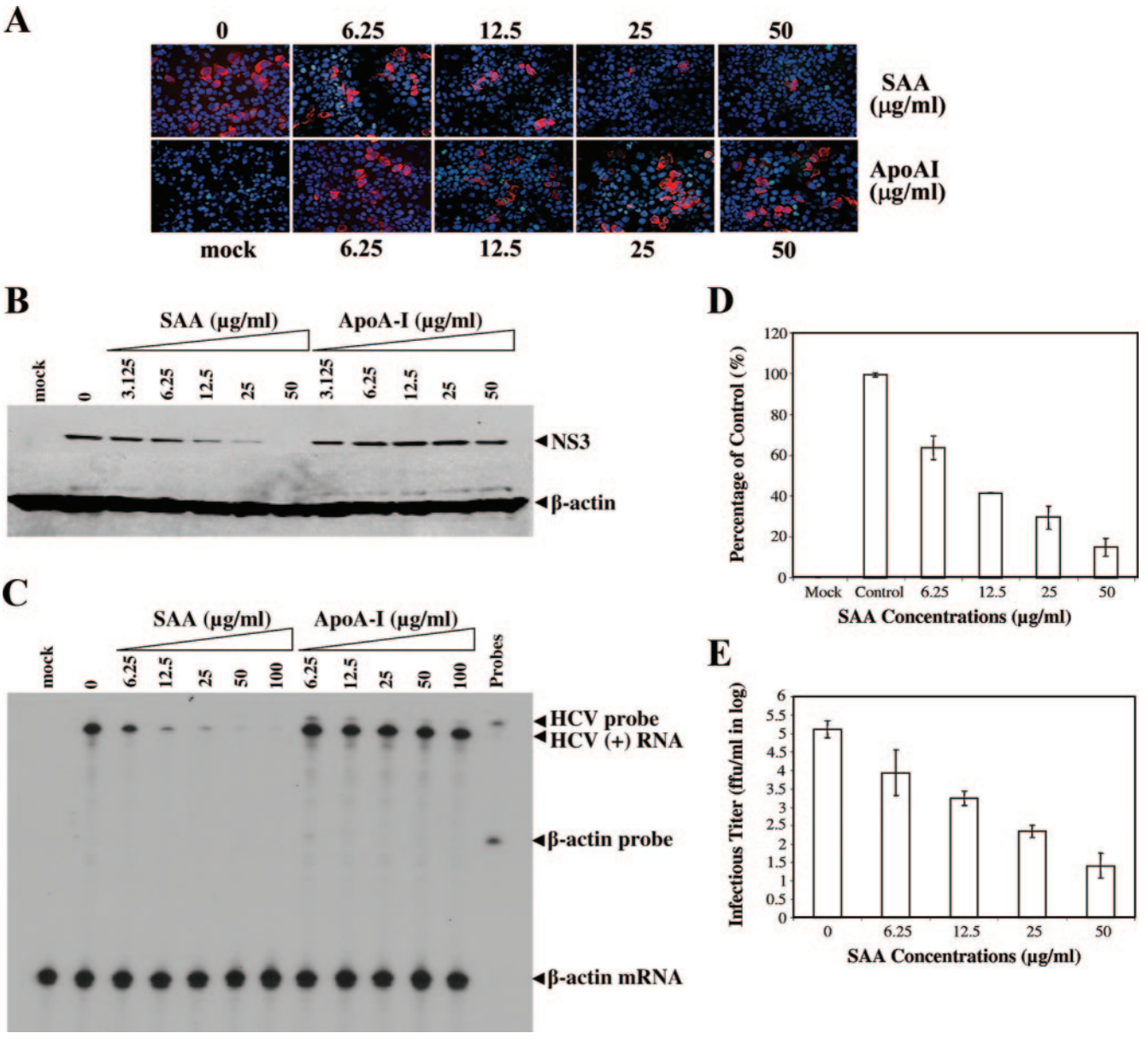

FIG. 1. Inhibition of HCV infection by SAA. (A) IFA for inhibition of HCV infection by SAA. A recombinant human SAA was purchased from Biodesign International (Saco, ME). The HCV-containing medium was used to dilute SAA to various concentrations, as indicated, and to infect Huh7.5 cells at an MOI of 0.1. At 2 h p.i., the cells were washed twice with PBS and then incubated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen). At 3 days p.i., the Huh7.5 cells were fixed, blocked, and stained for HCV NS3 expression using an NS3-specific monoclonal antibody, as described previously (8). (B) Western blot analysis of HCV NS3 protein in cells treated with SAA or ApoA-I. HCV was incubated with different concentrations (as indicated) of SAA (Biovision, Mountain View, CA) or ApoA-I (Biodesign) at room temperature for 1 hour and was then used to infect Huh7.5 cells (MOI, 0.5). At 2 h p.i., the cells were washed twice with PBS and then incubated with 10% FBS-containing DMEM for 3 days. The levels of HCV NS3 protein were determined by Western blotting, as described previously (8). The β-actin was used as an internal control for protein normalization. (C) Quantification of positive-strand HCV RNA by RPA. Huh7.5 cells in six-well culture plates were infected with HCV in the same way as for panel B. At 3 days p.i., total RNA was extracted with Trizol reagent (Invitrogen). A [^{32}P]UTP-labeled RNA probe containing negative-strand HCV 3′ untranslated region was used to hybridize with positive-strand HCV RNA. Upon digestion with RNase A/T1, HCV RNA products were analyzed in a 6% polyacrylamide-7.7 M urea gel (8). Positive-strand HCV RNA products protected from RNase digestion migrated faster, as the probe contained a 40-nucleotide mismatched region at the 3′ end. The levels of β-actin mRNA were used as controls. The probes and RNA products are highlighted by arrowheads on the right. (D) Dose-dependent reduction of HCV infection by SAA. The levels of positive-strand HCV RNA in at least three experiments were quantified by phosphorimager (C). The mean values obtained from different experiments were used to calculate the HCV RNA levels relative to the control, which was considered 100% without SAA treatment. The positive-strand HCV RNA levels relative to the control (*y* axis) are plotted against SAA concentrations (*x* axis). The error bars indicate standard deviations. (E) Dose-dependent reduction of infectious HCV titers by SAA. The infectious HCV titers (FFU per milliliter) in the supernatant of the same HCV-infected and SAA-treated Huh7.5 cells as in panel C were determined by serial 10-fold dilutions and IFA staining for NS3-positive foci using an NS3-specific monoclonal antibody, as described in our earlier studies (8). The infectious HCV titers (*y* axis) are plotted against SAA concentrations (*x* axis).

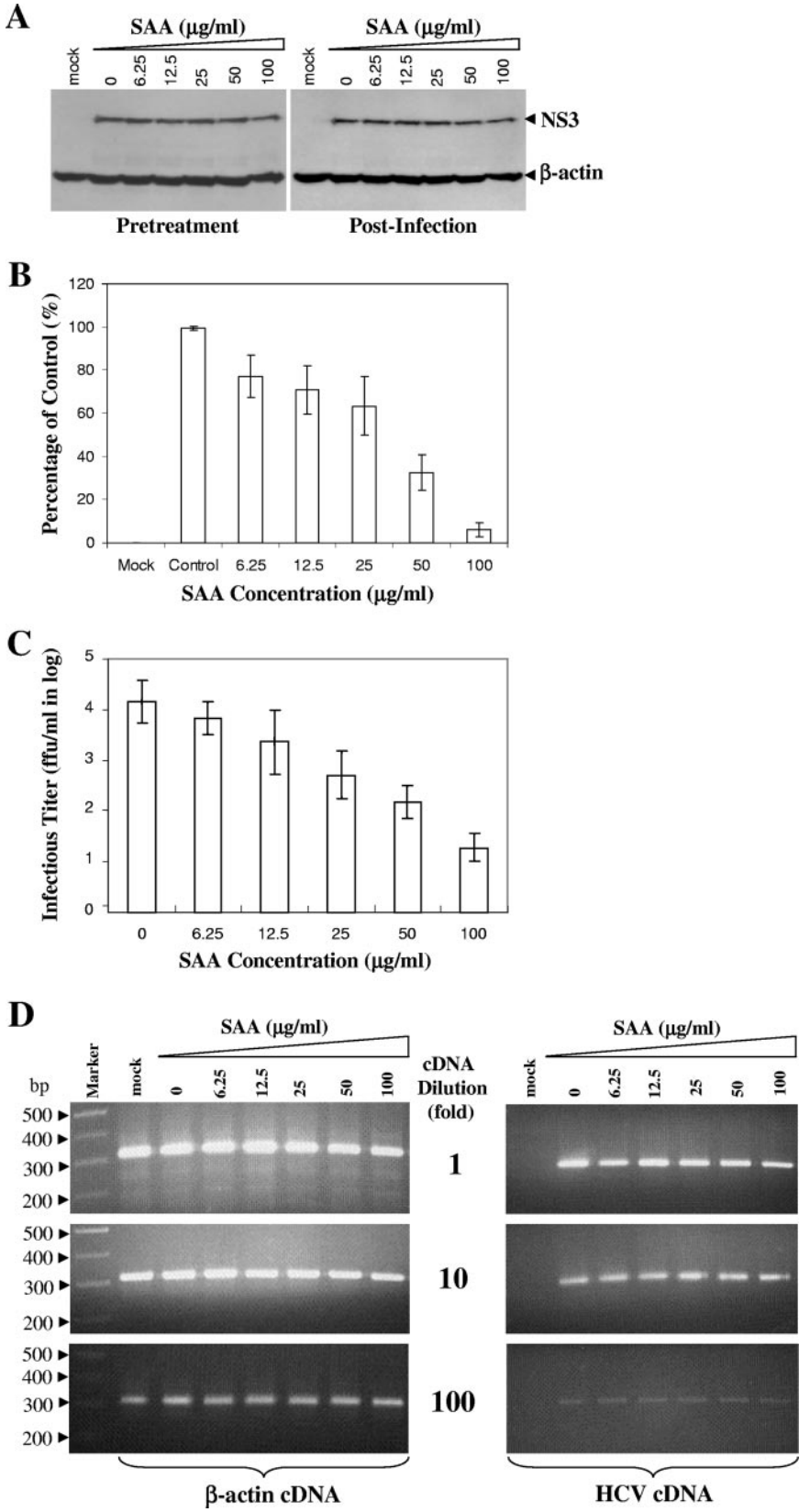

FIG. 2. Mechanism of action of SAA for inhibition of HCV infection. (A) Western blot analysis of HCV NS3 protein in Huh7.5 cells treated with SAA prior to HCV infection or p.i. Huh7.5 cells were pretreated with various concentrations of SAA at 37°C for 1 h. The cells were washed with PBS and then infected with HCV (Pretreatment). Alternatively, Huh7.5 cells were infected with HCV at 37°C for 2 h, washed with PBS, and then incubated with medium containing different amounts of SAA (Post-Infection). At 3 days p.i., the levels of HCV NS3 protein were determined

did vary depending on the source of the SAA, suggesting that the correct folding of SAA is important for its activity (data not shown). In contrast, ApoA-I did not affect HCV infectivity (Fig. 1B and C). We also determined the infectious HCV titer (focus-forming units [FFU] per milliliter) in the supernatant of the SAA-treated cells using a serial dilution and an IFA staining method, as described previously (8, 17). Similar to the reduction of HCV protein and RNA in the cell, the infectious HCV titers were progressively lowered by nearly 10,000-fold at 50 μg/ml of SAA (Fig. 1E). Taken together, SAA is a potent inhibitor of HCV infection in vitro.

SAA blocks HCV entry but does not affect virus attachment. SAA was previously shown to bind SR-BI (7), a putative receptor/coreceptor for HCV infection (3, 4, 13, 23). Thus, one possible explanation for SAA's inhibitory effect on HCV infectivity could be to its ability to block the SR-BI receptor/coreceptor. To address this question, we performed time-of-addition experiments (Fig. 2A). First, the Huh7.5 cells were treated with different amounts of SAA for 1 hour at 37°C prior to HCV infection. The SAA-treated Huh7.5 cells were then washed twice with PBS and subsequently infected with HCV at an MOI of about 0.1. The results show that the pretreatment of Huh7.5 with SAA did not affect HCV infectivity (Fig. 2A). The question arose as to whether SAA was rapidly internalized at 37°C and thereby failed to block HCV infection. We tested this possibility by incubating Huh7.5 cells with fluorescently labeled SAA either at 37°C or on ice (4°C). The internalization of SAA was determined by visualization under a fluorescence microscope. SAA was indeed internalized quickly into Huh7.5 cells at 37°C but failed to be at 4°C (data not shown). Therefore, we treated Huh7.5 cells with different amounts of SAA at 4°C for 1 hour prior to HCV infection. Again, HCV infectivity was unaffected by the pretreatment of Huh7.5 cells with SAA up to 100 μg/ml at 4°C (data not shown). Next, we examined whether HCV replication was inhibited by SAA at a stage after HCV entry. Huh7.5 cells were infected with HCV at 37°C for 2 hours, washed with PBS, and then incubated with various amounts of SAA at 37°C for 3 days. The findings from this experiment demonstrate that SAA had no effect on HCV replication when added after infection (Fig. 2A). Together, these findings demonstrate that SAA inhibited HCV infectivity only when it was present during HCV infection, but not after virus entry.

To further determine the specific step at which HCV infection was blocked by SAA, SAA was added after the binding of HCV to Huh7.5 cells. Huh7.5 cells were first incubated with HCV at 4°C for 2 hours. The unbound HCV was removed by washing the Huh7.5 cells twice with PBS, and SAA was then added to the HCV-bound Huh7.5 cells for an additional 1-hour incubation at 4°C prior to incubation at 37°C for 3 days. Similar to the inhibitory activity of SAA added during infection, SAA markedly reduced HCV infectivity when added after HCV binding to Huh7.5 cells at 4°C (Fig. 2B). Likewise, the infectious HCV titers in the supernatant of the HCV-infected and SAA-treated Huh7.5 cells were proportionally reduced by increasing amounts of SAA (Fig. 2C). To exclude possible effects of SAA on the binding of HCV to Huh7.5 cells, SAA was mixed with the HCV-containing medium prior to infection. The SAA-HCV mixture was incubated with Huh7.5 cells at 4°C for 2 hours. The unbound HCV virions were removed by washing them with PBS. The HCV virion RNA (vRNA), along with total cellular RNA, was then extracted with Trizol reagent (Invitrogen). The binding of HCV to Huh7.5 cells was determined by reverse-transcription (RT)-PCR quantification of the HCV vRNA. The results show that SAA did not significantly alter the binding of HCV to Huh7.5 cells, as the levels of HCV vRNA were unaffected by increasing concentrations of SAA (Fig. 2D). Collectively, these findings indicate that SAA specifically blocks HCV entry but does not affect HCV attachment.

SAA binds HCV virions. To examine whether SAA binds HCV virions, the HCV-containing medium was incubated with various amounts of SAA. The medium derived from normal Huh7.5 cells was used as a control. Following incubation, the media were subjected to ultracentrifugation (Fig. 3A). The HCV pellet was resuspended in the medium derived from normal Huh7.5 cells, while the control pellet was dissolved in an equal volume of the HCV-containing medium used for incubation with SAA. The resulting mixtures were first used for the determination of SAA by an enzyme-linked immunosorbent assay (ELISA) using an SAA-specific polyclonal antibody (Fig. 3B). SAA was detected only in the HCV pellet, not in the control pellet, indicating that SAA bound HCV (Fig. 3B). The levels of SAA are proportional to increasing amounts of SAA added prior to ultracentrifugation (Fig. 3B). Next, we determined the inhibitory effect of the HCV-associated SAA on HCV infectivity in cultured Huh7.5 cells. The resulting HCV mixtures were used to infect Huh7.5 cells at 37°C for 2 hours,

by Western blotting as described in the legend to Fig. 1B. (B) Blockage of HCV entry by SAA. Huh7.5 cells were first incubated with HCV at 4°C for 2 h. After HCV binding to Huh7.5 cells, the unbound HCV was removed by washing it twice with PBS. The HCV-bound Huh7.5 cells were then incubated with different concentrations of SAA at 4°C for 1 h prior to transfer to 37°C. At 3 days p.i., total RNA was extracted with Trizol reagent, and the levels of positive-strand HCV RNA were determined by RPA in the same way as for Fig. 1C. The relative levels of positive-strand HCV RNA to no-SAA-treatment control were quantified and calculated as percentages of the control, as described in the legend to Fig. 1D. The levels of HCV RNA relative to the control (*y* axis) are plotted against SAA concentrations (*x* axis). The error bars indicate standard deviations. (C) Reduction of infectious HCV titers by SAA. The infectious HCV titers in the supernatant of the Huh7.5 cells, as for panel B, were determined by serial dilutions and IFA staining, as described in the legend to Fig. 1E. The infectious HCV titers (FFU/ml in mean values ± standard deviations) are plotted against SAA concentrations. (D) Effect of SAA on HCV attachment. Huh7.5 cells were incubated with HCV (MOI, 0.5) and various concentrations of SAA at 4°C for 2 h. The unbound HCV was removed by washing the cells twice with PBS. Total cellular RNA was extracted with Trizol reagent. The HCV vRNA was quantified by RT-PCR using 2a/Heli/NcoI (5′-CATGCCATGGTTACAAGGTCTCCCACT TTC-3′) and 2a-4361R (5′-TGCTTGATCAAGGACCGTTC-3′) as primers. Human β-actin mRNA was used as a control and amplified using β-actin-F (5′-AGCGGGAAATCGTGCGTG-3′) and β-actin-R (5′-CAGGGTACATGGTGGTGCC-3′) as primers. The levels of HCV vRNA and β-actin mRNA were semiquantified using serial 10-fold dilutions of reverse-transcribed cDNAs as templates. RT-PCR products (409 bp for HCV cDNA and 305 bp for β-actin cDNA) were analyzed in a 2% agarose gel. The sizes of DNA markers are indicated on the left.

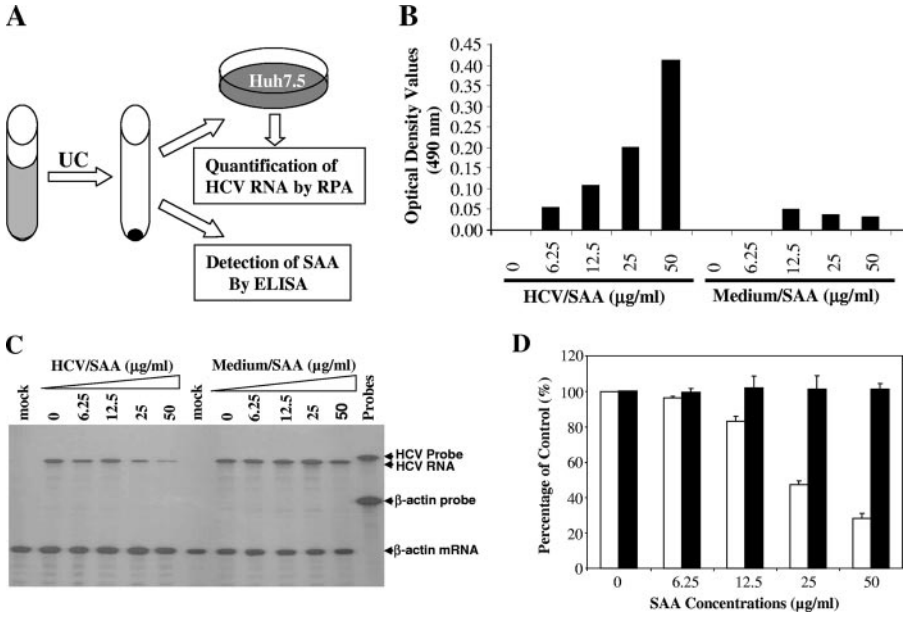

FIG. 3. Association of SAA with HCV virions. (A) Different amounts of SAA were incubated with either HCV-containing medium or normal medium from Huh7.5 cells. The mixtures were subjected to ultracentrifugation (UC) at 150,000 × *g* and 4°C for 12 h. The HCV pellet was resuspended in normal Dulbecco's modified Eagle's medium, while the control medium pellet was dissolved in an equal volume of the HCV-containing medium used for incubation with SAA. The resulting mixtures were used for detection of SAA by ELISA (B) or the determination of the effects of SAA on HCV infectivity (C). (B) Detection of SAA by ELISA. The resulting media were used to coat an ELISA plate at 4°C overnight. A goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase was used as the secondary antibody. The absorbance was determined at 490-nm wavelength. The optical density (*y* axis) was plotted against the amounts of SAA (*x* axis). (C) Inhibition of HCV infectivity by virion-bound SAA. The resulting media were used to infect Huh7.5 cells in six-well culture plates. At 3 days p.i., total cellular RNAs were extracted with Trizol reagent. The levels of positive-strand HCV RNA were determined by RPA in the same way as for Fig. 1C. (D) Correlation of HCV inhibition with HCV-bound SAA. The levels of positive-strand HCV RNA in panel C were quantified by phosphorimager and converted to percentages of the control. The level of HCV RNA in the absence of SAA was considered 100%. The levels (percentages) of HCV RNA relative to that without SAA treatment (100%) (mean values and standard deviations derived from different experiments) were plotted against the concentrations of SAA.

and the levels of positive-strand HCV RNA were determined by RPA at 3 days p.i. As shown in Fig. 3C, SAA in the HCV pellet inhibited HCV infectivity, similarly to SAA added during HCV infection (Fig. 1). The reduction of positive-strand HCV RNA levels was in proportion to increasing amounts of SAA present in the incubation media (Fig. 3D). In contrast, the control pellet did not affect the HCV infectivity (Fig. 3C and D). These results clearly indicate that SAA bound HCV virions and thus inhibited HCV infection.

In summary, several lines of evidence described above demonstrate that SAA is inhibitory to HCV infection by specifically blocking HCV entry, suggesting that SAA may play an important role in the host innate defense against HCV infection. Normally, the plasma level of SAA is around 1 μg/ml, but it can be induced up to 1,000-fold during acute-phase response to infections, suggesting a protective role of SAA against acute infections (15). In this study, the underlying molecular mechanism of HCV entry was not defined. In general, HCV infection is mediated by the binding of HCV virions to cell surface receptors/coreceptors and the subsequent fusion between the viral and cellular membranes (9). The HCV E2 glycoprotein was reported to interact with a number of cell surface molecules, including human CD81 (17, 22, 30), SR-BI (4, 13, 23), and the low-density lipoprotein receptor (1). SAA is known to bind SR-BI (7), and thus, the binding of SAA to SR-RI might interfere with HCV entry. Alternatively, SAA may replace apolipoprotein E, which is associated with HCV virions (20) and is required for HCV infection (K. S. Chang, J. Y. Jiang, Z. H. Cai, and G. Luo, unpublished data). A recent study demonstrated that SAA is able to dissociate apolipoprotein E from HDL (19). The exact mechanism of action of SAA for blocking HCV entry is not clear and warrants further investigation. It will also be interesting to determine whether the level of SAA response during acute HCV infection correlates with HCV clearance in patients.

Zhaohui Cai and Lei Cai contributed equally to this work.

We thank Charles M. Rice (Rockefeller University) for providing Huh7.5 cells.

This work was supported by NIH grants AI051592 and CA093712 (G.L.) and HL063763 (D.R.V.D.W.).

ADDENDUM IN PROOF

A similar work was published by M. Lavie et al. (Hepatology **44**:1626–1634, 2006) while this paper was under review.

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