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Original Article

Annexin A1 Negatively Regulates Viral RNA Replication of Hepatitis C Virus

Hiroki Hiramoto[a], Hiromichi Dansako[a], Midori Takeda[a], Shinya Satoh[a], Takaji Wakita[b], Masanori Ikeda[a], and Nobuyuki Kato[a]*

[a]Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700–8558, Japan, and
[b]Department of Virology II, National Institute of Infectious Disease, Shinjuku-ku, Tokyo 162–8640, Japan

Persistent infection with hepatitis C virus (HCV) often causes chronic hepatitis, and then shows a high rate of progression to liver cirrhosis and hepatocellular carcinoma. To clarify the mechanism of the persistent HCV infection is considered to be important for the discovery of new target(s) for the development of anti-HCV strategies. In the present study, we found that the expression level of annexin A1 (ANXA1) in human hepatoma cell line Li23-derived D7 cells was remarkably lower than that in parental Li23 cells, whereas the susceptibility of D7 cells to HCV infection was much higher than that of Li23 cells. Therefore, we hypothesized that ANXA1 negatively regulates persistent HCV infection through the inhibition of viral RNA replication. The results revealed that HCV production was significantly inhibited without a concomitant reduction in the amount of lipid droplets in the D7 cells stably expressing exogenous ANXA1. Further, we demonstrated that ANXA1 negatively regulated the step of viral RNA replication rather than that of viral entry in human hepatocytes. These results suggest that ANXA1 would be a novel target for the development of anti-HCV strategies.

Key words: HCV, annexin A1, Li23 cell line, Li23-derived D7 cells, HCV-JFH-1

Persistent infection with hepatitis C virus (HCV) frequently causes chronic hepatitis, and then shows a high rate of progression to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* family [1]. The HCV genome encodes a single polyprotein precursor of approximately 3,000 amino acids, which is cleaved by host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2–4].

The previous standard therapy for patients with chronic hepatitis C consisted of a combination of pegylated-interferon (PEG-IFN) and ribavirin (RBV), and achieved a sustained virological response (SVR) in about half of patients treated [5]. Recently, several direct-acting antiviral agents (DAAs) have been developed to inhibit the functions of HCV proteins such as NS3–4A (serine protease), NS5A, and NS5B (RNA-dependent RNA polymerase). Among these DAAs, an HCV NS3–4A protease inhibitor, telaprevir, is currently used in combination with PEG-IFN and RBV as

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*Corresponding author. Phone:+81-86-235-7385; Fax:+81-86-235-7392
E-mail:nkato@md.okayama-u.ac.jp (N. Kato)

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the standard therapy for patients possessing HCV of genotype 1b [6]. Although this therapy has improved the SVR by more than 70% [7], it has several problems, such as severe side effects, emergence of resistance viruses, and high treatment cost [8, 9]. Therefore, the identification of novel host factor(s) as a target of the anti-HCV agents is important for the further development of anti-HCV strategies.

We recently demonstrated that human hepatoma HuH-7 cell line-derived RSc cells were much more highly permissive for HCV-JFH-1 (genotype 2a) infection than parental HuH-7 cells [10]. Our previous studies using RSc cells demonstrated that DDX3 [11], ATM [12], and several proteins (TSG101, Alix, Vps4B, *etc.*) in the ESCRT system [13] were required for HCV RNA replication or HCV production. In addition, we recently found that Rab18 was also required for viral assembly of HCV in RSc cells [10].

On the other hand, in 2009, we found that a human hepatoma cell line, Li23, whose expression profile was distinct from that of HuH-7, also showed good susceptibility to HCV-JFH-1 as well as efficient HCV RNA replication [14, 15]. Furthermore, by serial subcloning of Li23 cells, we recently isolated D7 cells, which were highly permissive for HCV-JFH-1 infection [16]. These results suggest that some common host factor(s) are required for susceptibility to HCV as positive or negative regulator(s) in both RSc and D7 cells.

We recently identified annexin A1 (ANXA1) as one of the genes showing irreversible downregulated expression during the long-term (more than 2 years) replication of HCV RNA [17]. ANXA1, which belongs to the annexin family, was identified as a calcium ion (Ca^{2+})-dependent phospholipid-binding protein on the plasma membrane [18]. ANXA1 exerts anti-inflammatory activity through the inhibition of phospholipase A2 (PLA2), which is involved in the biosynthesis of inflammatory mediators [19]. ANXA1 is also a secreted protein, and blocks the inflammatory response by inhibiting the transmigration of leukocytes [20]. On the other hand, ANXA1 has also been shown to have the biological function of inhibiting infection by cytomegalovirus [21].

Based on the above results, we here decided to focus on clarifying the relation between the expression level of ANXA1 and the susceptibility to HCV. Here, we show that ANXA1 is required for regulation of the susceptibility to HCV as a negative regulator in human hepatocytes.

Materials and Methods

Cell culture. The human hepatoma Li23 cell line, which was established and characterized in 2009, consists of human hepatoma cells from a Japanese male (age 56) [14]. The Li23 cells and Li23-derived sOLc, ORL8c, and D7 cells were cultured in modified medium for human immortalized hepatocytes, as described previously [14, 16]. Human hepatoma Huh7.5 cells were provided by Apath LLC (Brooklyn, NY, USA). BOSC23 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum as previously described [22].

Ectopic expression of ANXA1 in D7 cells. ANXA1 cDNA containing a full-length open reading frame was introduced into D7 cells by pCX4bsr retroviral transfer, and subsequently D7 cells stably expressing exogenous ANXA1 (designated D7/ANXA1 cells) were selected by blasticidin as previously reported [23]. We also introduced the pCX4bsr vector into D7 cells, and then blasticidin-resistant D7 cells (designated D7/Control cells) were used as the control cells.

HCV infection. The cells (0.8×10^6 cells/well at 6-well plate) were cultured for 24 h before the infection with HCV-JFH-1 (genotype 2a) at a multiplicity of infection (MOI) of 1. Total cellular RNA was extracted from HCV-JFH-1-infected cells at each time point (6, 24, or 72 h) after the infection, as noted in the figure legends. Cell lysates were prepared from HCV-JFH-1-infected cells at 72 h after the infection for the Western blot analysis. HCV-JFH-1-containing culture media were also collected at 24 h after the infection, and then were passed through a 0.22-μm filter to exclude the cell debris. The obtained supernatant was used as the inoculum to monitor extracellular HCV infectivity. Quantitative RT-PCR was used to measure the levels of viral RNA replication in Huh7.5 cells at 72 h after the inoculation.

IFN-α treatment. IFN-α treatment was performed as described previously [24]. Briefly, D7/Control cells were infected with HCV-JFH-1 at an MOI of 1, and then the culture media were replaced

with fresh media containing IFN-α (0, 1.25, 2.5, or 5 IU/ml) at 6 h after the infection. At 24 h after the substitution of fresh media, the total cellular RNA was extracted from IFN-α-treated cells.

Quantitative RT-PCR analysis. To determine the RNA levels of ANXA1 and HCV, total cellular RNAs were prepared using the ISOGEN RNA extraction reagent (Nippon Gene, Toyama, Japan). A SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan) and LightCycler real-time PCR system (Roche Diagnostics, Basel, Switzerland) were used to perform quantitative RT-PCR. The primer sets for ANXA1 [17], HCV [25], and GAPDH [26] were used for PCR. The levels of ANXA1 mRNA and HCV RNA were normalized by the level of GAPDH mRNA. In vitro-transcribed HCV-JFH-1 RNA was used as the standard to calculate the amount of HCV RNA in HCV-JFH-1-infected cells. Data are shown as the means±SD from at least three independent experiments.

Flow cytometric analysis. D7/ANXA1 cells were treated with BODIPY 493/503 (Invitrogen) to label intracellular lipid droplets (LDs). The mean fluorescence intensity of BODIPY-labeled cells was measured by using a FACScan system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Western blot analysis. Preparation of cell lysates, SDS-polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described [27]. Anti-ANXA1 (Invitrogen), anti-Core (CP11; Institute of Immunology, Tokyo, Japan), and anti-β-actin (AC-15; Sigma-Aldrich, St. Louis, MO, USA) antibodies were used as primary antibodies. HRP-conjugated anti-mouse-IgG and anti-rabbit-IgG were used as secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). Immunocomplexes were detected by using a Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences, Wellesley, MA, USA) as previously described [28].

Statistic analysis. Determination of the significance of differences among groups was assessed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

Results

ANXA1 expression was extremely low in D7 cells, which are highly permissive for HCV-JFH-1 infection. We previously demonstrated that ORL8c cells were more permissive for HCV-JFH-1 infection than the parental Li23 cells (Fig. 1A and [14]). During the process of searching for a host factor that might alter this viral susceptibility, we carried out a cDNA microarray analysis using Li23 and ORL8c cells. Consequently, we noticed that the expression level of ANXA1 in ORL8c cells was remarkably lower than that in Li23 cells (data not shown). In a separate study performed around the same time, we have independently isolated D7 cells, which were highly permissive for HCV-JFH-1 infection, by the serial subcloning of ORL8c cells (Fig. 1A) [16]. To check whether the expression level of ANXA1 is correlated with viral susceptibility, we examined the levels of intracellular HCV RNA after HCV-JFH-1 infection and the expression levels of ANXA1 using Li23 cells and Li23-derived sOLc, ORL8c, and D7 cells (Fig. 1A). Susceptibility to HCV was evaluated with a quantity of the intracellular HCV RNA at 72 h after HCV-JFH-1 infection. The results revealed that the HCV susceptibility of these cells was highest in D7 cells, followed in order by ORL8c, sOLc, and Li23 cells (Fig. 1B). However, this order was reversed for the mRNA levels of ANXA1, which were highest in Li23, followed by sOLc, ORL8c, and D7 cells (Fig. 1C). We confirmed that the protein levels of ANXA1 were lower in sOLc, ORL8c, and D7 cells compared with Li23 cells (Fig. 1D). From these results, we hypothesized that ANXA1 negatively regulates the susceptibility to HCV, although it remains uncertain which step in the HCV life cycle ANXA1 regulates.

Exogenous expression of ANXA1 in D7 cells inhibited HCV production. To evaluate the above-described hypothesis, we first prepared D7 cells stably expressing exogenous ANXA1 (D7/ANXA1 cells). We confirmed that D7/ANXA1 cells exhibited sufficiently high-level expression of ANXA1 at both the mRNA (Fig. 2A) and protein (Fig. 2B) levels in comparison with the control D7/Control cells. We first examined the effect of ANXA1 expression on the cell growth rate of D7/ANXA1 cells, but found that ANXA1 had no effect on the growth rate (Fig. 2C). We next examined the quantity of LDs, which are important organelles for the production of HCV-JFH-1 [29], and which are induced by various cellular stresses, such as inflammation [30]. The results

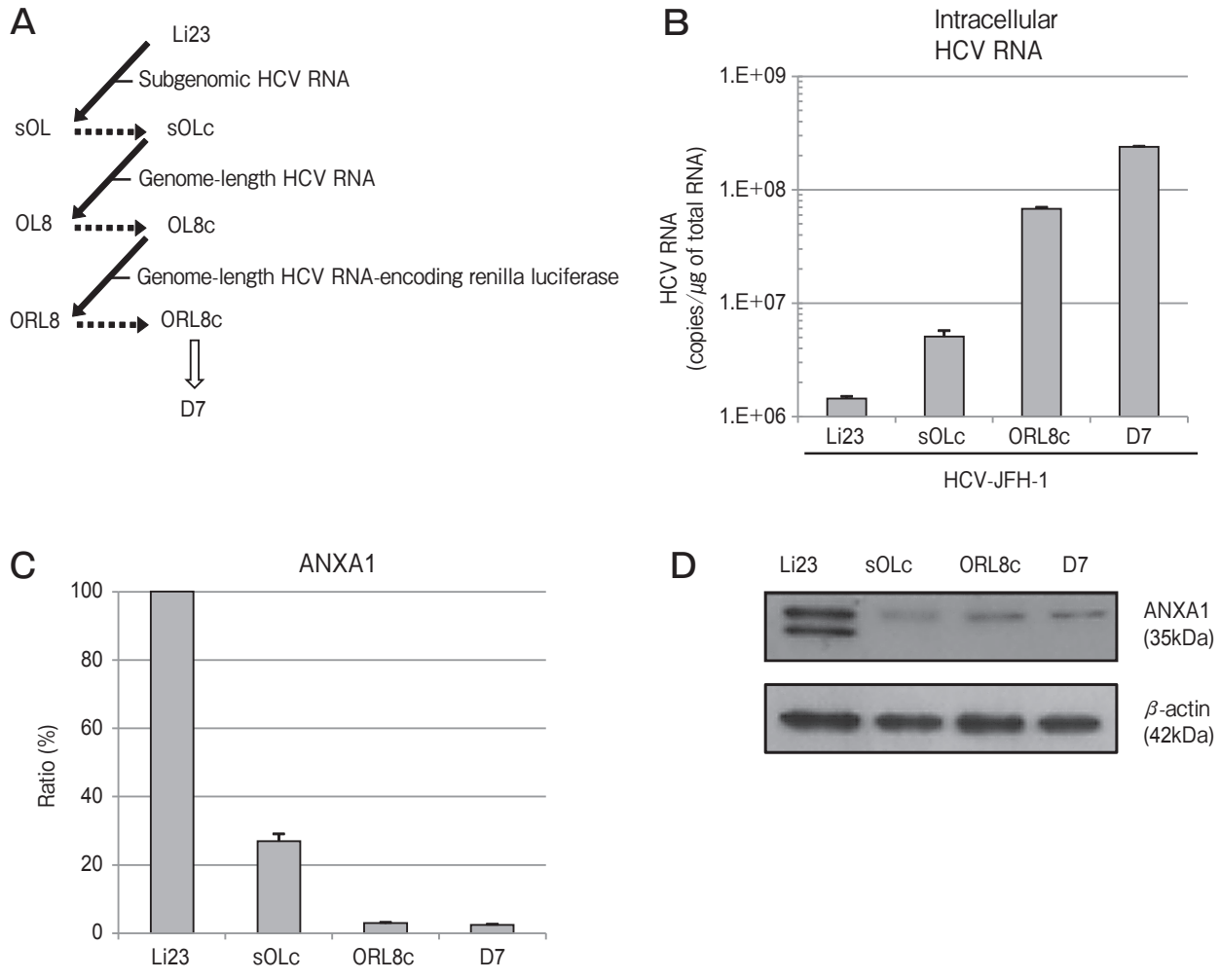

Fig. 1 Extremely low expression of ANXA1 in human hepatoma cell line Li23-derived D7 cells, which are highly permissive for HCV-JFH-1 infection. **A**, Lineage of Li23-derived cell lines. HCV RNA-replicating cell lines (sOL, OL8, and ORL8) are sequentially established by the transfection of subgenomic or genome-length HCV RNA as shown by the arrows with solid lines [14]. sOLc, OL8c, and ORL8c cells were prepared as cured cells by the elimination of HCV RNA from sOL, OL8, and ORL8 cells, respectively. The arrows with dashed lines show the elimination of HCV RNA by interferon treatment. D7 cells were isolated from ORL8c cells by serial subcloning with limited dilution [16]; **B**, Quantitative RT-PCR analysis of HCV RNA in Li23 cells and Li23-derived cells at 72 h after HCV-JFH-1 infection. The experiments were performed as described in the Materials and Methods; **C**, Quantitative RT-PCR analysis of ANXA1 mRNA in Li23 cells and Li23-derived cells. The levels of ANXA1 mRNA were calculated relative to the level in Li23 cells, which was assigned as 100%; **D**, Western blot analysis of Li23 cells and Li23-derived cells for ANXA1. The lower molecular weight band (corresponding to N-terminal-cleaved ANXA1 [37]) was not observed in Li23-derived cells because its expression was extremely low. β-actin was included as a loading control.

revealed that ANXA1 had no significant effect on the quantity of LDs (Fig. 2D). Interestingly, however, we found that the ANXA1 expression significantly inhibited the infectious viral production from the HCV-JFH-1-infected D7/ANXA1 cells compared with the HCV-JFH-1-infected D7/Control cells (Fig. 2E). These results indicate that ANXA1 negatively regulates the HCV production without affecting the cell growth and the quantity of LDs.

ANXA1 negatively regulates the viral RNA replication step after HCV infection. To clarify which step of the life cycle of HCV is prevented by ANXA1, we first examined whether ANXA1 inhibits the step of viral RNA replication. We compared the

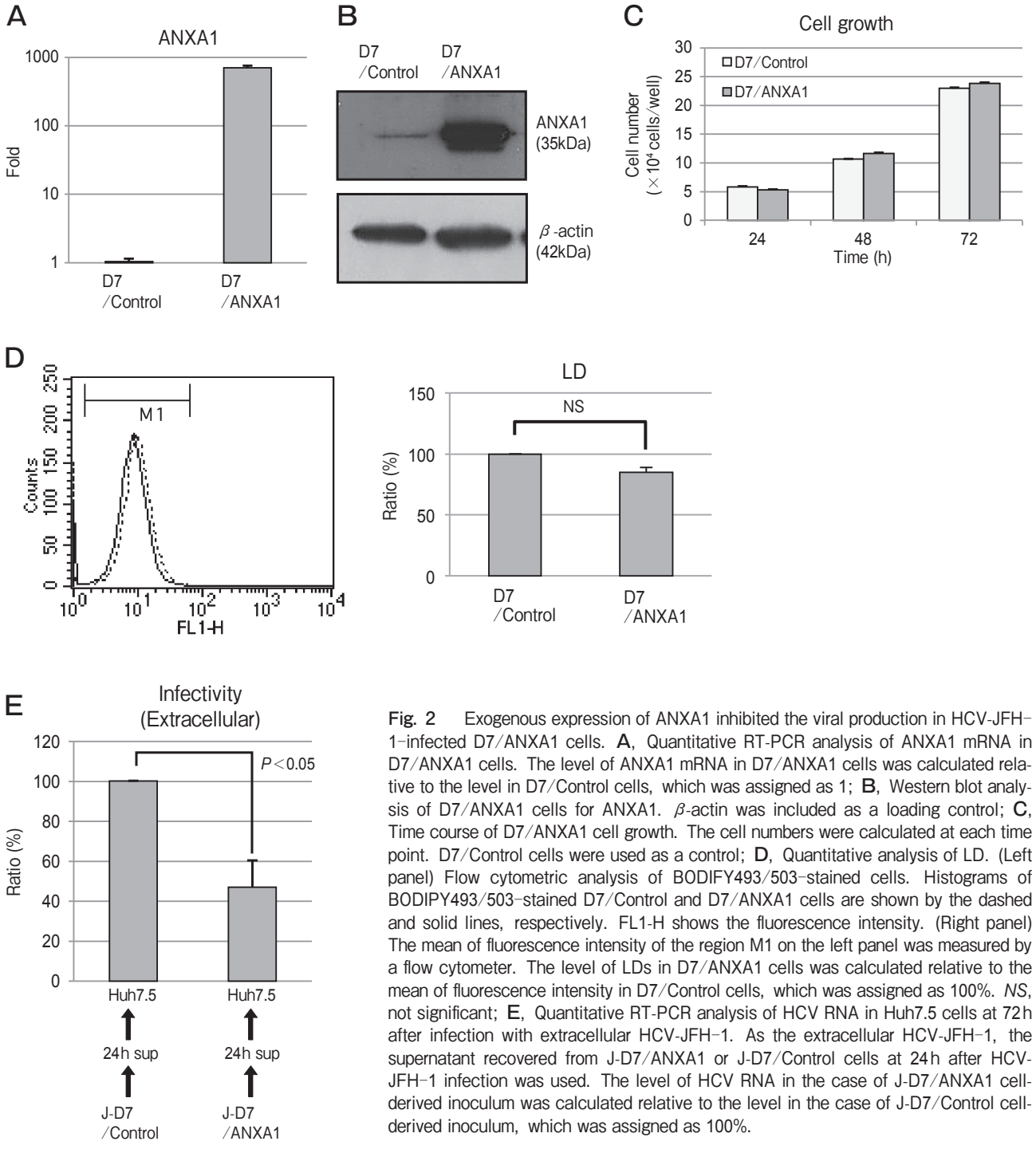

Fig. 2 Exogenous expression of ANXA1 inhibited the viral production in HCV-JFH-1-infected D7/ANXA1 cells. **A**, Quantitative RT-PCR analysis of ANXA1 mRNA in D7/ANXA1 cells. The level of ANXA1 mRNA in D7/ANXA1 cells was calculated relative to the level in D7/Control cells, which was assigned as 1; **B**, Western blot analysis of D7/ANXA1 cells for ANXA1. β-actin was included as a loading control; **C**, Time course of D7/ANXA1 cell growth. The cell numbers were calculated at each time point. D7/Control cells were used as a control; **D**, Quantitative analysis of LD. (Left panel) Flow cytometric analysis of BODIFY493/503-stained cells. Histograms of BODIPY493/503-stained D7/Control and D7/ANXA1 cells are shown by the dashed and solid lines, respectively. FL1-H shows the fluorescence intensity. (Right panel) The mean of fluorescence intensity of the region M1 on the left panel was measured by a flow cytometer. The level of LDs in D7/ANXA1 cells was calculated relative to the mean of fluorescence intensity in D7/Control cells, which was assigned as 100%. *NS*, not significant; **E**, Quantitative RT-PCR analysis of HCV RNA in Huh7.5 cells at 72 h after infection with extracellular HCV-JFH-1. As the extracellular HCV-JFH-1, the supernatant recovered from J-D7/ANXA1 or J-D7/Control cells at 24 h after HCV-JFH-1 infection was used. The level of HCV RNA in the case of J-D7/ANXA1 cell-derived inoculum was calculated relative to the level in the case of J-D7/Control cell-derived inoculum, which was assigned as 100%.

levels of intracellular HCV RNA between D7/ANXA1 and D7/Control cells at 24h after HCV-JFH-1 infection. The results revealed that the level of HCV RNA in the HCV-JFH-1-infected D7/ANXA1 cells was significantly lower than that in the infected D7/Control cells (Fig. 3A). The inhibitory effect of ANXA1 on HCV RNA replication was roughly equivalent to that of 1.25IU/ml of IFN-α (Fig. 3B). In addition, we noticed that the inhibition of HCV RNA replication by ANXA1 was at the same level as that of HCV production (Fig. 2E). Moreover, we confirmed by Western blot analysis that the level of core protein in the D7/ANXA1 cells was also decreased compared with that in the control cells (Fig. 3C). We next examined whether ANXA1 inhibits the step of viral entry. We compared the levels of

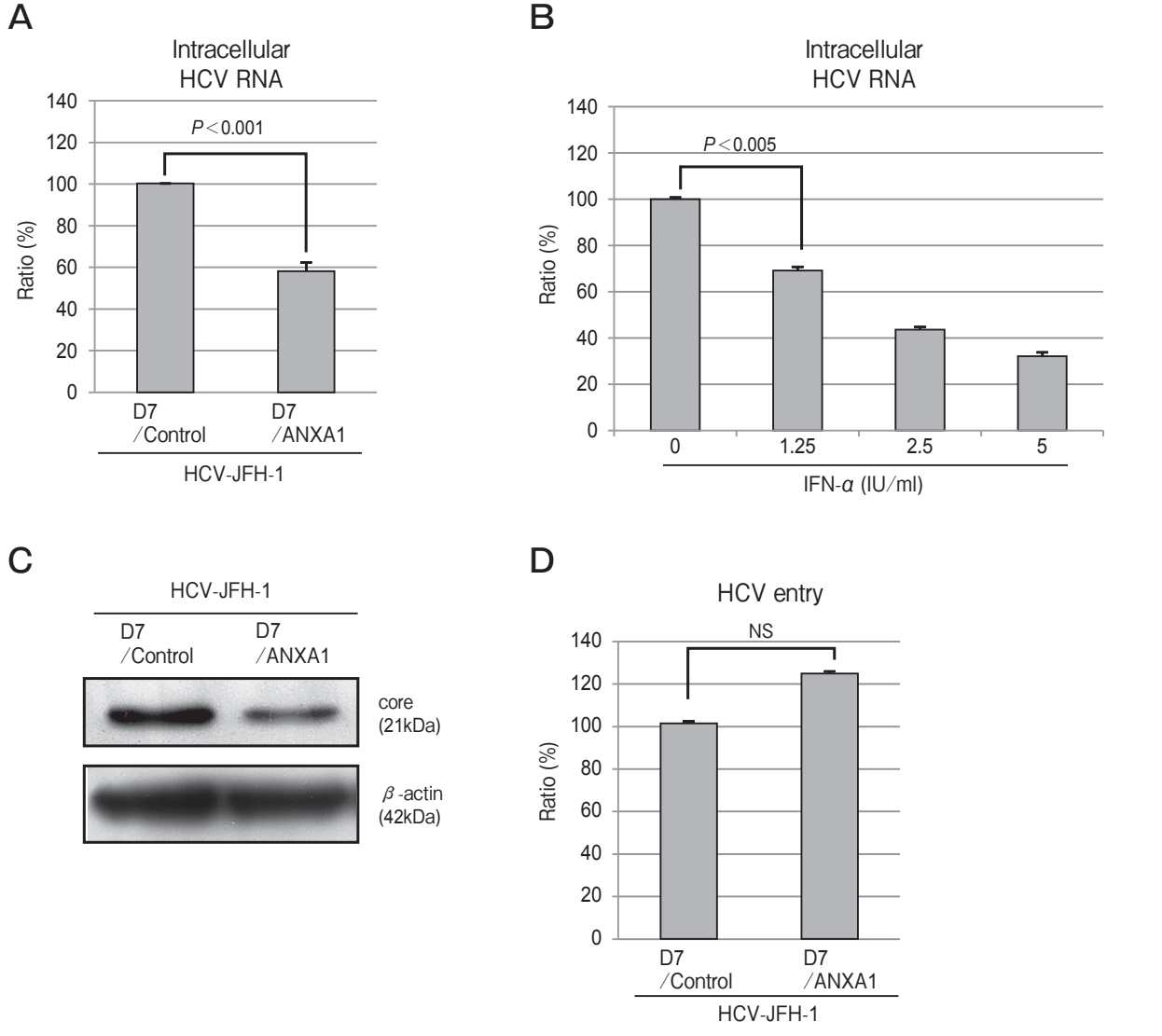

Fig. 3 Exogenous expression of ANXA1 inhibited viral replication after HCV infection. **A**, Quantitative RT-PCR analysis of HCV RNA in D7/ANXA1 cells at 24h after HCV-JFH-1 infection. The experiments were performed as described in the Materials and Methods; **B**, The effect of IFN-α on viral replication in HCV-JFH-1-infected D7/Control cells. HCV-JFH-1-infected D7/Control cells were treated with IFN-α as described in the Materials and Methods; **C**, Western blot analysis of D7/ANXA1 cells for core protein at 72h after HCV-JFH-1 infection. β-actin was included as a loading control; **D**, Quantitative RT-PCR analysis of HCV RNA in D7/ANXA1 cells at 6h after HCV-JFH-1 infection. NS, not significant.

intracellular HCV RNA between D7/ANXA1 and D7/Control cells at 6 h after HCV-JFH-1 infection. The results revealed that ANXA1 did not reduce the level of intracellular HCV RNA (Fig. 3D). These results indicate that ANXA1 negatively regulates the step of viral RNA replication rather than that of viral entry.

Discussion

In the present study, we found that HCV RNA replication was significantly inhibited by ANXA1 expression in Li23-derived D7 cells. Since this inhibitory phenomenon was not accompanied by an ANXA1-induced reduction of LDs, we were not able to clarify the mechanism by which ANXA1 negatively regulates HCV RNA replication. However, as one possibility, it is thought that ANXA1 blocks HCV RNA replication through the inhibition of PLA2 gamma (PLA2G4C), because it has been reported that PLA2 gamma is involved in viral RNA replication and assembly of HCV [31]. However, our cDNA microarray analysis revealed that the level of PLA2G4C expression was very low in Li23 cells or Li23-derived cells as well as HuH-7 cells (data not shown). From this fact, we were able to exclude the possibility that ANXA1 blocks HCV RNA replication through the inhibition of PLA2G4C. However, the members of the PLA2 superfamily are organized into 16 groups based on their amino acid sequences and 6 types based on their characteristics [32]. Therefore, we are currently examining whether ANXA1 inhibits other groups of PLA2.

On the other hand, ANXA1 was recently reported to be required for the production of interferon (IFN)-β through the association with TBK-1 [33]. We also recently reported that HCV-JFH-1 infection induced the production of IFN-β through the TLR3/TRIF/TBK-1 signaling pathway [25]. On the other hand, it was previously reported that HCV NS3 inhibited the production of IFN-β through the association with TBK-1 [34]. Therefore, further analysis will be needed to evaluate the hypothesis that ANXA1 inhibits HCV RNA replication through the production of IFN-β.

RSc and D7 cells were much more highly permissive for HCV infection than their parental HuH-7 and Li23 cells, respectively [14, 16]. Interestingly, the expression levels of ANXA1 in both RSc and D7 cells were extremely low compared with those of HuH-7 and Li23 cells (Fig. 1C and data not shown for RSc and HuH-7 cells), although the gene expression profile of Li23 cells or Li23-derived cells was distinct from that of HuH-7 cells or HuH-7-derived cells [14, 15, 35]. ANXA1 may be an important host factor for the blocking of HCV proliferation in human hepatocytes.

On the other hand, glucocorticoids were previously reported to increase the expression and function of ANXA1 [36]. The upregulation of ANXA1 by glucocorticoids may achieve both anti-HCV RNA replication and anti-inflammatory effects in the liver. Furthermore, since glucocorticoids are widely used as immunosuppressive agents, they may be useful to block the cellular immunity after liver transplantation. Therefore, we suggest that ANXA1 is a novel target for the development of anti-HCV strategies.

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