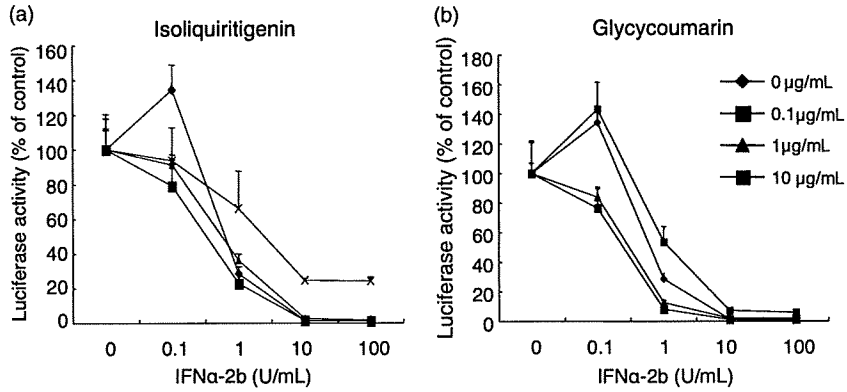

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

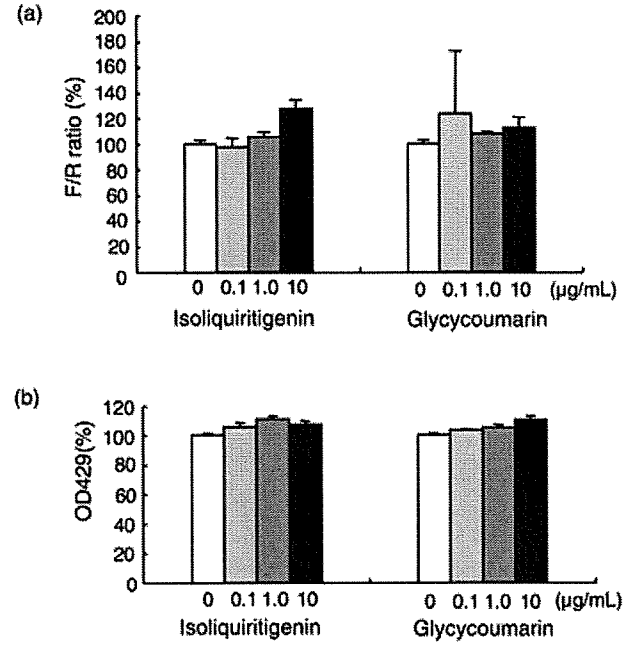

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.

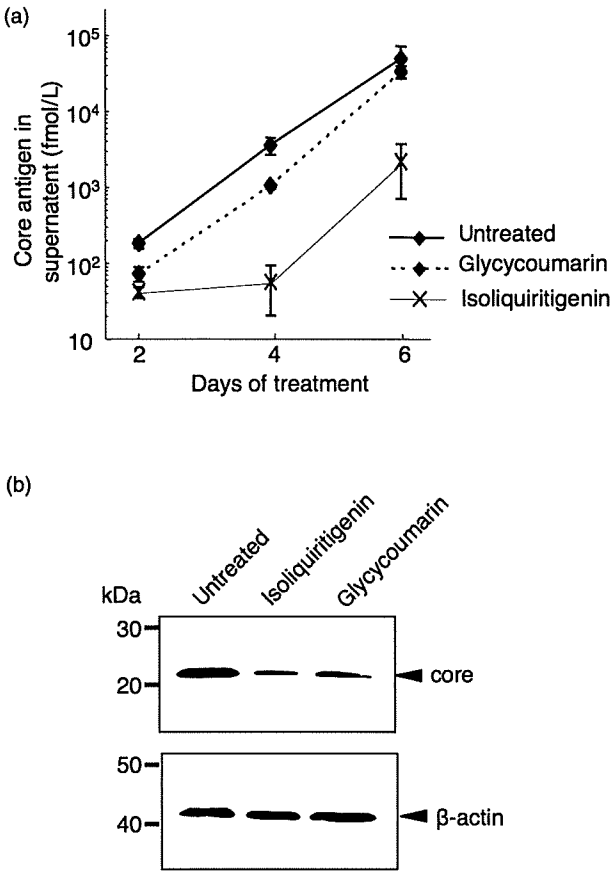

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin.* (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes, Haemophilus infuenzae* and *Moraxella catarrhalis,*[36] and methicillin-resistant *Staphylococcus aureus,*[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix.* However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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Original Article

Case–control study for the identification of virological factors associated with fulminant hepatitis B

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Background: Host and viral factors can promote the development of fulminant hepatitis B (FHB), but there have been no case–control studies for figuring out virological parameters that can distinguish FHB.

Methods: In a case–control study, virological factors associated with the development of FHB were sought in 50 patients with FH developed by transient hepatitis B virus (HBV) infection (FH-T) and 50 with acute self-limited hepatitis B (AHB) who were matched for sex and age. In addition, 12 patients with FH developed by acute exacerbation (AE) of asymptomatic HBV carrier (ASC) (FH-C) were also compared with 12 patients without FH by AE of chronic hepatitis B (AE-C).

Results: Higher HBV DNA levels, subgenotype B1/Bj, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent ($P < 0.05$), while hepatitis B e-antigen was less frequent in the FH-T patients than AHB. In multivariate analysis, G1896A mutation (odds ratio [OR], 13.53; 95% confidence interval [CI], 2.75–66.64), serum HBV DNA more than 5.23 log copies/mL (OR, 5.14; 95% CI, 1.10–24.15) and total bilirubin more than 10.35 mg/mL (OR, 7.81; 95% CI, 1.77–34.51) were independently associated with a fulminant outcome by transient HBV infection. On the other hand, in comparison with the patients between FH-C and AE-C groups, there was no significant difference of virological factors associated with the development of FHB.

Conclusion: A number of virological factors have been defined that may distinguish FH-T from AHB in a case–control study. The pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

Key words: acute exacerbation of asymptomatic hepatitis B virus carrier, fulminant hepatitis, genotypes, transient hepatitis B virus infection

INTRODUCTION

IN JAPAN, 634 patients with fulminant hepatitis (FH) were registered from 1998–2003. Of them, 41.8% were infected with hepatitis B virus (HBV) that is the most frequent cause of FH there.[1] HBV is classified into eight genotypes (A–H) based on a sequence divergence of more than 8% in the entire genome of approximately

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3200 nucleotides.[2–5] They have distinct geographical distributions and are associated with the severity of liver disease.[6,7] Furthermore, subgenotypes have been reported for HBV/A, B and C, and they are named A1/Aa (Asian/African type) and A2/Ae (European type),[8] B1/Bj (Japanese type) and B2/Ba (Asian type),[9] and C1/Cs (Southeast Asian type) and C2/Ce (East Asian type).[10,11] HBV genotypes/subgenotypes and mutations in the pre-core region and the core promoter can influence the viral replication and expression of hepatitis B e-antigen (HBeAg).[6,12]

Acute HBV infection in adulthood resolves in the most cases by far, but can induce FH or go on to become chronic in some. It has been reported that host and viral factors may influence the development of fulminant hepatitis B (FHB), but the pathogenesis of FHB remains unclear. As for virological factors associated with FHB, mutations in the core promoter (A1762T/G1764A)[13] and the pre-core region (G1896A)[14–16] have been reported in association with the development of FHB in Asia and the Middle East. Additional mutations, including T1753V, T1754V and A2339G in the core gene are implicated, also.[17,18] In regard of HBV genotypes, subgenotype B1/Bj is highly associated with the development of FHB in Japan.[15] In contrast, an association of HBV genotypes with the fulminant outcome has not been reproduced in patients from the USA and Europe.[19–22] Such a discrepancy would be attributed, at least in part, to distinct geographical distributions of HBV genotypes/subgenotypes over the world.

The original definition by Trey *et al.*[23] about fulminant hepatic failure is widely used all over the world. On the other hand, in Japan, the diagnosis of FH was contingent on a slight modification of Trey's original definition by the Inuyama Symposium (Aichi, Japan in 1981). Furthermore, the Intractable Liver Diseases Study Group of Japan modified the criteria for the etiology of FH and late-onset hepatic failure in 2002. According to the criteria of the Intractable Liver Diseases Study Group of Japan, there are two clinical entities of FHB that are induced, respectively, by transient HBV infection and acute exacerbation (AE) of an asymptomatic HBV carrier (ASC).[1]

Recently, FH developing in ASC who undergo AE is increasing in Japan.[1] In patients with hematological malignancy, in particular, rituximab and/or glucocorticoid, can reactivate HBV for the development of FHB.[24] The outcome is poor for FHB precipitating in ASC who undergo acute exacerbation,[1] but it has been difficult to identify it by clinical examinations.

As there have been no case–control studies for figuring out virological parameters that can distinguish FHB, a case–control study was conducted on the patients with FH by transient HBV infection and acute self-limited hepatitis B (AHB) in this study, for the identification of virological factors that influence a fulminant outcome. In addition, the patients with FH by AE of ASC, which is assumed as a different clinical condition from transient HBV infection, were also compared with the patients without FH by AE of chronic hepatitis B (CHB) in a case–control study.

METHODS

Patients

DURING 9 YEARS from 1998 to 2006, in twenty-six hospitals all over Japan, sera were obtained from the 50 FH patients by transient HBV infection (the FH-T group) and the 50 patients with AHB (the AHB group) who were controlled for age and sex. As the elder patients with FHB were enrolled in this study (mean age, 42.8 years), the mean age of AHB patients became relatively high (42.9 years, Table 1). Furthermore, the 12 FH patients developed by AE of ASC (the FH-C group) were also compared with the 12 patients without FH by AE of CHB who were matched by age and sex (the AE-C group).

All the serum samples tested for this study were collected at hospitalization. All 124 patients had hepatitis B surface antigen (HBsAg) in serum. Infection with hepatitis A virus and hepatitis C virus, as well as alcoholic hepatitis, were excluded in them.

The diagnosis of acute hepatitis B was based on sudden manifestation of clinical symptoms of hepatitis and detection of high-titered immunoglobulin (Ig)M anti-hepatitis B core (HBC). Patients with initial high-titered anti-HBC (>90% inhibition by a 1:200 diluted serum) were excluded. The diagnosis of FH was contingent on a slight modification by Inuyama Symposium (Aichi, Japan in 1981) of the original definition by Trey *et al.*:[23] (i) coma of grade II or higher; and (ii) a prothorombin time less than 40% developing within 8 weeks after the onset of hepatitis. To exclude AE of ASC in FH-T and AHB groups, we confirmed the negativity of HBsAg before onset of FHB or AHB and no family histories of hepatitis were found among all the patients. Furthermore, serum HBsAg in all patients with FH-T or AHB became naturally seronegative within 24 weeks. AE of ASC or CHB was defined as the elevation of alanine aminotransferase (ALT >300 IU/L) or total bilirubin (T.bil >3.0 mg/dL).[25] All 24 patients with AE of ASC or CHB could be confirmed positive for serum HBsAg before the onset of acute liver injury.

Table 1 Baseline characteristics between fulminant hepatitis B patients by transient infection (FH-T) and acute self-limited hepatitis B (AHB) patients

Features	FH-T ($n = 50$)	AHB ($n = 50$)	Differences *P*-value
Age (years)	42.8 ± 16.1	42.9 ± 14.6	Matched
Men	25 (50%)	25 (50%)	Matched
ALT (IU/L)	3788 ± 2856	2170 ± 1350	<0.001
AST (IU/L)	3131 ± 3673	1676 ± 1851	<0.05
Total bilirubin (mg/dL)	14.8 ± 8.6	9.5 ± 9.8	<0.01
Prothrombin time (%)	16.9 ± 11.2	72.8 ± 26.0	<0.001
HBeAg positive	15 (30%)	28 (56%)	<0.01
Core protein (log U/mL)	3.21 ± 1.28	3.01 ± 1.00	NS
HBcrAg (log U/mL)	5.30 ± 1.32	5.95 ± 1.13	<0.01
HBV DNA (log copies/mL)	5.97 ± 1.87	4.98 ± 1.17	<0.005
Deceased	19 (38%)	0 (0%)	<0.001

AHB, acute self-limited hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FH-T, fulminant hepatitis B by transient HBV infection; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Serological markers of HBV infection

Hepatitis B surface antigen, HBeAg and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBC of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan). Core protein constituting the viral nucleocapsid and HBV core-related antigen (HBcrAg), both of which correlate with HBV DNA in serum, were measured by CLEIA as described elsewhere.[26,27]

Quantification of serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol[28] with a slight modification;[8] it has a detection limit of 100 copies/mL.

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100 μL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/pre-core/core regions [nt 1628–2364], as described previously.[29] The target of PCR covered several mutations which were associated with FHB. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions.

Hepatitis B virus genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).[30] Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Statistical differences were evaluated by the Mann–Whitney *U*-test, Fisher's exact probability test and χ^2-test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate analyses with logistic regression were utilized to sort out independent risk factors for FHB. STATA Software ver. 8.0 was employed for all analyses.

RESULTS

Baseline characteristics of the patients with FHB by transient HBV infection and AHB

TABLE 1 COMPARES baseline clinical characteristics of the 50 FH-T patients and the 50 AHB who

were matched for age and sex. The peak ALT, AST and T.bil levels were significantly higher (3788 ± 2856 vs 2170 ± 1350 IU/L, $P < 0.001$; 3131 ± 3673 vs 1676 ± 1851 IU/L, $P < 0.05$; and 14.8 ± 8.6 vs 9.5 ± 9.8 mg/dL, $P < 0.01$, respectively), while HBeAg was less frequent (30% vs 56%, $P < 0.01$) in the FH-T patients than AHB. The level of HBcrAg was significantly lower (5.30 ± 1.32 vs 5.95 ± 1.13 log U/mL, $P < 0.01$), while HBV DNA loads were higher (5.97 ± 1.87 vs 4.98 ± 1.17 log copies/mL, $P < 0.005$), in the FH-T patients than AHB. The level of core protein in sera tended to be higher in the FH-T patients than AHB (3.21 ± 1.28 vs 3.01 ± 1.00 log U/mL). Death occurred more often in the FH-T patients than AHB (38% vs 0%, $P < 0.001$).

HBV Genotypes and enhancer II/core promoter/pre-core/core Mutations in Patients with FHB by transient HBV infection and AHB

Figure 1(a) compares the distribution of HBV genotypes/subgenotypes between the FH-T and the AHB patients. The subgenotype C2/Ce was most prevalent in both patients with FH-T and AHB (66% and 62%, respectively), whereas B1/Bj was more frequent in the FH-T patients than AHB (22% vs 6%, $P < 0.05$). Likewise, mutations in enhancer II/core promoter/pre-core/core regions are compared between the FH-T and AHB patients in Figure 1(b). A1762T/G1764A, G1896A, G1899A and A2339G mutation were more frequent in the FH-T patients than AHB (48% vs 16%, $P < 0.001$; 62% vs 6%, $P < 0.001$; 24% vs 4%, $P < 0.001$; and 8% vs 0%, $P < 0.05$, respectively).

Figure 2(a) compares various mutations between the 11 FH-T patients and the three AHB patients who were infected with B1/Bj. Only G1896A was significantly more frequent (73% vs 0%, $P < 0.05$), while the lack of any mutations was less common (0% vs 33%, $P < 0.05$) in the FH-T patients than AHB. In comparison with the 33 FH-T patients and the 31 AHB patients who were infected with C2/Ce (Fig. 2b), A1762T/G1764A (70% vs 19%, $P < 0.001$), G1896A (61% vs 6%, $P < 0.001$) and the combination of all three mutations (A1762T/G1764A and G1896A) (45% vs 6%, $P < 0.001$) were significantly more frequent, while the lack of any mutations was less common (9% vs 70%, $P < 0.001$) in the FH-T patients than AHB. Interestingly, all the AHB patients with both G1896A and A1762T/G1764A mutations suffered acute severe hepatitis B that was defined by prothrombin time less than 40% but without coma of grade II or higher.

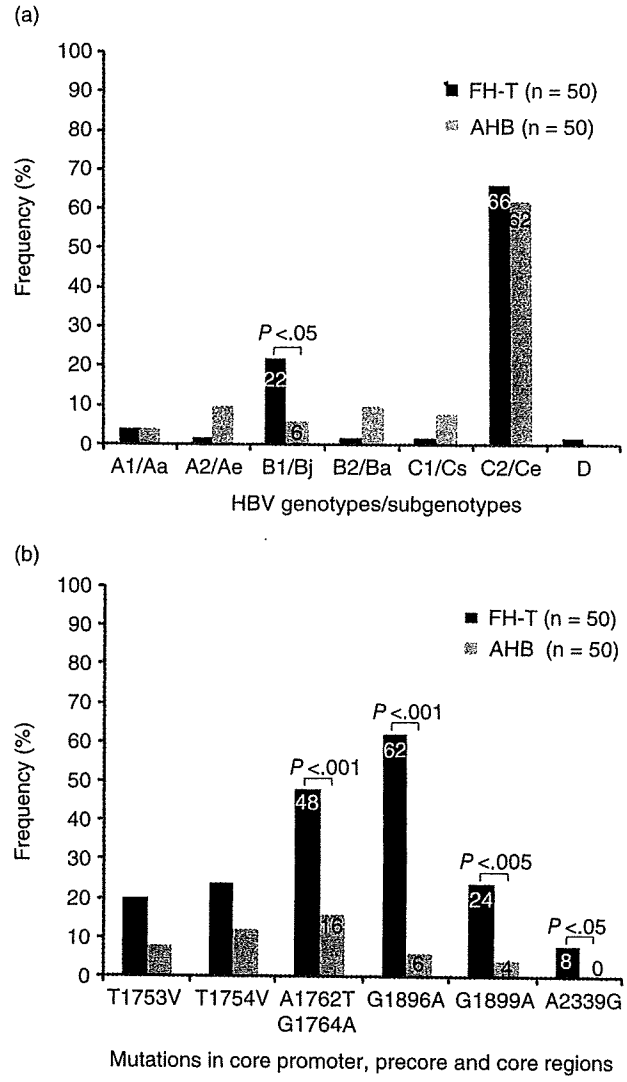

Figure 1 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 50 transient hepatitis B virus infection (FH-T) and the 50 acute self-limited hepatitis B (AHB) patients.

Factors independently associated with the development of FHB by transient HBV infection

The following independent factors, promoting the development of FHB, were evaluated by multivariate analysis: ALT, AST, T.bil, HBeAg, HBV DNA, core protein, HBcrAg, genotypes/subgenotypes (B1/Bj or not) and mutations (T1753V, T1754V, A1762T/G1764A, G1896A, G1899A and A2339G). T.bil more than 10.35 mg/dL (OR, 7.81 [95% CI, 1.77–34.51], $P = 0.0067$), G1896A mutation (OR, 13.53 [95% CI,

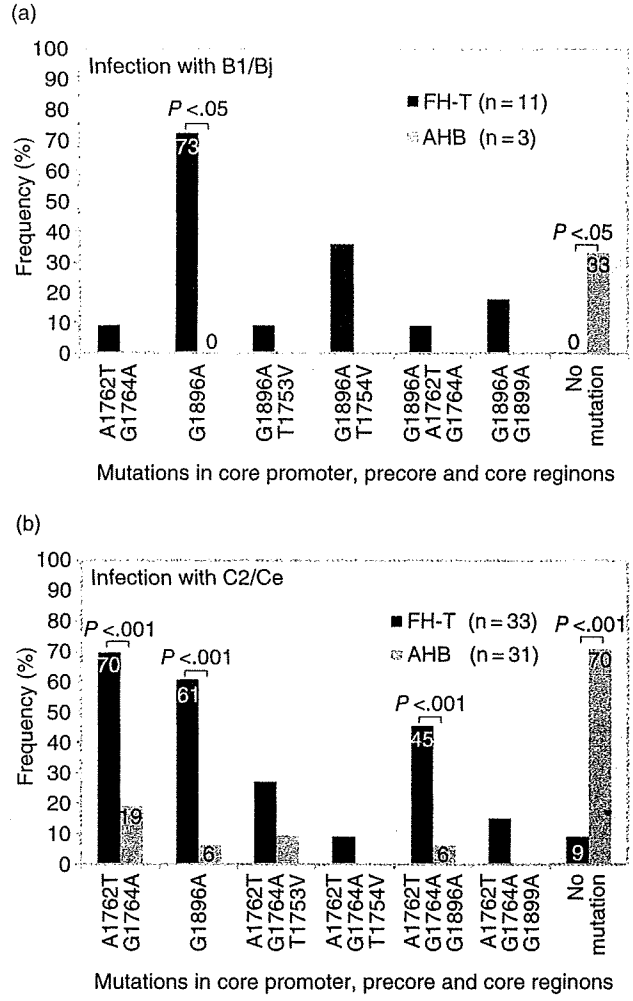

Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs. 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	*P*-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C ($n = 12$)	AE-C ($n = 12$)	Differences *P*-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.[31] Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.[13–16] Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.[19–21] It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.[22]

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,[32] and downregulated by the A1762T/G1764A double mutation at the transcription level.[33,34] Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.[35] Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,[35] the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tollerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.[36,37] In the absence or decrease of HBeAg, therefore, hosts would mount vigor cytotoxic T-cell responses to core epitopes excessively presented on hepatocytes, and develop severe liver injuries culminating in FHB.[38]

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case–control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.[8] These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;[13–16] these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.[18] Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient

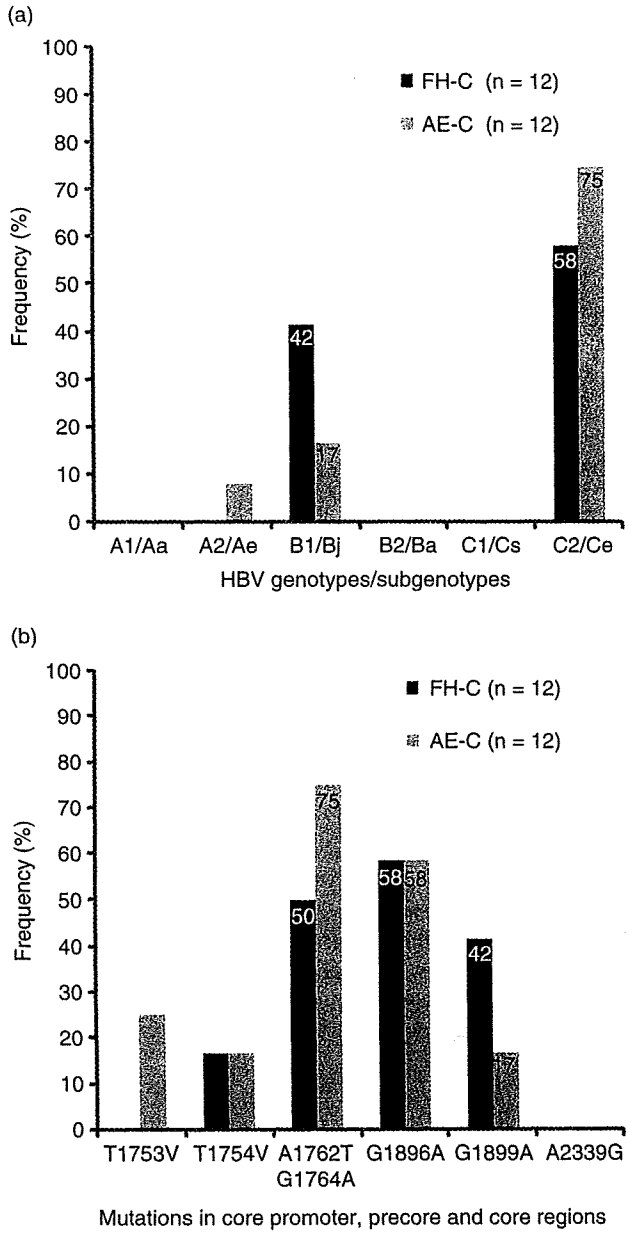

Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

HBV infection (Table 2). In particular, G1896A mutation was the most important factor associated with the development of FHB. Host responses, represented by T.bil, contributed to the development of FHB as well.

As for HBV genotypes, B1/Bj alone was significantly more frequent in the FH-T patients in univariate analysis. In the patients infected with B1/Bj, G1896A was more frequent in those with FH-T than AHB. In *in vitro* replication analysis, Ozasa *et al.*[15] observed extremely high expressions of intra- and extracellular HBV DNA in culture transfected with an HBV clone of B1/Bj genotype having the G1896A mutation; a high replication would be induced by this pre-core mutation for the induction of FHB. Our clinical results stand in support of this *in vitro* analysis. Taken altogether, chances for developing severe acute or FH would be high in the patients with acute hepatitis who are infected with HBV/B1 having the pre-core mutation. By contrast, in patients infected with C2/Ce, G1896A or A1762T/G1764A, or both was much more frequent in the FH-T patients than AHB. Of note, the co-occurrence of G1896A and A1762T/G1764A mutations was invariably accompanied by either FHB or acute severe hepatitis B in this study. Hence, these pre-core and core-promoter mutations might have addictive or synergetic effects for exacerbating hepatitis, when they emerge in the patients infected with C2/Ce. Such high-risk patients deserve special care and surveillance for signs and symptoms of fulminant or severe acute hepatitis B.

In the present study, serum levels of HBV DNA were significantly higher in the patients with FH-T than AHB. High serum levels of HBV DNA have been reported in patients with FHB;[39] they are followed by rapid decrease as the sequel of virus elimination operated by vigorous immune responses. Because of rapid and extensive elimination of HBV by the host immune system, HBV DNA in serum, in general, has decreased to low levels in patients with FHB at the presentation.[40] HBV DNA levels may be subject to the time that has elapsed from the onset of hepatitis to its measurement.[39] Also, serum levels of core protein (the product of the C gene) closely correlate with serum HBV DNA levels in patients with hepatitis B,[27] and they were compared between the FH-T patients and AHB. The core protein was determined by the newly developed CLEIA method; it is much easier and less expensive than the determination of HBV DNA. The level of core protein has turned out to be marginally higher in the FH-T patients than AHB (Table 1), and therefore might not contribute to an early diagnosis of FHB by transient infection.

Fulminant hepatitis B by AE of ASC is assumed as a different clinical condition from FHB by transient HBV infection. In this study, as there was no case–control study on virological factors associated with FHB for the patients with AE of ASC, we also attempted to identify virological factors associated with the development of FHB in the 12 FH-C and the 12 AE-C patients who were

matched for age as well as sex. Disappointingly, no differences of virological factors such as HBV genotypes and pre-core mutations, which were strongly associated with the development of FHB by transient infection, were found between the FH-C and AE-C patients (Fig. 3a,b). Furthermore, there were also no significant differences about HBeAg-positive rate and the levels of serum HBV DNA or core protein (Table 3), suggesting that several host factors may play a more important role in the development of FHB in ASC instead of virological factors. In this case–control study, however, there seems to be some problems: a small number of patients, different duration of HBV infection, different clinical stage (ASC or CHB) at the onset of AE, and HBV quasispecies complexity. Further investigations are needed to identify factors associated with FHB precipitating in asymptomatic HBV carriers.

In conclusion, virological factors associated with enhancement of viral replication seemed to be important for the development of FHB in the patients by transient HBV infection. But no virological factors were identified for differentiation of the FH-C patients from the AE-C patients. Hence, the pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

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Clinical Brief Report

Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles

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Abbreviations: Atg, autophagy related genes and their products; Beclin 1, autophagy-related bcl2-interacting Atg6 homologue; BECN1, *beclin 1* gene and mRNA; HCV, hepatitis C virus; IFNA1, interferon alpha1 gene; IFNA2, interferon alpha2 gene; IFNB1, interferon beta1 gene; NS, nonstructural protein; LC3, wild-type human microtubule-associated protein 1 light chain 3; LC3-I, soluble unlipidated form of LC3; LC3-II, LC3-phospholipid conjugate

Key words: autophagy, Atg7, LC3, Beclin 1, Hepatitis C virus, infectious HCV particles

Hepatitis C virus (HCV) is a positive-strand RNA virus, and classified within the Flaviridae family. Atg7-knockdown decreases the amount of HCV replicon RNA, when HCV JFH1 RNA and HCV subgenomic replicon are transfected into Huh7.5 cells. However, when infectious naive HCV particles are directly infected into Huh7.5.1 cells, it is still unclear whether Atg7-knockdown decreases the production of intracellular HCV-related proteins, HCV mRNA and infectious HCV particles. When Atg7 protein in HCV-infected Huh7.5.1 cells was knocked down by RNA-interference, the levels of intracellular HCV core, NS3, NS5A proteins, HCV mRNA and secreted albumin remained unchanged compared with those in the control HCV-infected cells. However, the level of infectious HCV particles released in the medium was decreased by the Atg7-knockdown. Similar results were obtained when Beclin 1 was knocked down by RNA-interference. The colocalization of endogenous LC3-puncta with HCV core, HS5A proteins and lipid droplets was also investigated. However, little endogenous LC3-puncta colocalized with HCV core, NS5A proteins or lipid droplets. These results suggested that autophagy contributed to the effective production of HCV particles, but little to the intracellular production of HCV-related proteins, HCV mRNA and the secretory pathway, in a naive HCV particles-infection system.

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Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus with a genome size of 9.6 kb, and is classified within the Flaviridae family.[1] HCV is spread by blood-to-blood contact, and infection can lead to liver cirrhosis and heaptocellular carcinoma. More than 150 million people in the world are infected with HCV. The HCV genome encodes polyproteins that are processed into structural proteins (core protein and envelope glycoproteins) and nonstructural (NS) proteins (NS1-NS5A including a protease and RNA helicase).[2] HCV replicates in association with intracellular membrane structures called "the membranous web."[3] HCV-infected cells accumulate lipid droplets, and the lipid droplets play an important role in the assembly of HCV particles.[4] HCV core protein recruits NS proteins and replication complexes to lipid droplets-associated membranes. This recruitment is critical for producing infectious viruses.

Recently, when HCV JFH1 RNA and HCV subgenomic replicon were transfected into Huh7.5 cells, HCV infection leads to incomplete autophagic flux, and Atg7-knockdown decreases the amount of HCV replicon RNA.[5-7] Interestingly, endogenous LC3 can associate with cytoplasmic lipid droplets in uninfected Huh7.5.1 cells.[8] Considering that HCV core and NS5A proteins associate with lipid droplets for the assembly of HCV particles, it is possible that Atg7-knockdown will result in a decreased production of these particles. Therefore, the contribution of Atg7 to the production of intracellular HCV-related proteins and the release of infectious HCV particles was investigated. In addition, the authors investigated whether endogenous LC3 colocalizes to HCV lipid droplets, HCV core and NS5A proteins.

Results

Human *ATG7* and *BECN1* RNAi resulted in decreased release of HCV particles in the medium without a decrease of the intracellular production of HCV-related proteins and HCV

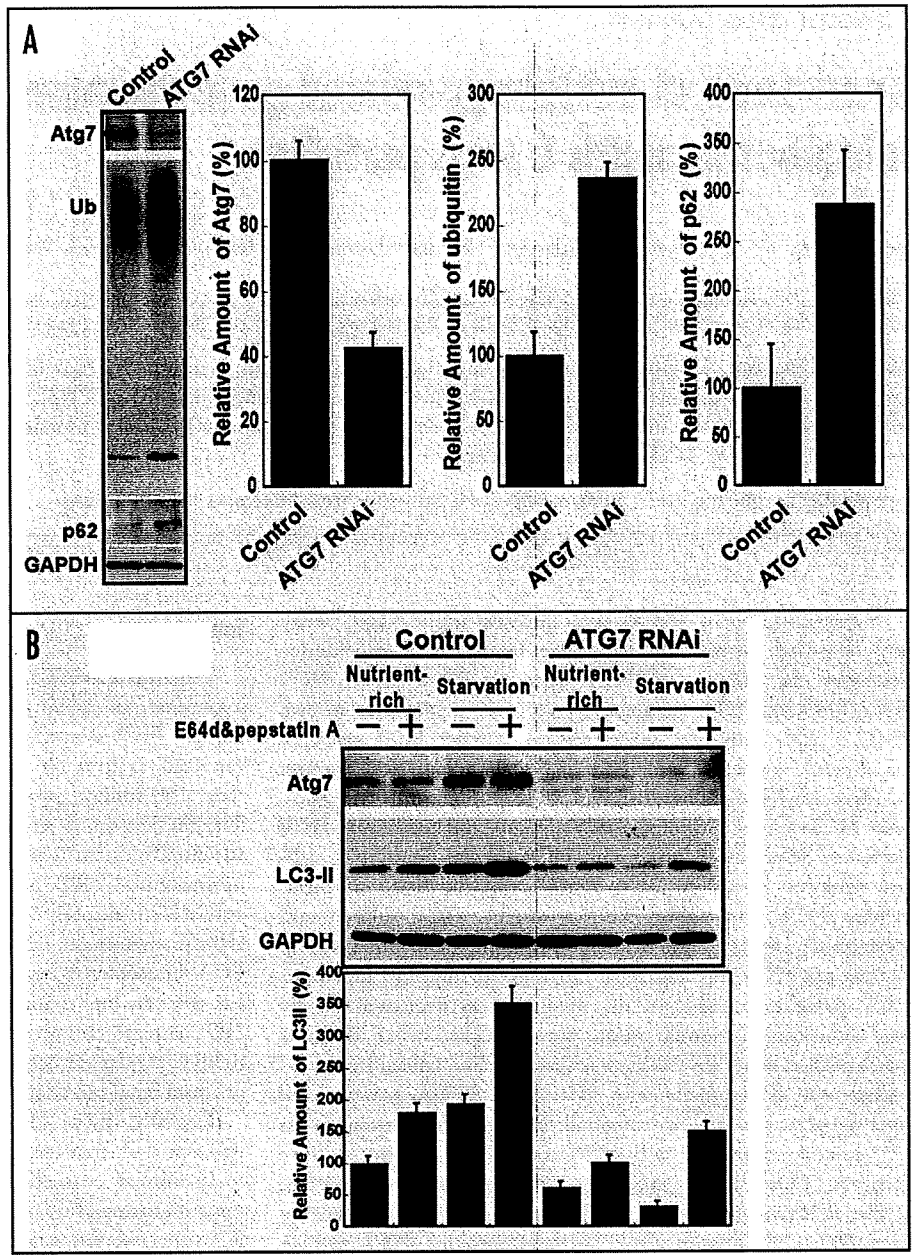

Figure 1. Effect of *ATG7*-RNAi on p62, ubiquitinated proteins and lysosomal turnover of LC3-II. (A) Increase of p62 and ubiquitinated proteins in Huh7 cells by *ATG7*-RNAi. dsRNA for *ATG7*-RNAi was transfected into Huh7.5.1 cells (ATG7 RNAi). As a negative control, scrambled dsRNA was employed (Control). Cells were harvested after incubation at 37°C for 48 h, and total proteins (20 μg) were separated on SDS-PAGE. Atg7, p62 and ubiquitinated proteins (ubiquitin) in the lysate were recognized by immunoblotting. GAPDH was employed as a loading control. Right: indicated the relative levels of intensity of each band of three independent experiments estimated by densitometry. Error bars indicate standard errors. (B) Effect of *ATG7*-RNAi on lysosomal turnover of LC3-II. Atg7-knockdown was performed as described in (A), and cells were cultured for 48 h. For Nutrient-rich conditions, cells were cultured in DMEM medium containing 10% FCS. Where indicated, cells were treated with the protease inhibitors, E64d (10 μg/ml) and pepstatin A (10 μg/ml) (E64d & pepstatin A +) for 4 h, or, as a negative control (Inhibitors -), with the solvent, dimethylsulfoxide. For Starvation conditions, cells were incubated for 4 hrs in Krebs-Ringer medium (KRB) in the presence (+) or absence (-) of the protease inhibitors. The cells were lysed, total proteins (10 μg per lane) were separated by SDS-PAGE, and endogenous LC3 and Atg7 in the lysates was recognized by immunoblotting. LC3-II, membrane-bound form of LC3. Lower: indicated the relative levels of intensity of each band estimated by densitometry. Error bars indicate standard errors.

mRNA. The initial investigation was on whether Atg7-knockdown leads to a decreased HCV core, NS3 and NS5A proteins in an in vitro naive HCV particle-infection system. Atg7 in Huh7.5.1 cells was knocked down by double-stranded RNAs for *ATG7* RNAi compared with scrambled double-stranded RNAs (Fig. 1A). The Atg7-knockdown in Huh7.5.1 cells resulted in an increase of p62 and ubiquitinated proteins (Fig. 1A), and led to an insufficiency in lysosomal turnover of LC3-II under starvation conditions (Fig. 1B).[14-18] HCV was infected into Huh7.5.1 cells, and the *ATG7* RNAi was performed at day 1 and day 3 post-infection. At day 5 post-infection, cells were harvested, and Atg7 and HCV core, NS3 and NS5A proteins in the cell lysate were analyzed by SDS-PAGE and immunoblotting. Under these conditions, the Atg7 protein was significantly decreased in HCV-infected Huh7.5.1 cells (Fig. 2A). However, the levels of HCV core, NS3 and NS5A proteins in Atg7-knockdown HCV-infected cells remained unchanged as compared with control HCV-infected cells (Fig. 2A). In addition, the levels of HCV mRNA in Atg7-knockdown HCV-infected cells also remained unchanged as compared with control HCV-infected cells (Fig. 2B). These results indicated that the *ATG7* RNAi has little effect on the intracellular production of HCV-related proteins and HCV mRNA.

The next investigation was on whether Atg7-knockdown affects the release of HCV particles. The amount of HCV core protein in the medium at day 5 post-infection was estimated by an ELISA for HCV core antigen. Interestingly, the amount of released HCV core protein derived from Atg7-knockdown cells decreased by about 40 ± 8% compared with control scrambled RNA-treated cells at day 5 post-infection ($p < 0.03$) (Fig. 2E). Under these conditions,

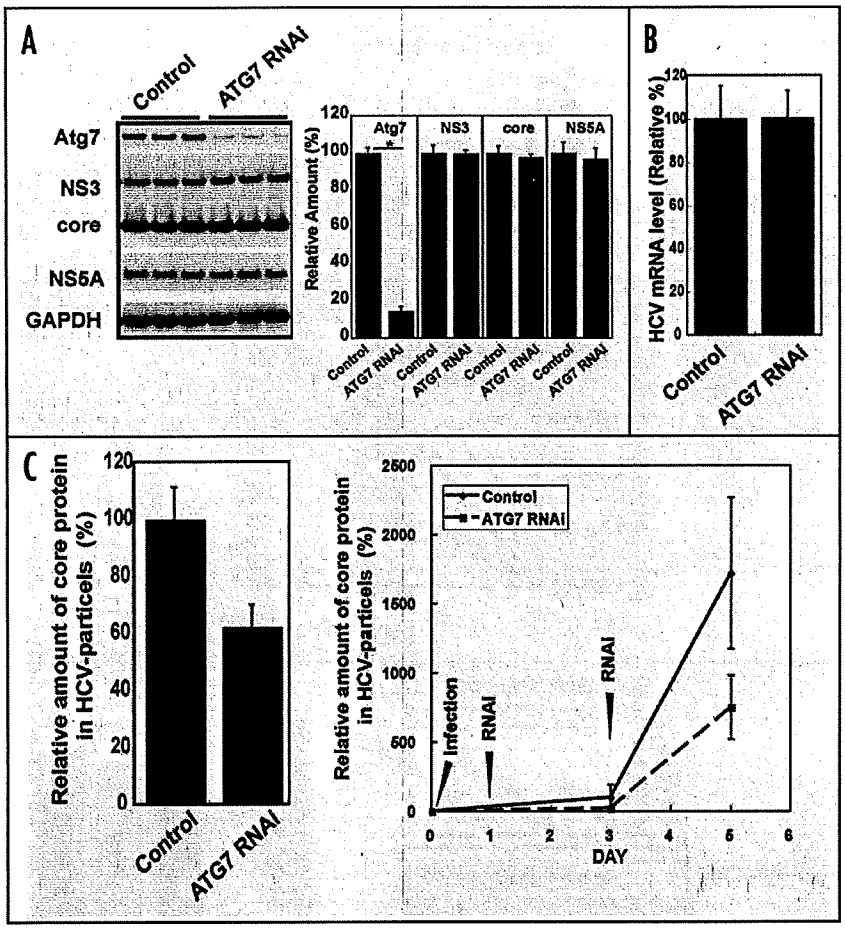

Figure 2. Decrease of infectious HCV particles in the medium by *ATG7*-RNAi. (A) Minor effect of *ATG7* RNAi on the intracellular production of HCV core, NS3 and NS5A proteins. HCV particles were infected into Huh7.5.1 cells. After incubation at 37°C for 2 h, cells were washed and cultured in the DMEM containing 10% fetal calf serum. At day 1 and 3 after HCV infection, dsRNA for *ATG7*-RNAi was transfected into the cells (ATG7 RNAi). As a negative control, scrambled dsRNA was employed (Control). Cells were harvested on day 5 after HCV infection, total proteins (10 μg) were separated by SDS-PAGE, and Atg7, HCV core, NS3 and NS5A proteins in the lysate were recognized by immunoblotting. Three independent experiments are shown. Note that HCV-related proteins remained unchanged, whereas Atg7 protein decreased by the RNAi treatment. Right: indicated the relative levels of intensity of each band estimated by densitometry. Asterisks indicated that the p-value of a Student's t-test is <0.03. The average intensity of "control" in each protein was regarded as 100%. Error bars indicate standard errors. (B) Relative amount of HCV mRNA in Atg7-knockdown cells and control cells. 140 ng of total RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of GAPDH mRNA. Error bars indicate standard errors. (C) Decrease in the amount of the core protein of HCV particle released in the medium by *ATG7*-RNAi. Atg7-knockdown was performed as described in (A). The amount of HCV core protein in HCV particles released into the medium was estimated by a HCV core ELISA kit. The data are from three independent experiments. Relative amount of HCV core protein in Atg7-knockdown cells compared to scrambled RNA infected cells is shown, and the p-value of a Student's t-test is <0.03. Error bars indicate standard errors. Right: indicates the time course of the relative amount of HCV particles. The average amount of HCV particles from control HCV-infected cells at day 3 post-infection was set as 100%.

there was little difference in viability between Atg7-knockdown cells and control cells (Fig. 3A). The amount of HCV mRNA in Huh7.5.1 cells after the reinfection of HCV particles derived from Atg7-knockdown and control cells increased in a time-dependent manner, indicating that the HCV particles derived from Atg7-knockdown cells have a reinfectivity (Fig. 3B). It would be possible that Atg7-knockdown could inhibit the secretory pathway in addition to the release of HCV particles. However, the levels of secreted human albumin, one of the major secreted proteins of hepatocytes, in the medium of Atg7-knockdown cells remained unchanged as compared with that in the medium of control cells, suggesting that there was little, if any, effect on the secretory pathway in HCV-infected cells by the RNAi (Fig. 3C).

To clarify whether or not the decrease of released HCV particles in the medium by *ATG7* RNAi is caused by a defect in autophagy, we investigated the effect of Beclin 1-knockdown on the production of naive HCV-particles (Figs. 3B and C; and 4). Beclin 1, a yeast Atg6 homologue, was isolated as a Bcl-2-interacting protein,[12] and is a subunit of the class III PtdIns 3-kinase lipid-kinase complex essential for autophagy.[19,20] HCV was infected into Huh7.5.1 cells, and *BECN1* RNAi was performed at day 1 and day 3 post-infection. At day 5 post-infection, Beclin 1 protein in HCV-infected Huh7.5.1 cells was knocked down by *BECN1* RNAi compared with scrambled double-stranded RNAs (Fig. 4A). The levels of HCV core, NS3 and NS5A proteins in Beclin 1-knockdown HCV-infected cells remained unchanged as compared with control HCV-infected cells (Fig. 4A). The levels of HCV mRNA in Beclin 1-knockdown HCV-infected cells also remained unchanged as compared with control HCV-infected cells (Fig. 4B). Under these conditions, the amount of released HCV core protein derived from Beclin 1-knockdown cells decreased by about 60 ± 9% compared with control scrambled RNA-treated cells at day 5 post-infection ($p < 0.03$) (Fig. 4C). The HCV particles released from Beclin 1-knockdown cells have reinfectivity (Fig. 3B). There was little difference in viability between Beclin 1-knockdown cells and control cells (Fig. 4D). The levels of secreted human albumin in the medium of Beclin 1-knockdown cells remained unchanged as compared with that in the medium of control cells (Fig. 3C). These results suggested that autophagy contributes to an effective production of infectious HCV particles from the cells without a decrease of the intracellular levels of HCV core, NS3, NS5A or HCV mRNA.

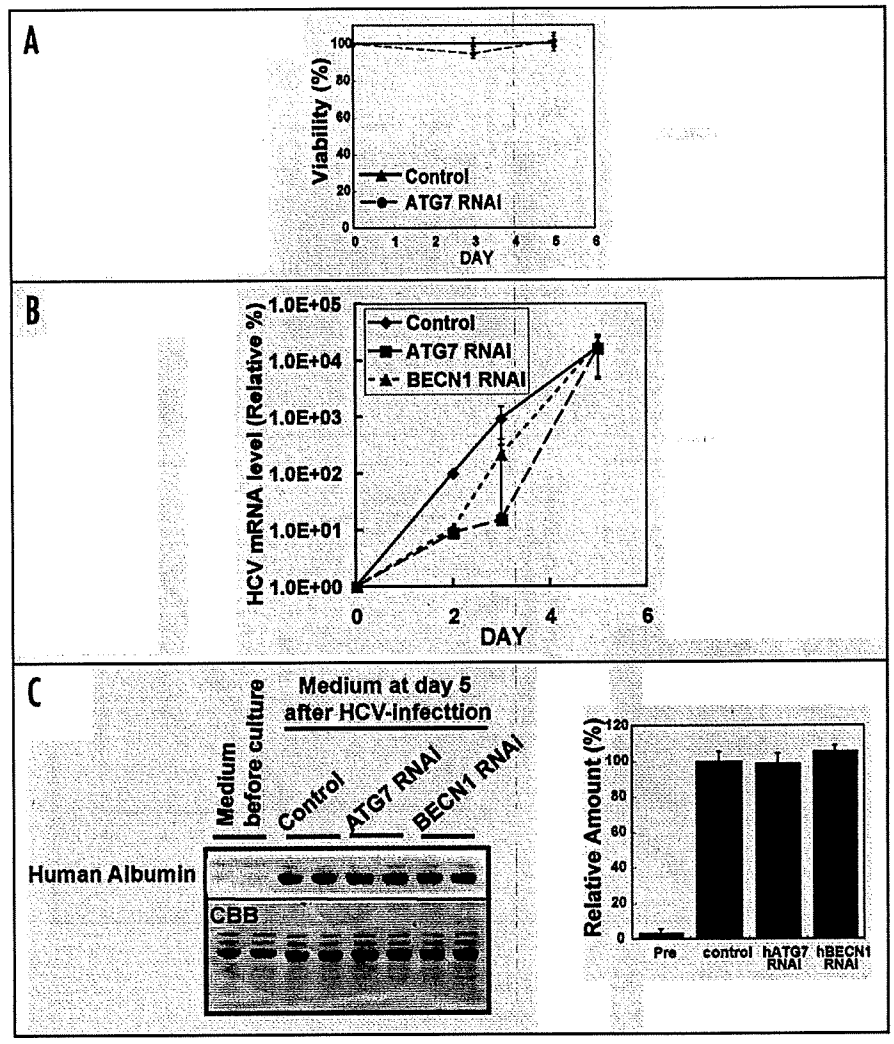

Figure 3. Effects of Atg7-knockdown on cell viability, reinfectivity of HCV particles, and secretion of human albumin. (A) Cell viability of Atg7-knockdown HCV-infected cells. As a negative control for *ATG7* RNAi, scrambled RNA was employed. Error bars indicate standard errors. (B) Reinfectivity of HCV particles derived from Atg7-knockdown HCV-infected cells. HCV-infection, *ATG7*-RNAi and *BECN1*-RNAi were performed as described in Figure 2. At day 5 post-infection, the medium containing HCV particles was harvested. The same volume of the medium containing HCV particles was added to the medium, in which Huh7.5.1 cells were cultured to semiconfluency. After incubation at 37°C for 2 h, cells were washed twice with culture medium, and incubated at 37°C for 2, 3 and 5 days. After RNA-preparation, 140 ng of total RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of GAPDH mRNA. Error bars indicate standard errors. The average amount of HCV mRNA in the control HCV-infected cells at day 2 post-infection was set to 100%. (C) Minor effect of Atg7- and Beclin1-knockdowns on secretion of human albumin in HCV-infected cells. Media from cultured Atg7-(ATG7 RNAi) and beclin1-(BECN1 RNAi) knockdown HCV-infected cells was harvested. As a negative control for RNAi, scrambled RNA was employed (Control). The medium before cell culture was used as a non-incubation control (Medium before culture). Total proteins in the medium were separated by SDS-PAGE, and human albumin in the medium was recognized by immunoblotting with anti-human albumin IgG. To avoid cross-reactivity of the antibody against bovine albumin, the IgG was pre-absorbed with bovine albumin. As a loading control, the membrane was stained with Coomassie Brilliant Blue (CBB). Right: indicates the average relative levels of each bands estimated by densitometry.

Endogenous LC3-puncta displayed little colocalization with HCV core, NS5A or lipid droplets. Intracellular colocalization of endogenous LC3-puncta with HCV core, NS5A and lipid droplets in the HCV-infected cells was investigated next. GFP-LC3-puncta increase in HCV-infected cells.[5-7] However, there are some problems concerning the estimation of autophagy using GFP-LC3.[21-23] Therefore, using affinity purified anti-LC3 IgG, intracellular distribution of endogenous LC3 in the HCV-infected Huh7.5.1 cells was investigated (Figs. 5–9). As positive controls, endogenous LC3 in uninfected Huh7.5.1 cells were stained under starvation conditions in the absence and presence of E64d and pepstatin A (Fig. 6A–F). When uninfected Huh7.5.1 cells were cultured under nutrient-rich conditions, few puncta of LC3 were recognized (Fig. 6A and B). When the uninfected Huh7.5.1 cells were incubated under starvation conditions for 2 h, fluorescence of LC3-positive puncta was increased at a perinuclear region (Fig. 6C and D). The LC3-positive puncta increased in the presence of E64d and pepstatin A under starvation conditions (Fig. 6E and F). In HCV-infected Huh7.5.1 cells, the LC3-positive puncta increased significantly at day 5 post-infection (Figs. 5A vs. 6A, C and E). Deconvolution of high-magnification images (Fig. 8) indicated that LC3-positive puncta in HCV-infected cells were larger than those in starved cells even in the presence of these inhibitors (Fig. 8C and D vs. B). Quantitative analyses of the intensity of the fluorescence of LC3-puncta in each cell indicated that the total intensity of fluorescence of LC3-puncta per cell in HCV-infected cells was about 4.5-fold higher than in uninfected cell under starvation conditions in the presence of E64d and pepstatin A (Fig. 9). Further investigation was made on whether endogenous LC3-puncta colocalized with HCV core, NS5A and lipid droplets. However, there was little colocalization between LC3 and these structures (Figs. 7 and 8).

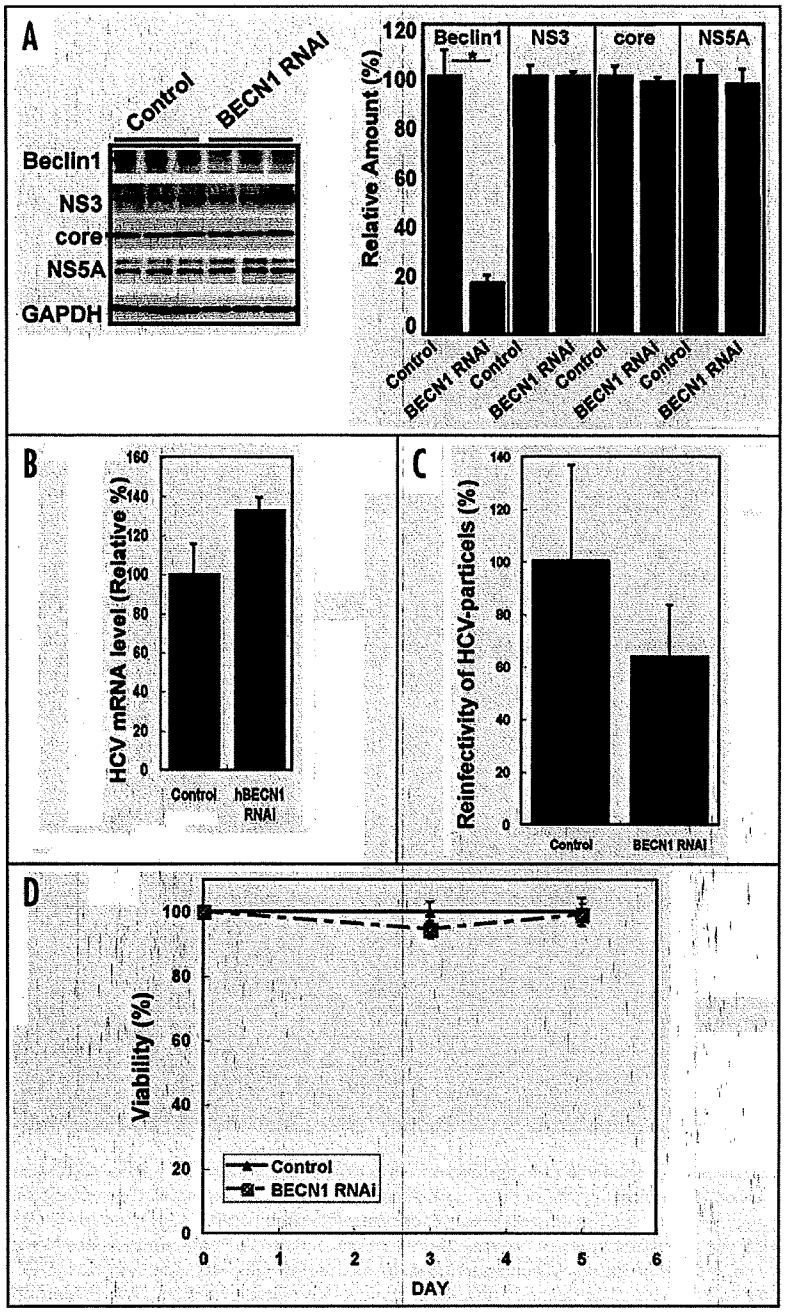

Figure 4. Decrease of infectious HCV particles in the medium by Beclin1 knockdown. (A) Minor effect of Beclin1-knockdown on the intracellular production of HCV core, NS3 and NS5A proteins. HCV-infection, *BECN1* RNAi and immunological analyses were performed as described in Figure 2. BECN1 RNAi, Beclin1-knockdown; Control, scrambled dsRNA. Right: indicates the relative levels of intensity of each band estimated by densitometry. Asterisks indicated that the p-value of a Student's t-test is <0.03. The average intensity of "control" in each protein was set to 100%. Error bars indicate standard errors. (B) Relative amount of HCV mRNA in Beclin1-knockdown cells and control cells. 140 ng of toal RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of ACTB mRNA. Error bars indicate standard errors. (C) Decrease in the amount of the core protein of HCV particle released in the medium by Beclin 1-knockdown. Beclin 1-knockdown-dependent estimation of core protein in HCV particles was performed as described above. The data are from three independent experiments. Relative amount of HCV core protein in Beclin1-knockdown cells compared to scrambled RNA infected cells is shown, and the p-value of a Student's t-test is <0.03. Error bars indicate standard errors. (D) Cell viability of Beclin 1-knockdown HCV-infected cells. As a negative control for *BECN1* RNAi, scrambled RNA was employed. Error bars indicate standard errors.

Discussion

Results of this study showed that Atg7- and Beclin 1-knockdowns in HCV-infected cells resulted in a decrease in the production of infectious HCV particles in the medium, whereas the intracellular production of HCV mRNA and HCV proteins examined remained unchanged. Few endogenous LC3-puncta in HCV-infected cells were colocalized to lipid droplets, core and NS5A proteins.

Using transfection of HCV JFH1 mRNA into Huh7.5 cells, Atg7-knockdown decreases the amount of HCV replicon RNA.[5-7] However, using an in vitro naive HCV JFH1 particle-infection system, Atg7-knockdown decreased the level of infectious particles in the medium by about 40%, whereas intracellular HCV mRNA and HCV proteins remained unchanged. This discrepancy results from the difference between the naive HCV particle-infection system and the transfection of HCV mRNA.

Against some pathogens, autophagy plays a role in intracellular immunity. Initially, it was hypothesized that if autophagy plays a protective role against HCV infection, Atg7- and Beclin 1-knockdowns could lead to an increase of HCV particles and/or intracellular HCV-related proteins. However, contrary to the hypothesis, these knockdowns decreased the production of HCV particles. Atg7 is a key enzyme essential for formation of autophagosomes, and Beclin 1 is a subunit of the class III PtdIns 3-kinase lipid-kinase complex that induces autophagy. Considering that Atg7 and Beclin 1 have different functions in autophagy, these results indicated that autophagy contributes to the effective production of HCV particles in the medium, but little to the intracellular levels of HCV mRNA and HCV-related proteins. It is possible that this knockdown would inhibit the secretory pathway. However, there was little difference in the level of secreted albumin in the medium between autophagy-knockdown cells and control cells. In yeast, no *atg* mutants have defects in the secretory pathway, and there is no report that

ATG gene-knockout influences the secretory pathway in animals and plants. Therefore, it is unlikely that these knockdowns inhibit the secretory pathway. Considering these results, autophagy will contribute to the release and/or assembly of HCV particles, but little to the intracellular production of HCV mRNA and HCV-related proteins.

LC3-II can associate with lipid droplets,[24] and lipid droplets are induced by HCV infection.[4] However, little endogenous LC3-puncta associated with lipid droplets in the HCV-infected Huh7.5.1 cells. These results show that LC3-II cannot always associate with lipid droplets. There is a certain mechanism that allows LC3-II to associate with lipid droplets, which will be the subject of future research.

Materials and Methods

Cells, media, materials and antibodies. Huh7.5.1 cells derived from the Huh7 cell line (ATCC CCL-185) were cultured in Dulbecco's modified Eagle medium (DMEM; Wako, 045-30285) containing 10% fetal calf serum (JRH biosciences/SIGMA, 12603C) and 1% nonessential amino acids (Invitrogen, 11140050). Polyclonal antibodies against Atg7 and LC3 were described previously.[9-11] For the preparation of antiserum against human Beclin 1, rabbits were immunized with a glutathione S-transferase-human Beclin 1 fusion protein. The anti-Beclin 1 IgG was affinity-purified using recombinant human Beclin 1-conjugated Sepharose. The monoclonal antibody against HCV core protein was purchased from Anogen (MO-I40015B), the monoclonal antibody against HCV NS5A protein (HCM-131-5) was from Austral, the monoclonal antibodies against GAPDH (ab8245) and HCV NS3 proteins (ab18664) were from Abcam, and the polyclonal antibody against human albumin (126584) was from Calbiochem. For RNA-interference for human *ATG7* (*ATG7* RNAi) and *BECN1* (*BECN1* RNAi),[12] lipofectamine RNAi MAX, and Stealth™ select RNAi sets (Invitrogen, 1299003), respectively, and Stealth™ RNAi negative control (Invitrogen, 935300) were used. Little interferon mRNAs (IFNA1, IFNA2 and IFNB1) were activated by the transfection of these double-stranded RNAs (Table 1). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, 23225). E64d (4321-v) and pepstatin A (4397-v) were purchased from Peptide Institute.

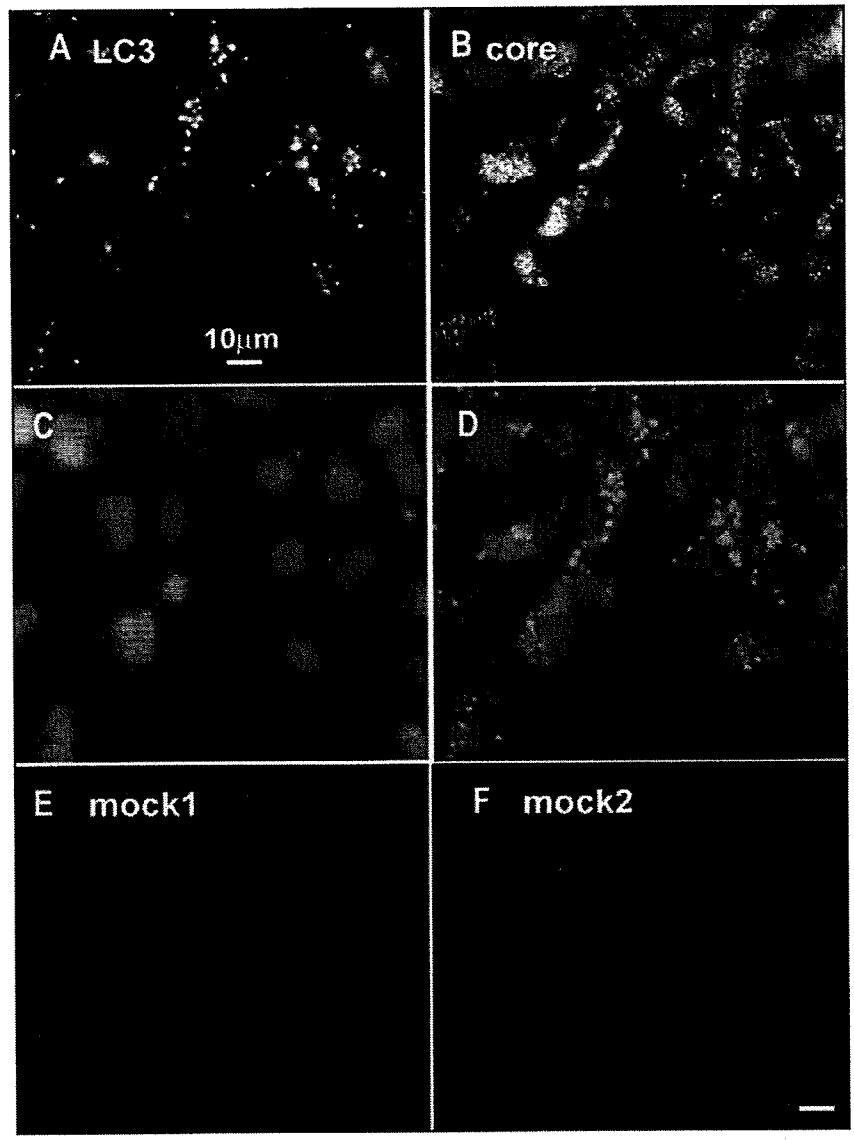

Figure 5. Intracellular distribution of endogenous LC3 in HCV-infected Huh7.5.1 cells. Cells were fixed in 4% paraformaldehyde/PBS at day 5 post-infection (A–F), and permeabilized in 50 μg/ml of digitonin. Endogenous LC3 in the cells was recognized with rabbit anti-LC3 IgG and Alexa488-conjugated goat anti-rabbit IgG (A). Intracellular HCV core protein was recognized with mouse anti-HCV core IgG and Alexa594-conjugated goat ant-mouse IgG (B). DAPI staining was in (C). As a negative control for LC3-staining, cells were stained with normal rabbit IgG and Alexa488-conjugated goat anti-rabbit IgG (E mock 1). As a negative control for HCV core-staining, cells were stained with normal mouse IgG and Alexa594-conjugated goat anti-mouse IgG (F mock 2). Fluorescence of Alexa488, Alexa594 and DAPI was monitored by fluorescence microscopy. Merged pseudo color images (LC3, green: HCV core, red: and DAPI, blue) was shown in (D). Bars indicate 10 μm.

Infection of Huh7.5.1 cells with HCV. Infectious HCV (JFH1 strain) particles were produced in Huh7.5.1 cells as described in an earlier study.[13] Culture supernatant containing infectious HCV particles was collected and stored at -80°C until use. Subconfluent Huh7.5.1 cells in 24-well or 48-well plates were exposed to

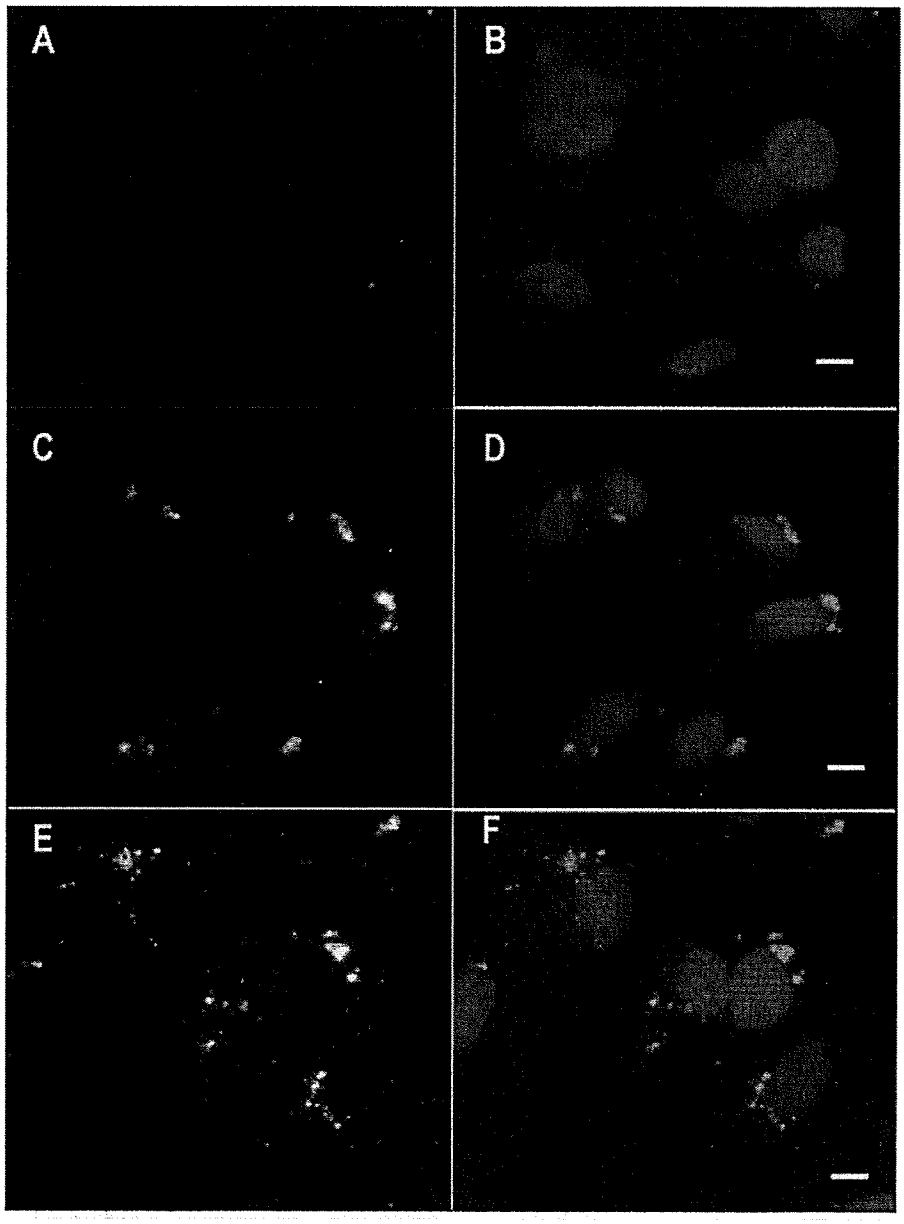

Figure 6. Intracellular distribution of endogenous LC3 in starved Huh7.5.1 cells. Huh7.5.1 cells were cultured in the nutrient-rich medium (A and B). For starvation-induced autophagosomes and autolysosomes, cells were incubated in the Krebs-Ringer buffered medium for 4 h in the absence (C and D) or presence (E and F) of E64d and pepstatin A. Endogenous LC3 in the cells was recognized with rabbit anti-LC3 IgG and Alexa488-conjugated goat anti-rabbit IgG (A, C and E). Merged pseudo color images (LC3, green; and DAPI, blue) were shown in (B, D and E). Bars indicate 10 μm.

normal culture medium containing HCV particles (8 fmoles of core protein/well, corresponding to moi = 0.1) for 6 h at 37°C. Cells were then washed and maintained in 500 μl (24-well) or 250 μl (48-well) of normal culture medium for 6–7 days at 37°C. To determine HCV production activity, the amounts of HCV core protein in the culture medium was quantified with an enzyme-linked immunosorbent assay (ELISA) (Ortho® HCV antigen ELISA test, Ortho-Clinical Diagnostics, 601002).

Immunoblotting analyses. After HCV infection, cells were washed twice in phosphate-buffered saline, lysed in lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 1% sodium dodecyl sulfate) containing a Complete® protease-inhibitor cocktail (Roche Diagnostics, 1697498). Proteins (10 μg) of the lysate were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% Bis-Tris, Invitrogen, NP0322BOX). After transferring the proteins to a polyvinylidine difluoride membrane using a Trans-Blot SD transfer cell (Bio-Rad, 170-3940), HCV core protein, HCV NS3 protein, HCV NS5A protein, and Atg7 in the lysate were recognized with appropriate antibodies. A chemiluminescent method was carried out according to standard protocols with SuperSignal West Dura Extended Duration Substrate (Pierce, 34075) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077).

Immunofluorescence analyses. Indirect immunofluorescence analysis was basically performed as described in the literature.[11,14] Briefly, cells were fixed in a fixation solution (phosphate buffered saline containing 4% paraformaldehyde) at room temperature for 5 min, and permeabilized in phosphate-buffered saline containing 1% digitonin. Rabbit polyclonal anti-LC3 antibody (10 μg/ml), mouse monoclonal anti-HCV core antibody (10 μg/ml), and mouse monoclonal anti-HCV NS5A antibody were used for recognizing LC3, HCV core protein, and HCV NS5A protein, respectively. As secondary antibodies, Alexa488-conjugated goat anti-rabbit IgG (Invitrogen, A11008) and Alexa594-conjugated goat anti-mouse IgG (Invitrogen, A11005) were used. Nile Red (Invitrogen, N1142) was used for lipid droplet staining. Fluorescence of Alexa488 and Alexa594 was monitored with Biozero BZ-8000 (KEYENCE, Tokyo, Japan).

Other techniques. Densitometric analyses of images were performed with an ImageJ program (http://rsbweb.nih.gov/ij/) on a PowerMac G4 computer. Cell viability was measured with a CellTiter 96 nonradioactive cell proliferation assay kit (Promega,