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Upregulation of nuclear PA28γ expression in cirrhosis and hepatocellular carcinoma

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Abstract. We previously reported that proteasome activator 28γ (PA28γ) is an oncogenic protein in hepatitis C virus (HCV) core protein transgenic mice. The aim of this study was to determine the role of PA28γ expression at the protein level in the development and progression of human hepatocarcinogenesis and hepatocellular carcinoma (HCC). Samples from tissues representing a wide spectrum of liver disease were analyzed, including histologically normal livers (n=5), HCV-related chronic hepatitis (CH) (n=15) and cirrhosis (n=31). The level of nuclear PA28γ increased with the progression of liver disease from CH to cirrhosis. The majority of cirrhotic livers (68%; 21/31) displayed high nuclear PA28γ expression. However, in half of the HCCs (50%; 18/36), little or no nuclear PA28γ expression was observed, while the remaining 50% (18/36) of the cases displayed high levels of nuclear PA28γ expression. A clinicopathological survey demonstrated a significant correlation between nuclear PA28γ expression and capsular invasion in HCC (P=0.026); a striking difference was found between nuclear PA28γ expression in non-tumor tissues and shorter disease-free survival (P<0.01). Moreover, nuclear PA28γ expression in non-tumor tissues correlated with the expression of molecules related to the genesis of hepatic steatosis and HCC, such as sterol regulatory element binding protein-1c mRNA. The findings suggest the involvement of nuclear PA28γ expression in the progression and relapse of HCC, and suggest that nuclear PA28γ is a potentially suitable target for the prevention and/or treatment of HCC.

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Abbreviations: CH, chronic hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PA, proteasome activator; PBGD, porphobilinogen deaminase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: proteasome activator 28γ, hepatocellular carcinoma, cirrhosis, western blotting, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for approximately 6% of all human carcinomas and 1 million deaths annually, with an estimated number of new cases of over 500,000/year (1). Clinical and experimental evidence suggests a link between infection with hepatitis C virus (HCV) and/or hepatitis B virus (HBV), chronic hepatitis (CH) and cirrhosis, as well as the progression of HCC. Liver cirrhosis is observed in up to 90% of patients with HCC, and HCV is the causative factor in 80% and HBV in 10% of cases in Japan (2-5). In the United States, almost 4 million individuals are infected with HCV each year which progresses to chronic hepatitis C, which could potentially progress to liver cirrhosis. The results are often liver failure or HCC. Chronic hepatitis C is the nation's leading cause of HCC, and according to the American Liver Foundation, is also the leading reason for liver transplantation. In Japan, HCV and/or HBV-based hepatitis and cirrhosis are also serious problems since they progress to HCC at a ratio of 5 to 7% per year (4,5). These findings strongly suggest the existence of a link between hepatocarcinogenesis and HCV/HBV infection and chronic liver inflammation.

Various therapies are currently in use for HCC. These include surgical resection, percutaneous ethanol injection (PEI), systemic or arterial chemotherapy using either single or combination drugs, transcatheter arterial chemoembolization (TACE), hormonal therapy and selective radiotherapy. However, the prognosis of patients with HCC remains poor, as they often develop intrahepatic and/or multicentric tumor recurrence, at a rate of 20-40% within 1 year, and ~80% within 5 years of therapy even when curative treatment is applied (6-9). Liver transplantation offers the best prognosis for patients with small HCC, although its use is limited due to the scarcity of donor organs. Therefore, an effective therapeutic strategy against HCC is required.

In a previous study, we reported that proteasome activator 28γ (PA28γ) directly enhances the degradation of the HCV core protein and plays a key role in the genesis of hepatic steatosis and HCC in HCV core protein transgenic mice (10). Furthermore, the above events were not observed in PA28γ-knockout mice. The present study is an extension of our previous study and was designed to assess the utility of PA28γ expression as a biological marker for HCV-related human liver disease and HCC. The findings showed the presence of high levels of nuclear PA28γ in multistep hepatocarcinogenesis and HCC invasion, suggesting that selective inhibitors of nuclear PA28γ may be useful in the prevention and/or treatment of this disease.

Materials and methods

Tissue samples. The study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each subject for the use of tissue samples for medical research. Tissue samples were obtained from 51 patients with liver tumors, who underwent hepatectomy at the Department of Gastroenterological Surgery, Osaka University Hospital. All patients had HCV infection (28 patients) and some had HCV plus HBV infection (18 patients), but none had only HBV infection. The mean post-treatment follow-up period was 6.2±2.5 years ± standard deviation (SD). The excised hepatic tissue samples were examined immunohistochemically for PA28γ expression, including 46 paired HCCs. Non-tumor tissues were also examined, which comprised 15 CH-based livers (5 chronic active hepatitis and 10 chronic inactive hepatitis) and 31 cirrhotic livers. Prior to hepatectomy for HCC, 10 patients were treated with transarterial embolization (TAE). In these cases, histopathological examination showed complete hepatic necrosis. Histologically normal livers were also obtained from patients negative for hepatic viral infections who had liver metastasis secondary to colorectal cancer.

For immunohistochemistry, the tissue samples were fixed in 10% neutral buffered formalin, processed through graded ethanol and embedded in paraffin. The samples were frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis by reverse transcription-polymerase chain reaction (RT-PCR).

Histopathological examination. Tissue sections (4 μm thick) were deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin solution. Separation of the tissues into non-tumor and tumor tissues was determined by a pathologist (K.W.) who was blinded to the clinical background. For non-tumor tissues, the presence of inflammation or cirrhotic nodules was examined. Tumor tissues were examined for the following characteristics: cell differentiation (well, moderate, poorly differentiated), number of tumors, capsular formation, septal formation, capsular invasion, portal vein tumor thrombus formation and hepatic vein invasion.

Preparation of anti-human PA28γ antibody. Chicken anti-human PA28γ antibody was prepared by immunization using the synthetic peptides of residues from 75 to 88, SHDGLDGPTYKKRR, of human PA28γ. The antibody was purified by affinity chromatography using beads conjugated with the antigen peptide.

Immunohistochemistry and evaluation of PA28γ immunostaining. Formalin-fixed tissues were embedded in paraffin according to the standard procedures. For immunohistochemistry, formalin-fixed tissue sections were boiled in Target Retrieval Solution (Dako, Glostrup, Denmark) and then treated with 3% H_2O_2. The activated sections were washed twice with phosphate-buffered saline (PBS), blocked with PBS containing 5% bovine serum albumin, and incubated overnight with the purified chicken antibody to PA28γ, followed by incubation with horseradish peroxidase-conjugated anti-chicken IgG antibody (ICN, Biomedicals, Inc., Aurora, OH, USA) as a secondary antibody. Immunoreactive antigen was visualized with 3,3'-diaminobenzidine substrate. The resulting sections were counterstained with hematoxylin. Staining of endogenous PA28γ with the antibody was identified in normal mouse liver sections but not in the liver sections from PA28γ-deficient mice. Pre-immune purified antibody did not react with any other antigen in these sections under the experimental conditions.

For evaluation of PA28γ immunostaining, each section was scored for nuclear and cytoplasmic staining using a scale from 0 to 2 where 0 represented negative or faint staining, 1 represented moderate staining, and 2 represented strong staining. In general, the nuclei of the bile ducts faintly expressed PA28γ (Fig. 1a). Thus, the staining level was used as a nuclear inner control within the sample, which was designated arbitrarily as intensity level 0. Also, slightly higher expression was designated arbitrarily as intensity level 1 and clearly higher expression was designated arbitrarily as intensity level 2. PA28γ expression was very faint or undetectable in the vascular epithelia and nuclei (Fig. 1a), whereas the cytoplasm of bile duct epithelial cells and nuclei devoid of significant inflammation generally expressed faint levels of PA28γ (Fig. 1a). For semi-quantitative analysis, the latter level of staining was used as a cytoplasmic inner control within the sample, and designated arbitrarily as intensity level 0. Furthermore, a slightly higher expression was designated arbitrarily as intensity level 1 whereas clearly higher expression was designated arbitrarily as intensity level 2. PA28γ expression was generally heterogeneous in each sample. For assessment of nuclear and cytoplasmic PA28γ, 4 high-power fields in each specimen were selected at random, and staining was examined under high power magnification. More than 1,000 cells were counted to determine the labeling index, which represented the percentage of immunostained cells relative to the total number of cells. The tissue samples were also categorized as positive (levels 1 and 2) and negative (level 0) for evaluation of the relationship between immunostaining and various clinicopathological factors.

Semi-quantitative RT-PCR. RNA extraction was carried out with TRIzol reagent using the single-step method, and the cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described previously (11). Sterol regulatory element binding protein-1c (SREBP-1c) mRNA expression was analyzed semi-quantitatively using the multiplex RT-PCR method. In this assay, the

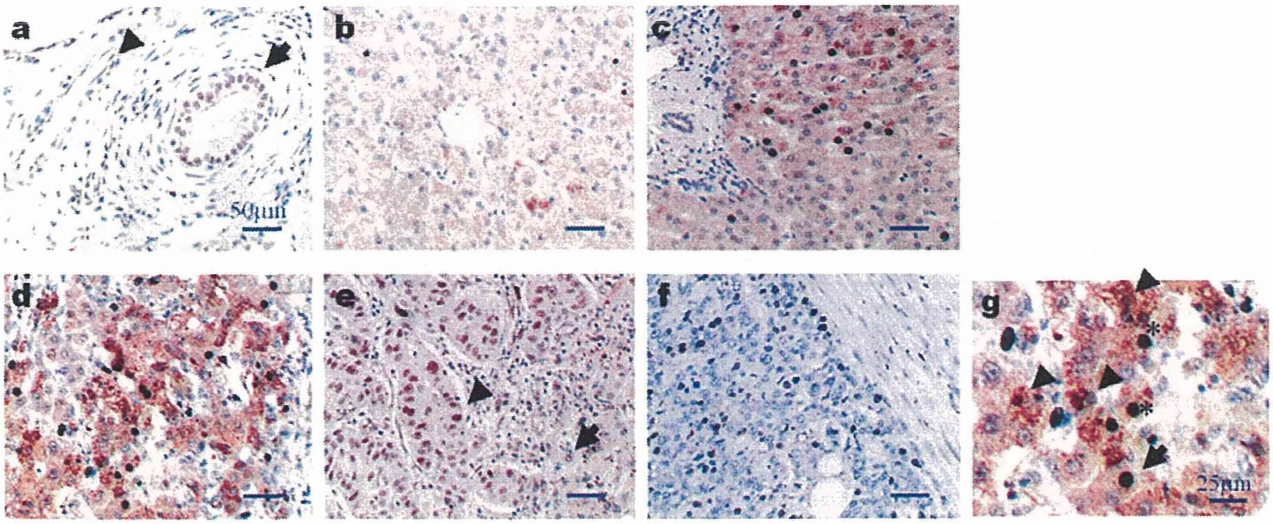

Figure 1. Immunohistochemical staining for PA28γ. (a-f) Representative samples for bile duct (inner control), vascular epithelium and various liver pathologies; (a) bile duct (arrow), vascular epithelium (arrowhead); (b) normal liver; (c) chronic hepatitis; (d) cirrhotic liver; (e) HCC with high nuclear PA28γ expression (arrowhead; left side) and non-tumor liver tissue with low nuclear PA28γ expression (arrow; right side); (f) HCC with low expression of nuclear PA28γ. Magnification, x200. (g) High-power view of liver section shown in (d). Note the faint staining of hepatocytes with high expression of nuclear PA28γ (arrow; hepatocytes, level 0 and nucleus, level 2), moderate staining of hepatocytes with high expression of nuclear PA28γ (asterisk; hepatocyte, level 1 and nucleus, level 2) and strong staining of hepatocytes with low expression of nuclear PA28γ (arrowhead; hepatocyte, level 2 and nucleus, level 0). Magnification, x400. No staining was observed when the primary antibody was substituted by non-immunized rabbit IgG or TBS (data not shown). PA28γ, proteasome activator 28γ; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; TBS, Tris-buffered saline.

housekeeping gene, porphobilinogen deaminase (PBGD), was used as the internal control. This gene is favored over β-actin or glyceraldehyde-3-phosphate dehydrogenase as a reference gene for competitive PCR amplification as the presence of pseudogenes for the latter housekeeping genes may produce false-positive signals from genomic DNA contamination (12,13). In addition, in order to minimize possible inter-PCR differences, PCR was performed with SREBP-1c and PBGD primers in an identical tube, under unsaturated conditions. PCR was performed in a 25-μl reaction mixture containing 1 μl of the cDNA template, 1X Perkin-Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.8 μM of each primer for SREBP-1c and 80 nM PBGD, and 1 unit of TaqDNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc.). The PCR primers used for the detection of SREBP-1c and PBGD cDNAs were synthesized as described previously (14,15). The conditions for multiplex PCR were one cycle of denaturation at 95˚C for 12 min, followed by 40 cycles at 95˚C for 1 min, 62˚C for 1 min and 72˚C for 1 min, and a final extension at 72˚C for 10 min. The electrophoresed PCR products were scanned by densitometry, and the relative value of the SREBP-1c band relative to that of PBGD was calculated for each sample.

Statistical analysis. Data were expressed as the means ± SD. The Chi-square test and Fisher's exact probability test, or the log-rank test, were used to examine the association between PA28γ expression and the clinicopathological parameters or prognosis. A P-value of <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using the StatView-J-5.0 program (SAS Institute, Cary, NC, USA).

Results

Immunohistochemical analysis of PA28γ. Immunohistochemical assays were performed on a series of 46 paired HCCs and their matched non-tumor tissues, and 5 normal livers. The labeling index of nuclear PA28γ showed a wide spectrum and increased from low in the normal livers to strong in the cirrhotic livers (Fig. 1b-d). Specifically, the nuclear PA28γ labeling index was generally low in the normal liver tissues, but was moderate-strong in HCV-related liver tissues. The nuclear labeling index was markedly higher in the majority of cirrhotic liver tissues. Fig. 2 summarizes the above results and the analysis of cytoplasmic expression of PA28γ. The difference in the PA28γ-nuclear labeling index between normal and cirrhotic livers was significant (P<0.0001) as was that between CH and cirrhosis (P<0.0001) (Fig. 2A). Also, the difference in the proportion of the PA28γ-cytoplasmic expression labeling index between normal and cirrhotic livers was significant (P<0.05) (Fig. 2B). The mean labeling indexes of nuclear PA28γ expression was 42% in both HCC and HCV-related livers.

To evaluate the relationship between immunohistochemical staining and various clinicopathological factors, we divided the samples into nuclear PA28γ high index (≥42%) and low index (<42%) groups. The labeling index was low in half of the examined HCC cases (50%; 18/36) and markedly high in the other half (50%; 18/36) (Table I). The labeling index was low in 30% (14/46) of HCV-related cases and markedly higher in the remaining 70% (32/46) (Table II). The samples were also divided into 2 groups according to the labeling index of cytoplasmic staining. The mean PA28γ-labeling index of the HCC and HCV-related cases was 58 and 80%, respectively. The labeling index was low in 47% (17/36) and high in 53% (19/36)

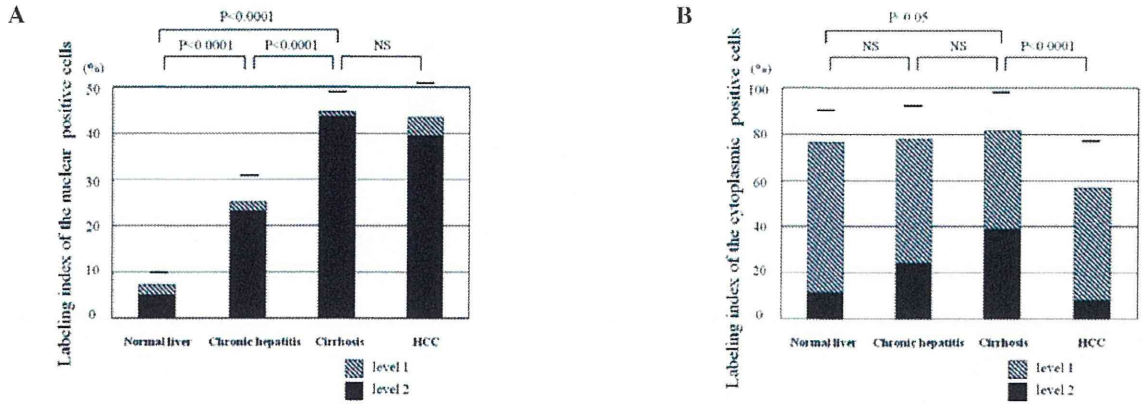

Figure 2. (A) Nuclear PA28γ expression in multistep hepatocarcinogenesis. The labeling index increased in a stepwise manner with the severity of liver damage and carcinogenesis. Quantitative analysis showed that 25, 10 and 1% of cells of the normal liver, CH and cirrhosis, respectively, were moderately positive (level 1). In HCCs, 10% of cells were evaluated as moderately positive (level 1). (B) Cytoplasmic PA28γ expression in multistep hepatocarcinogenesis. The expression increased slightly in a stepwise manner. Quantitative analysis showed that 80, 68 and 50% of cells of the normal liver, CH and cirrhosis, respectively, were moderately positive (level 1). In HCCs, 82% of cells were evaluated as moderately positive (level 1). PA28γ, proteasome activator 28γ; CH, chronic hepatitis; HCC, hepatocellular carcinoma. NS, not significant.

Table I. Correlation between nuclear PA28γ expression and various clinicopathological parameters in patients with HCC.

		PA28γ		
	n	Low (<42%)	High (≥42%)	P-value
Age (years)				
≥60	15	7	8	
<60	21	11	10	NS
Gender				
Male	21	10	11	
Female	15	8	7	NS
Tumor size				
≤2 cm	8	4	4	
>2 cm	28	14	14	NS
Histological type				
Well/moderately differentiated	5	2	3	
Poorly differentiated	31	16	15	NS
Hepatic vein invasion				
Yes	6	2	4	
No	30	16	14	NS
Portal vein tumor thrombus				
Yes	5	2	3	
No	31	16	15	NS
Number of tumors				
Multiple[a]	3	1	2	
Solitary	33	17	16	NS
Septum formation				
Yes	15	8	7	
No	21	10	11	NS
Capsular formation				
Yes	14	6	8	
No	22	12	10	NS
Capsular invasion				
Yes	8	1	7	
No	6	5	1	0.026

[a]This category includes intrahepatic metastasis and multicentric carcinogenesis. PA28γ, proteasome activator 28γ; HCC, hepatocellular carcinoma; NS, not significant.

Table II. Correlation between nuclear PA28γ expression and various clinicopathological parameters in non-tumor liver tissues.

		PA28γ		
	n	Low (<42%)	High (≥42%)	P-value
Age (years)				
≥60	22	5	17	
<60	24	9	15	NS
Gender				
Male	27	6	21	
Female	19	8	11	NS
HCV	28	9	19	
HBV	0			
HCV plus HBV	18	5	13	NS
Inflammatory status (HAI score)				
Absent-mild (0-3)	22	12	10	
Moderate-severe (>4)	24	2	22	0.0007
Degree of fibrosis (HAI score)				
Absent-moderate (0-2)	12	11	1	
Severe-cirrhosis (>3)	34	3	31	<0.0001

NS, not significant; PA28γ, proteasome activator 28γ; HCV, hepatitis C virus; HBV, hepatitis B virus; HAI, histological activity index.

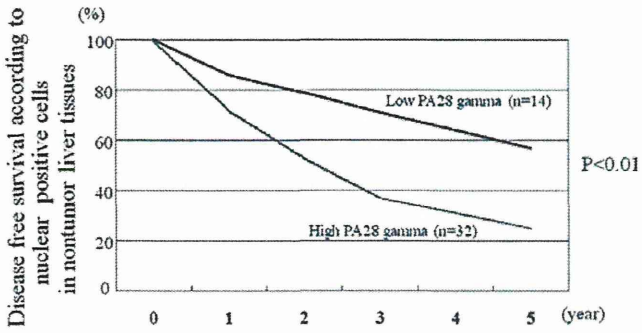

Figure 3. Disease-free survival based on nuclear PA28γ expression in non-tumor tissues. The disease-free survival was significantly different between patients with high nuclear PA28γ expression (levels 1 and 2) and those with low nuclear PA28γ expression (level 0) (P<0.01). PA28γ, proteasome activator 28γ.

of the HCC cases. The respective values for HCV-related cases were 28% (13/46) and 72% (33/46). All cut-off values used were according to the mean labeling index.

Correlation between nuclear PA28γ expression and clinicopathological parameters. We examined the correlation between PA28γ nuclear expression analyzed in 36 HCCs (10 samples with complete necrosis by TAE were excluded from this analysis) and various clinicopathological features (Table I). The cases were divided into two groups based on the labeling index of nuclear expression of PA28γ, using a cut-off mean value of 42%. There was a significant difference in PA28γ expression based on capsular invasion (Table I). We also analyzed the relationship between nuclear PA28γ expression in non-tumor tissues (15 CH and 31 cirrhosis) and disease-free survival, as the pathologic status of non-tumor tissues has been shown to correlate with the relapse of HCC (16-18). The disease-free survival, but not overall survival (P=0.052), was significantly different between high and low nuclear PA28γ expressors (P<0.01) (Fig. 3). In addition, PA28γ expression in non-tumor tissues correlated closely with active inflammation and fibrosis (Table II).

In univariate analysis, PA28γ expression in non-tumor liver tissues, portal vein tumor thrombus, inflammatory status and degree of fibrosis in the non-cancerous liver tissue were significant factors for disease-free survival. These variables were subsequently entered into multivariate analysis. The results identified nuclear PA28γ expression level [95% confidence interval (CI), 1.82-3.22; P<0.01], portal vein tumor thrombus (95% CI, 1.33-6.38; P=0.023), inflammatory status (95% CI, 2.11-3.58; P=0.012) and degree of fibrosis (95% CI, 1.99-7.21; P<0.01) as independent factors for disease-free survival (Table III).

SREBP-1c expression. Five CH and five cirrhotic liver tissues were selected to analyze the correlation between nuclear PA28γ expression and SREBP-1c gene expression in non-tumor liver tissues. Fig. 4 shows a clear correlation between nuclear PA28γ expression and SREBP-1c gene expression.

Discussion

The present study shows that non-tumor liver tissues commonly express high levels of nuclear PA28γ protein relative to those of carcinoma tissues. These results are contradictory to those from other studies on other types of cancer, such as thyroid carcinoma; the nuclear PA28γ level was higher in these tumors compared to non-tumor tissues (19). While the exact reason for

Table III. Multivariate analysis of clinicopathological factors for disease-free survival in patients with HCC.

	n	Relative risk	95% confidence interval	P-value
PA28γ				
High	32	2.67	1.82-3.22	<0.01
Low	14			
Portal vein tumor thrombus				
Yes	5	2.21	1.33-6.38	0.023
No	31			
Inflammatory status (HAI score)				
Absent-mild (0-3)	22	2.59	2.11-3.58	0.012
Moderate-severe (>4)	24			
Degree of fibrosis (HAI score)				
Absent-moderate (0-2)	12	2.68	1.99-7.21	<0.01
Severe-cirrhosis (>3)	34			

HCC, hepatocellular carcinoma; PA28γ, proteasome activator 28γ; HAI, histological activity index.

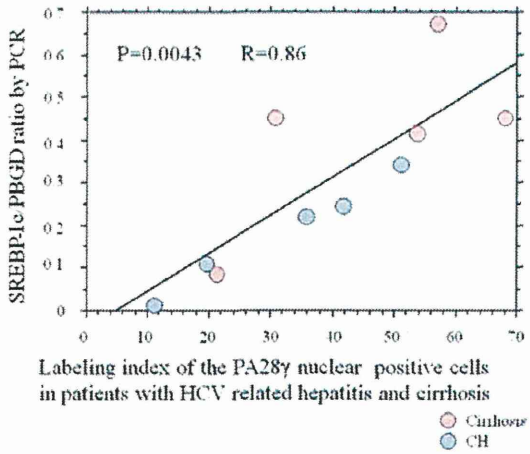

Figure 4. Linear correlation analysis of nuclear PA28γ expression and SREBP-1c gene expression in patients with cirrhosis and chronic hepatitis (CH) (P=0.0043). PA28γ, proteasome activator 28γ; HCV, hepatitis C virus. SREBP-1c, sterol regulatory element binding protein-1c.

the different results is not known at present, it is likely to be related to the type of control tissue used in the present study; the non-tumor tissues were mostly not normal, consisting of HCV-infected CH or cirrhotic tissues. In support of this conclusion, normal liver tissues from patients with metastatic liver tumors from patients with colorectal carcinoma who were negative for HCV/HBV showed low expression of nuclear PA28γ.

In non-neoplastic liver tissues, we found a wide spectrum of nuclear PA28γ expression from normal liver to cirrhosis. Our results also show that active inflammation with hepatitis virus induces nuclear PA28γ in CH and cirrhotic livers (Table II). This is reasonable considering the fundamental action of nuclear PA28γ as a mediator of inflammation. Another mechanism for the high induction of nuclear PA28γ in cirrhosis might be related to the degradation of the HCV core protein by PA28γ and its translocation from the cytoplasm to the nucleus, based on the results of our previous study (10). In fact, nuclear PA28γ-expressing cells had no or faint-to-moderate cytoplasmic PA28γ expression (Fig. 1c and g). Furthermore, the nuclear overexpression could be due to the relatively hypoxic microenvironment in the cirrhotic liver. In this regard, we hypothesized that hypoxia might directly induce PA28γ, which in turn enhances angiogenesis via the enhanced release of a battery of angiogenic growth factors, such as vascular endothelial growth factor (VEGF). Since the VEGF level is increased in cirrhosis (20), it is possible that nuclear PA28γ may improve the ischemic/hypoxic microenvironment in the cirrhotic liver through upregulation of angiogenesis. Although cirrhotic nodules occasionally show p53 mutation and increased telomerase activity (21,22), cirrhosis is not considered a premalignant lesion. However, it is apparent from a number of etiological studies that cirrhosis is a strong risk factor for HCC. In this context, nuclear PA28γ expression in cirrhosis might be a prerequisite for the genesis of premalignant dysplastic nodules or early cancer.

From a clinical point of view, it is interesting to note the correlation between high nuclear PA28γ expression in non-tumor tissues and the relapse of HCC. The prognosis of HCC is generally unfavorable. Although primary tumors are curatively resected, 50-60% of patients develop relapse within 5 years. This is due to either a newly established tumor from the remnant liver, a process termed multicentric carcinogenesis, or recurrence of the original tumor. One possible mechanism for a link between nuclear PA28γ and disease relapse is that high expression of PA28γ in the remnant liver may contribute to carcinogenesis. Nuclear PA28γ expression highly correlated with the presence of active inflammation (P<0.0001). Furthermore, active inflammation in non-tumor tissues has been reported to be associated with relapse of HCC (17,23,24).

In the present study, a clinicopathological survey demonstrated a significant correlation between nuclear PA28γ protein expression and capsular invasion of the cancer tissue. This

finding is in agreement with a recent study that showed increased expression of PA28γ protein during cancer progression and its correlation with PCNA labeling index (19). Thus, the results suggest the possible involvement of PA28γ in HCC progression. Further studies of larger population samples are required to confirm the clinical significance of nuclear PA28γ in HCC. This is particularly important, as the overall survival of patients with high nuclear PA28γ expression was worse than that of those with low expression level (P=0.052) (data not shown).

Also in our series, the labeling index of cytoplasmic expression of PA28γ significantly increased from normal liver to cirrhotic liver (Fig. 2b). Further extended studies are required to determine the importance of cytoplasmic expression of PA28γ in HCC and HCV-related liver.

In conclusion, the present study demonstrates a close correlation between nuclear PA28γ expression in liver tissue and the development and progression of HCC, as well as its possible involvement in HCC relapse. Further studies are required to examine the therapeutic benefits of the suppression of nuclear PA28γ expression in HCV-related CH, cirrhosis or HCC.

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Dysfunction of Autophagy Participates in Vacuole Formation and Cell Death in Cells Replicating Hepatitis C Virus▽§

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Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma. Infection with the HCV genotype 1 in particular is considered a clinical risk factor for the development of hepatocellular carcinoma, although the molecular mechanisms of the pathogenesis are largely unknown. Autophagy is involved in the degradation of cellular organelles and the elimination of invasive microorganisms. In addition, disruption of autophagy often leads to several protein deposition diseases. Although recent reports suggest that HCV exploits the autophagy pathway for viral propagation, the biological significance of the autophagy to the life cycle of HCV is still uncertain. Here, we show that replication of HCV RNA induces autophagy to inhibit cell death. Cells harboring an HCV replicon RNA of genotype 1b strain Con1 but not of genotype 2a strain JFH1 exhibited an incomplete acidification of the autolysosome due to a lysosomal defect, leading to the enhanced secretion of immature cathepsin B. The suppression of autophagy in the Con1 HCV replicon cells induced severe cytoplasmic vacuolation and cell death. These results suggest that HCV harnesses autophagy to circumvent the harmful vacuole formation and to maintain a persistent infection. These findings reveal a unique survival strategy of HCV and provide new insights into the genotype-specific pathogenicity of HCV.

Hepatitis C virus (HCV) is a major causative agent of bloodborne hepatitis and currently infects at least 180 million people worldwide (58). The majority of individuals infected with HCV develop chronic hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma (25, 48). In addition, HCV infection is known to induce extrahepatic diseases such as type 2 diabetes and malignant lymphoma (20). It is believed that the frequency of development of these diseases varies among viral genotypes (14, 51). However, the precise mechanism of the genotype-dependent outcome of HCV-related diseases has not yet been elucidated. Despite HCV's status as a major public health problem, the current therapy with pegylated interferon and ribavirin is effective in only around 50% of patients with genotype 1, which is the most common genotype worldwide, and no effective vaccines for HCV are available (35, 52). Although recently approved protease inhibitors for HCV exhibited a potent antiviral efficacy in patients with genotype 1 (36, 43), the emergence of drug-resistant mutants is a growing problem (16). Therefore, it is important to clarify the life cycle and pathogenesis of HCV for the development of more potent remedies for chronic hepatitis C.

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HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a single positive-stranded RNA genome with a nucleotide length of 9.6 kb, which encodes a single polyprotein consisting of approximately 3,000 amino acids (40). The precursor polyprotein is processed by host and viral proteases into structural and nonstructural (NS) proteins (34). Not only viral proteins but also several host factors are required for efficient replication of the HCV genome, where NS5A is known to recruit various host proteins and to form replication complexes with other NS proteins (39). In the HCV-propagating cell, host intracellular membranes are reconstructed for the viral niche known as the membranous web, where it is thought that progeny viral RNA and proteins are concentrated for efficient replication and are protected from defensive degradation, as are the host protease and nucleases (38).

Autophagy is a bulk degradation process, wherein portions of cytoplasm and organelles are enclosed by a unique membrane structure called an autophagosome, which subsequently fuses with the lysosome for degradation (37, 60). Autophagy occurs not only in order to recycle amino acids during starvation but also to clear away deteriorated proteins or organelles irrespective of nutritional stress. In fact, the deficiency of autophagy leads to the accumulation of disordered proteins that can ultimately cause a diverse range of diseases, including neurodegeneration and liver injury (12, 29, 30), and often to type 2 diabetes and malignant lymphoma (9, 32).

Recently, it has been shown that autophagy is provoked upon replication of several RNA viruses and is closely related to their propagation and/or pathogenesis. Coxsackievirus B3

utilizes autophagic membrane as a site of genome replication, whereas influenza virus attenuates apoptosis through the induction of autophagy (10, 59). Moreover, several groups have reported that HCV induces autophagy for infection or replication (5, 49); however, the role(s) of autophagy in the propagation of HCV is still controversial and the involvement of autophagy in the pathogenesis of HCV has not yet been clarified. In this study, we examined the biological significance of the autophagy observed in cells in which the HCV genome replicates.

MATERIALS AND METHODS

Plasmids. The plasmids pmStrawberry-C1, pmStrawberry-Atg4B^{C74A}, pmRFP-GFP-LC3, pEGFP-LC3, and pEGFP-Atg16L were described previously (7, 8, 24). The plasmids pFGR-JFH1 and pSGR-JFH1 were kind gifts from T. Wakita.

Cell culture. All cell lines were cultured at 37°C under a humidified atmosphere with 5% CO_2. Huh7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. For the starvation, the cells were cultivated with Earle's balanced salt solution (EBSS) (Sigma) for 6 h. HCV replicon cells were established as described previously (53). The plasmid pairs pFK-I$_{389}$ neo/NS3-3′/NK5.1 and pFK-I$_{389}$ neo/FGR/NK5.1 and pFGR-JFH1 and pSGR-JFH1 were linearized with ScaI or XbaI. The plasmids pFGR-JFH1 and pSGR-JFH1 were treated with mung bean exonuclease. The linearized DNA was transcribed *in vitro* by using the MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The transcribed RNA was electroporated into cells under conditions of 270 V and 960 mF using a Gene Pulser (Bio-Rad). All HCV replicon cells were maintained in DMEM containing 10% FBS, nonessential amino acids, and 1 mg/ml G418 (Nacalai).

Reagents and antibodies. Concanamycin A and bafilomycin A1 were purchased from Sigma and Fluka, respectively. E64D and pepstatin A were from Peptide Institute Inc. Rabbit anti-HCV NS5A polyclonal antibody was described previously (45). Mouse monoclonal anti-JEV NS3 antibody was prepared by immunization using the recombinant protein spanning amino acid residues 171 to 619 of JEV NS3. Rabbit polyclonal anti-LC3 (PM036), mouse monoclonal anti-RFP (8D6), and anti-62/SQSTM1 (5F2) antibodies were purchased from Medical & Biological Laboratories. Rabbit polyclonal anti-cathepsin B (FL-339) and mouse monoclonal anti-LAMP1 (H4A3) antibodies were from Santa Cruz Biotechnology. Mouse monoclonal anti-HCV NS5A (HCM-131-5), rabbit polyclonal anti-β-actin, and mouse monoclonal anti-Golgin97 (CDF4) antibodies were from Austral Biologicals, Sigma, and Invitrogen, respectively. Mouse monoclonal and rabbit polyclonal anti-cathepsin B antibodies were from Calbiochem. Mouse monoclonal anti-p62/SQSTM1 (5F2) and anti-ATP6V0D1 (ab56441) antibodies were from Abcam. Rabbit polyclonal anti-Atg4B antibody was from Sigma. Mouse anti-double-stranded RNA (dsRNA) IgG2a (J2 and K1) antibodies were from Biocenter Ltd. (Szirak, Hungary).

Transfection, infection, and immunoblotting. Transfection and infection were carried out as described previously (53). Each lysosome-enriched fraction was isolated by using the Lysosome Enrichment Kit for Tissue and Cultured Cells (Pierce) according to the manufacturer's protocol. Samples were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and detected by an LAS-3000 image analyzer system (Fujifilm). The protein bands of LC3 and β-actin were quantified by Multi Gauge software (Fujifilm), and the values of LC3 were normalized to those of β-actin.

Fluorescence microscopy. Cells were cultured on glass slides and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After being washed twice with PBS, the cells were permeabilized at room temperature for 20 min with PBS containing 0.25% saponin and then blocked with PBS containing 0.2% gelatin (gelatin-PBS) for 60 min at room temperature. The cells were incubated with gelatin-PBS containing appropriate antibodies at 37°C for 60 min and washed three times with PBS containing 1% Tween 20 (PBST). The resulting cells were incubated with gelatin-PBS containing corresponding fluorescent-conjugated secondary antibodies at 37°C for 60 min and then washed three times with PBST. The stained cells were covered with Vectashield mounting medium containing DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories Inc.) and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus). Time-lapse video microscopy was performed at 37°C with a DeltaVision microscope system (Applied Precision Inc.) equipped with a ΔTC3 culture dish system (Bioptechs) for temperature control.

Quantification of pro-cathepsin B. Each cell line was seeded on 12-well type I collagen-coated dishes (IWAKI) and cultured for 48 h. The supernatant and the cells were harvested and subjected to quantification of pro-cathepsin B by using Quantikine human pro-cathepsin B immunoassay (R&D Systems) according to the manufacturer's protocol.

Statistical analysis. Estimated values were represented as the means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

RESULTS

Autophagy is induced in the HCV replicating cell in a strain-dependent manner. To determine whether autophagy is induced during the replication of HCV, we investigated the phosphoethanolamine (PE) conjugation of LC3 in HCV replicon cells in which HCV RNA was autonomously replicating. As shown in Fig. 1A, the amounts of PE-conjugated LC-3 (LC3-II), a conventional marker for an autophagosomal membrane, in Huh7 cells were slightly increased by starvation, in conjunction with a reduction of the unmodified LC-3 (LC3-I). In contrast, the amount of LC3-II was significantly increased in the subgenomic and full genomic HCV replicon cells of the genotype 1b strain Con1 (SGR^{Con1} and FGR^{Con1}), whereas a small amount of LC3-II was detected in the full genomic replicon cells of the genotype 2a strain JFH1 (FGR^{JFH1}). We also examined the subcellular localization of LC3 by using confocal microscopy. Although LC3 was diffusely detected in the cytoplasm of naïve Huh7 cells, small foci of the accumulated LC3 appeared after starvation (Fig. 1B), whereas many LC3 foci that were larger in size than those in the starved cells appeared in the cytoplasm, particularly near the nucleus, in both SGR^{Con1} and FGR^{Con1} cells. However, a low level of LC3 focus formation comparable to that in the starved cells was observed in the FGR^{JFH1} cells. Most of the LC3 foci were not colocalized with NS5A, an HCV protein of the viral replication complex, in the HCV replicon cells, as reported previously (49). Elimination of HCV RNA from the SGR^{Con1} cells by treatment with alpha interferon (SGR^{cured}) abrogated the lipidation and accumulation of LC3 (Fig. 1C and D). Interestingly, overexpression of the HCV polyprotein of genotype 1b by an expression plasmid induced no autophagy (data not shown), suggesting that replication of viral RNA is required for induction of autophagy. Furthermore, neither lipidation nor accumulation of LC3 was observed in SGR^{JEV} cells harboring subgenomic replicon RNA cells of Japanese encephalitis virus (JEV), which is also a member of the family *Flaviviridae* (Fig. 1C and D). These results suggest that replication of HCV but not that of JEV induces autophagy.

The autophagy flux is impaired in the replicon cells of HCV strain Con1 after a step of autophagosome formation. To further examine the autophagy induced in the HCV replicon cells in more detail, Huh7 and SGR^{Con1} cells were treated with pepstatin A and E64D, inhibitors of aspartic protease and cysteine protease, respectively. In this assay, treatment of intact cells capable of inducing autophagy with the inhibitors increases the amount of LC3-II, whereas no increase is observed in cells impaired in the autophagic degradation. The amount of LC3-II was significantly increased in the naïve Huh7