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Specific interaction of the envelope glycoproteins E1 and E2 with liver heparan sulfate involved in the tissue tropismatic infection by hepatitis C virus

Fumi Kobayashi · Shuhei Yamada · Shuhei Taguwa · Chikako Kataoka · Satomi Naito · Yoshiki Hama · Hideki Tani · Yoshiharu Matsuura · Kazuyuki Sugahara

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Abstract The first step in the process of infections by the hepatitis C virus (HCV) is attachment to the host cell, which is assumed to be mediated by interaction of the envelope glycoproteins E1 and E2 with cell surface glycosaminoglycans. In this study, a variety of glycosaminoglycans, heparan sulfate (HS) from various bovine tissues as well as chondroitin sulfate (CS)/dermatan sulfate from bovine liver, were used to examine the direct interaction with recombinant E1 and E2 proteins. Intriguingly, among HS preparations from various bovine tissues, only liver HS strongly bound to both E1 and E2. Since HS from liver, which is the target tissue of HCV, contains highly sulfated structures compared to HS from other tissues, the present results suggest that HS-proteoglycan on the liver cell surface appears to be one of the molecules that define the liver-specific tissue tropism of HCV infection. The interaction assay with chemically modified heparin derivatives provided evidence that the binding of the viral proteins to heparin/HS is not only mediated by simple ionic interactions, but that the 6-*O*-sulfation and *N*-sulfation are important. Heparin oligosaccharides equal to or larger than 10-mer were required to inhibit the binding. Notably, a highly sulfated CS-E preparation from squid cartilage also strongly interacted with both viral proteins and inhibited the entry of pseudotype HCV into the target cells, suggesting that the highly sulfated CS-E might be useful as an anti-HCV drug.

Keywords Heparan sulfate · Chondroitin sulfate · Hepatitis C virus · Glycosaminoglycan · Tissue tropism

The contributions of Fumi Kobayashi and Shuhei Yamada should be considered equal.

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F. Kobayashi · S. Yamada (✉) · Y. Hama · K. Sugahara
Laboratory of Proteoglycan Signaling and Therapeutics, Hokkaido University Graduate School of Life Science,
West-11, North-21, Kita-ku,
Sapporo 001-0021, Japan
e-mail: shuheiy@meijo-u.ac.jp

S. Yamada · S. Naito · K. Sugahara (✉)
Department of Biochemistry, Kobe Pharmaceutical University,
Higashinada-ku, Kobe 658-8558, Japan
e-mail: k-sugar@sci.hokudai.ac.jp

S. Taguwa · C. Kataoka · H. Tani · Y. Matsuura
Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University,
Yamada-oka 3-1,
Suita-shi, Osaka 565-0871, Japan

Present Address:
S. Yamada
Department of Pathobiochemistry, Faculty of Pharmacy,
Meijo University,
Yagotoyama 150,
Tempaku-ku, Nagoya 468-8503, Japan

Abbreviations

2AB	2-aminobenzamide
CDNA	completely desulfated and *N*-acetylated heparin
CDNS	completely desulfated and *N*-sulfated heparin
NDNA	*N*-desulfated and *N*-acetylated heparin
2ODS	2-*O*-desulfated heparin
6ODS	6-*O*-desulfated heparin
CS	chondroitin sulfate
DS	dermatan sulfate
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HCV	hepatitis C virus

ΔHexA	4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid
HPLC	high performance liquid chromatography
HS	heparan sulfate
PG	proteoglycan
VSV	vesicular stomatitis virus
NS	2-*N*-sulfate
2S	2-*O*-sulfate
4S	4-*O*-sulfate
6S	6-*O*-sulfate

Introduction

Hepatitis C virus (HCV) is classified in the genus *Hepacivirus* within the family *Flaviviridae*, which includes classical flaviviruses (ex. yellow fever, dengue and tickborne encephalitis viruses) and animal pestiviruses (ex. bovine viral diarrhoea virus). Nearly 170 million people worldwide are infected with HCV [1]. Chronic HCV infections can lead to liver cirrhosis and hepatocellular carcinoma [2]. However, no specific antiviral drug is available for treatment. Therefore, a better understanding of the mechanism of HCV infection and the development of an effective anti-HCV drug are high priority tasks in medical and pharmaceutical communities.

The first step in the HCV infection process is the attachment of the virus to the host cell, which is assumed to be mediated by interactions of the envelope glycoproteins E1 and E2 with heparan sulfate (HS)-proteoglycan (PG) [3, 4] and a low-density lipoprotein receptor [5, 6]. Subsequently, specific binding between the viral glycoproteins and entry receptor proteins induces receptor-mediated endocytosis and the ingress of HCV particles across the plasma membrane of cells. There are several candidate receptor proteins on the host cell including a member of the tetraspanin protein family, CD81 [7], the scavenger receptor BI [8], and the tight-junction proteins human claudin-1 [9] and occludin [10]. Little is known about how these factors co-ordinate to facilitate the actual viral entry process. One current model predicts a multistep process that includes attachment, receptor binding, post-binding association with tight-junction proteins, and then internalization by endocytosis, which is followed by a pH-dependent step that results in the fusion of membranes and the release of the viral RNA into the cytoplasm of the host cells [11]. Several studies have demonstrated the role of glycosaminoglycans (GAGs) in the HCV adsorption and the binding of the E2 protein [3, 12, 13]. Recently, the interaction of glypican-3, a cell surface HS-PG, with CD81 in the liver has been demonstrated [14], suggesting the HCV particles to be transferred from the cell surface HS to CD81. However, little is known about the structural features of GAGs required for the binding of HCV to host cells.

GAGs are linear polymers composed of alternating amino sugar and hexuronic acid residues and distributed as side chains of PGs in the extracellular matrix or at the cell surface of animal tissues. GAG chains play important roles in various biological functions such as cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection, and wound repair [15–17] by interacting with bioactive molecules. Major GAGs include chondroitin sulfate/dermatan sulfate (CS/DS) and HS/heparin. Although the polysaccharide backbones of these GAGs are simple, repetitive linear chains, these structures acquire a considerable degree of variability by extensive modifications involving sulfation and uronate epimerization, which are the basis for a wide variety of their biological activities [17–19].

Many bacteria, parasites, and viruses exploit cell surface GAGs as receptors [17, 20, 21]. Among several GAG types present in animal cells, HS has been the most studied, and demonstrated to associate with various pathogens including dengue virus, herpes simplex virus type 1, human papillomavirus, and HCV [22–25]. Most interactions between adherent microorganisms and cell surface GAGs are considered to be nonspecific and ionic because of the high charge density of GAGs due to a cluster of sulfate groups. However, in some cases, unique sugar sequences in GAG chains appear to be involved in microbial adherence [20].

Detailed investigations of GAG structure not only should provide a better understanding of the mechanism of HCV attachment, but may also lead to the potential application of GAG as an anti-HCV drug. In the present study, specific binding of the E1 and E2 proteins to the HS from liver among the HS preparations from various bovine tissues was demonstrated, which revealed the tissue tropism of HCV infection. To characterize the structure of GAG chains involved in the HCV infection process, the sulfation and chain length required for binding to the E1 and E2 proteins were studied using heparin oligosaccharides. Moreover, the inhibition of HCV infectivity by highly sulfated CS/DS preparations was unveiled.

Materials and methods

Materials Chondroitinase ABC from *Proteus vulgaris*, standard unsaturated disaccharides, CS-C and CS-D from shark cartilage, CS-E from squid cartilage, chemically modified heparin derivatives (*CDNS*, completely desulfated and *N*-sulfated heparin; *CDNA*, completely desulfated and *N*-acetylated heparin; *NDNA*, *N*-desulfated and *N*-acetylated heparin), and HS from bovine kidney were obtained from Seikagaku Corp., Tokyo, Japan. 2-*O*-Desulfated heparin (*2ODS*) and 6-*O*-desulfated heparin (*6ODS*) derivatives were kindly provided by

Prof. Masayuki Ishihara (National Defense Medical College, Tokorozawa, Japan) [26, 27]. HS preparations from bovine intestine, aorta, and lung were kindly provided by Keiichi Yoshida (Seikagaku Corp., Tokyo, Japan) [28]. HS and CS/DS from bovine liver were prepared as described previously [29]. Recombinant heparinases I and III from *Flavobacterium heparinum* were from IBEX Technologies, Montreal, Canada. Anti-myc antibody, anti-V5 antibody, and ECL anti-mouse IgG horseradish peroxidase-linked whole antibody (from sheep) were obtained from Invitrogen Co., Carlsbad, CA. Phosphatase-labeled anti-mouse IgG+IgM (H+L) antibody was from Kirkegaard & Perry Laboratories, Inc., Geithersburg, MA. A soluble form of the recombinant envelope glycoprotein E1 of genotype 1b (comprising amino acids 192–340) with a V5 and His6 tag fusion protein or E2 of genotype 1b (comprising amino acids 384–711) with a myc and His6 tag fusion protein was generated using a baculovirus/HighFive cell system at 27 °C with Sf-900 II SFM insect cell medium (GIBCO) containing 10 % (v/v) fetal bovine serum (FBS). The expressed proteins were purified using a QIAexpress Protein Purification System (QIAGEN), following a protocol provided by the manufacturer. Concentration of the purified E1 and E2 proteins was estimated on the basis of silver staining using bovine serum albumin as the protein standard.

Analysis of the disaccharide composition of various GAGs An aliquot of the GAG samples was digested with chondroitinase ABC or a mixture of recombinant heparinases I and III as described previously [30, 31]. Each digest was labeled with 2-aminobenzamide (2AB) [32], and excess 2AB reagents were removed by extraction with chloroform [33]. The 2AB-labeled digest was analyzed by anion-exchange HPLC on a PA-03 silica column (YMC Co., Kyoto, Japan) [32]. Identification and quantification of the resulting disaccharides were achieved by comparison with the elution positions of authentic unsaturated disaccharides.

Western blotting The purified E1 and E2 proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5 % polyacrylamide gels (Ready Gels J, Bio-Rad laboratories Inc., Tokyo, Japan), and transferred to a hydrophobic polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). The recombinant E1 and E2 proteins were detected with anti-V5 and anti-myc mouse monoclonal IgG antibodies (diluted 1:1,000 in 25 mM Tris-buffered saline containing 2 % blocking reagent), respectively, using Amersham ECL Advance reagents (GE Healthcare).

Enzyme-linked immunosorbent assay (ELISA) GAG preparations (250 μg) were biotinylated using EZ-Link Biotin-LC-Hydrazide as recommended by the manufacturer or Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific Inc., Rockford, IL) [34]. Excess reagent was removed by dialysis against distilled water. ELISA plates (Thermo Fisher Scientific Inc.) were coated with 0.5 or 1 μg of biotinylated GAG per well at 4 °C overnight and then incubated with blocking buffer, 3 % bovine serum albumin in phosphate-buffered saline (PBS), for 1 h at room temperature. The recombinant E1 or E2 protein was added and incubated for 1 h at 37 °C. Since the molecular weight of E1 protein is approximately one sixth of that of E2 protein, the amount of the latter used was six times that of the former for the incubation to perform the assays at a similar molar concentration. After washing, the bound protein was detected by the addition of anti-V5 or anti-myc antibody (diluted 1:200 in PBS for 1 h at 37 °C) for the detection of E1 and E2, respectively, and then alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody (diluted 1:3,000 in Tris-buffered saline for 1 h at 37 °C). *p*-Nitrophenyl phosphate was used as the substrate for alkaline phosphatase.

For inhibition experiments, the recombinant E1 or E2 protein was preincubated for 30 min at room temperature with inhibitors (CS-E, CS-D, heparin, or heparin oligosaccharides) before being added to the biotinylated heparin-coated plate. After washing, the bound protein was detected using anti-V5 or anti-myc antibody and alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody as described above.

Effects of GAGs on infectivity of pseudotype vesicular stomatitis virus (VSV) possessing HCV envelope proteins (HCVpv) The construction of HCVpv and infection experiments were carried out as reported previously [35]. Briefly, HEK293T cells were transfected with an expression plasmid encoding the E1 and E2 proteins and incubated for 24 h at 37 °C. To incorporate these proteins into VSV, the cells were then infected with a VSVG-complemented pseudotype virus, in which the G envelope gene was replaced with the luciferase gene [35]. After 2 h of incubation at 37 °C, the cells were extensively washed four times with DMEM and harvested after incubation for 24 h at 37 °C. The HCVpv secreted in the conditioned medium of the infected cells was used for the infection experiment.

HCVpv was preincubated with various concentrations of CS-E, CS-D, HS, or heparin (0, 5, and 50 μg/ml) at 37 °C for 1 h and inoculated into the culture medium of Huh7 cells. After incubation for 1 h at 37 °C, the cells were washed with DMEM containing 10 % FBS three times and incubated at 37 °C for 24 h and the luciferase activity was measured.

Results

Characterization of GAGs in bovine liver tissue Hepatocytes are the main target cells of HCV. GAGs in liver tissue may contain unique structures required for the attachment of

HCV. To characterize the structural features in detail, GAGs were extracted from bovine liver, and the proportion of HS and CS/DS in GAGs derived from bovine liver was quantified to be 65 % and 35 %, respectively. HS was the major component in the GAG preparation from liver tissue. Their disaccharide composition was analyzed and the data are summarized in Tables 1 and 2. The major disaccharide unit in bovine liver HS was the trisulfated disaccharide ΔHexA (2S)-GlcN(NS, 6S) (43 %). The proportion of highly sulfated HS disaccharides (di- and trisulfated disaccharides) in bovine liver HS was 57 % (Table 1), whereas that in bovine aorta, lung, intestine, or kidney was 10 %, 21 %, 26 %, or 19 %, respectively [28], indicating the bovine liver HS to be more highly sulfated than HS from other bovine organs. The major disaccharide unit in CS/DS from bovine liver was ΔHexA-GalNAc(4S) (71 %), followed by the 4- and 6-*O*-disulfated disaccharide ΔHexA-GalNAc(4S, 6S) (25 %) (Table 2). The proportion of highly sulfated CS/DS disaccharides (di- and trisulfated disaccharides) found in the bovine liver (25 %) was significantly higher, compared with that of CS/DS preparations from bovine lung (13 %), trachea (0 %), and heart (15 %) [37, 38].

Interaction of the recombinant E1 and E2 proteins with GAGs derived from bovine liver The direct interaction of various GAGs with the recombinant E1 and E2 proteins was analyzed. HS from bovine liver, kidney, intestine, aorta, and lung as well as CS/DS from bovine liver were biotinylated and immobilized on a streptavidin-coated plate for ELISA. The recombinant proteins were produced by insect High Five cells and detected by Western blotting (Supplementary Data 1). Although their expected sizes were 10 and 40 kDa, respectively, E2 protein was larger than expected, consistent with a previous study indicating the posttranslational modification of the proteins [39]. Only bovine liver HS bound to both E1 and E2 proteins (172 % and 123 %, respectively, compared to the binding to heparin) (Fig. 1) in a concentration-dependent manner (data not shown). The interaction was confirmed using the BIAcore system. E1 and E2 proteins were individually injected at different concentrations onto the surface of the bovine liver HS-immobilized sensor chip. Overlaid sensorgrams are shown in Supplementary Data 2. Both E1 and E2 proteins bound to the bovine liver HS preparation in a concentration-dependent manner. In contrast, no significant binding of E1 or E2 to HS from other tissues or bovine liver CS/DS was observed (Fig. 1), indicating that the E1 and E2 proteins interact specifically with bovine liver HS, which appears to play the major role in the binding of HCV to liver cells, in consistent with the tissue tropism of the infection of HCV.

Determination of the sulfation structure required for the binding to E1 and E2 proteins To study whether the binding of the E1 and E2 proteins to heparin requires structurally defined HS oligosaccharides, the size effect of heparin oligosaccharides (ranging from di- to dodecasaccharides and polysaccharides) on the binding of E1 or E2 to immobilized heparin was analyzed. Although the reactivity of the E1 and E2 proteins with the immobilized heparin was strongly inhibited by free heparin polysaccharide chains, heparin oligosaccharides did not exhibit as much inhibitory activity as heparin polysaccharides. However, the 10-mer and 12-mer forms showed some inhibition (Fig. 2), indicating the minimum length required for the inhibition to be 10-mer. This result is consistent with a report that HCV pseudoparticles required heparin oligosaccharides of at least 10-mer for binding [40].

The structure required for the binding was characterized further. Chemically modified heparin derivatives were used to analyze the direct interaction. Both the E1 and E2 proteins bound strongly to *2ODS* (84 % and 58 %, respectively, compared to the binding to heparin) and moderately to *6ODS* (38 % and 23 %) and *NDNA* (34 % and 19 %), whereas no significant interaction of either protein with *CDNS* or *CDNA* was observed (Fig. 3), indicating that sulfation at the C2 (amino group) and C6 positions of GlcN residues is more important for the interaction than sulfation at the C2 position of uronic acid residues.

Table 1 Disaccharide composition of HS derived from various tissues (%)

	ΔHexA-GlcNAc	ΔHexA-GlcN(NS)	ΔHexA-GlcNAc(6S)	ΔHexA (2S)-GlcNAc	ΔHexA-GlcN(NS, 6S)	ΔHexA(2S)-GlcN(NS)	ΔHexA(2S)-GlcNAc(6S)	ΔHexA(2S)-GlcN(NS, 6S)	S/unit[b]	Ref. No.
Bovine Aorta	63	20	6	1	2	5	ND	3	0.50	28
Bovine Lung	45	16	17	1	9	8	ND	4	0.80	28
Bovine Intestine	45	19	9	1	8	11	ND	7	0.88	28
Bovine Kidney	53	16	11	1	6	7	ND	6	0.72	28
Bovine Liver	20	17	6	ND[a]	8	3	3	36	1.80	–
Human Liver	37	15	10	1	8	6	1	22	1.22	36

[a] *ND* not detected

[b] *S/unit* the number of sulfate groups per disaccharide unit

Table 2 Disaccharide composition of bovine liver CS/DS

CS/DS disaccharide	Proportion (%)
ΔHexA-GalNAc	ND[a]
ΔHexA-GalNAc(6S)	4
ΔHexA-GalNAc(4S)	71
ΔHexA(2S)-GalNAc(6S)	ND
ΔHexA(2S)-GalNAc(4S)	ND
ΔHexA-GalNAc(4S, 6S)	25
ΔHexA(2S)-GalNAc(4S, 6S)	ND
S/unit[b]	1.25

[a] *ND* not detected

[b] *S/unit* the number of sulfate groups per disaccharide unit

Effect of highly sulfated CS preparations on the infectivity of pesudotype HCV Since a highly sulfated structure is required for interaction with the E1 and E2 proteins, some CS preparations derived from marine animals, which are more highly sulfated than those from mammalian tissue, may bind the proteins. To investigate the potential of such highly sulfated CS from marine animals to inhibit the entry of HCV into host cells, effects on the infection by pesudotype HCV (HCVpv) of Huh7 cells were examined. Highly sulfated CS (CS-D and CS-E), heparin, and bovine liver HS preparations showed dose-dependent inhibition of HCVpv infection, whereas no significant effect was observed on the addition of low sulfated HS from bovine kidney (Fig. 4), indicating that highly sulfated CS and HS/heparin can inhibit the infection of Huh7 cells by HCVpv.

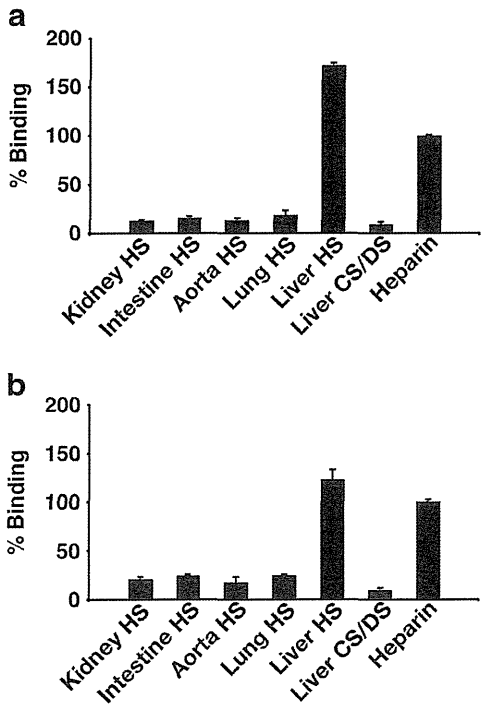

Fig. 1 Interaction of the recombinant E1 or E2 protein with GAGs from various bovine tissues. ELISA plates were coated with 1 μg/well of biotinylated GAGs from various bovine tissues or porcine intestinal heparin as described under "Materials and methods". Recombinant E1 (**a**) or E2 (**b**) protein (3 or 18 μg, respectively) was added and incubated for 1 h at 37 °C. After a wash with PBS/0.05 % Tween 20, the bound E1 and E2 proteins were detected using monoclonal anti-V5 and anti-myc antibodies, respectively, and then alkaline phosphatase-conjugated anti-mouse IgG/IgM secondary antibody. Data are shown as a percentage of the binding of the E1 or E2 protein to heparin. Values represent the mean±standard deviation (SD) (n=2)

Interaction of the recombinant E1 and E2 proteins with highly sulfated CS preparations To examine whether the highly sulfated CS preparations (CS-D and CS-E) bind directly to the recombinant E1 and E2 proteins, the interaction of the proteins with immobilized CS-D and CS-E was examined. Both the E1 and E2 proteins bound strongly to CS-E (69 % and 85 %, respectively, compared to heparin), but very weakly to CS-D (4 % and 19 %, respectively) (Fig. 5). These results may reflect the difference in their total negative charge as represented by the number of sulfate groups per disaccharide unit. CS-D and CS-E contain 1.2 and 1.6 sulfate groups per disaccharide [41], respectively. To further characterize the binding of the recombinant E1 and E2 proteins to highly sulfated CS, the inhibitory effect of CS-E and CS-D on the binding of the E1 and E2 proteins to immobilized heparin was examined. The binding to E1 or E2 was weakly inhibited by CS-D and CS-E or CS-D, respectively (11 % and 22 % or 31 % inhibition at 10 μg/well, respectively, compared to the binding in the absence of inhibitors), whereas the binding to E2 was strongly inhibited by CS-E (54 % inhibition at 5 μg/well, compared to the binding in the absence of inhibitors) (Fig. 6), supporting the higher affinity of CS-E than CS-D for the E1 and E2 proteins.

Discussion

Several lines of evidence have demonstrated that GAGs play an important role in the attachment of HCV to host cells [3, 12, 13, 40]. Among GAGs, heparin has been well studied for its interaction with the envelope glycoproteins E1 and E2 [3, 40]. Heparin is distributed in the cytoplasmic granules of mast cells *in vivo* and more highly sulfated than HS, which is ubiquitous on the cell surface [42]. HS at the cell surface in human liver was predicted to interact with the HCV envelope proteins based on reports by Barth *et al.* [3, 40, 43]. However, no direct interaction between the viral proteins and GAGs derived from liver tissue has been shown. In this study, we characterized the disaccharide composition of the GAGs derived from bovine liver and demonstrated the direct interaction of the highly sulfated HS from bovine liver with both E1 and E2 proteins for the first time, although Barth *et al.* have demonstrated inhibition of the cellular binding of E1 and E2 proteins to human

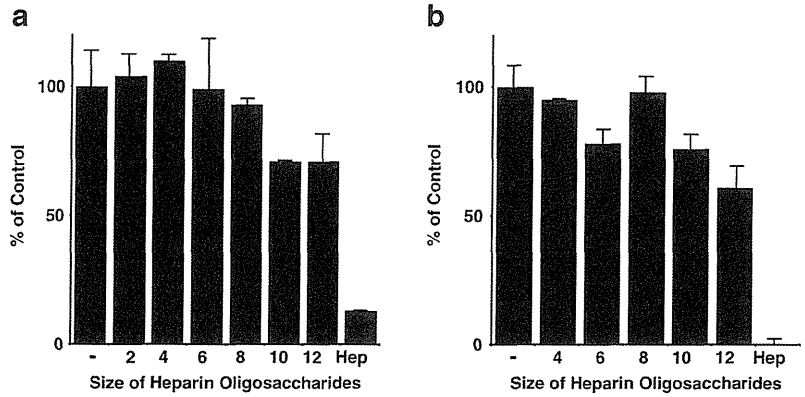

Fig. 2 Inhibition of the interaction of the E1 or E2 protein with heparin by heparin oligosaccharides. The E1 (**a**) or E2 (**b**) protein (0.75 or 4.5 μg, respectively) was preincubated with size-defined heparin oligosaccharides ranging from di- to dodecasaccharides and polysaccharides (15 μg each) for 30 min at room temperature and then the mixture was added to an ELISA plate coated with biotinylated heparin (0.5 μg per well). The bound E1 and E2 proteins were detected using anti-V5 and anti-myc antibodies, respectively. Data are shown as a percentage of the binding in the absence of heparin oligosaccharides. Values represent the mean±SD (n=2)

hepatoma cell lines by liver-derived HS [40]. Intriguingly, among the HS preparations from various tissues, only liver HS bound to E1 and E2 proteins strongly. Since HS from human liver, which is the target tissue of HCV, contains highly sulfated structures (di- and trisulfated disaccahrides) accounting for 37 % of all disaccharides (Table 1) [36], the present results provide further evidence that HCV utilizes cellular HS for attachment to the target tissue. HS-PG on the liver cell surface appears to be one of the molecules that define the liver-specific tissue tropism of HCV, in addition to the internal ribosome entry site (IRES)-dependent HCV tropism [44]. The participation of HS in virus tropism to different tissues has been suggested [21, 45].

Although bovine liver HS was used for the interaction with the E1 and E2 proteins, HCV infects only human and chimpanzee but not bovine liver. The species tropism of HCV is modulated at the level of cell entry, and recently it has been reported that the expression of CD81 and occludin, which are important for HCV entry, is sufficient to allow the HCV infection of mice [46]. Since the primary structure of HS is common among mammals, and liver HS is generally highly sulfated [47], HS in liver is likely involved in the enrichment of HCV particles before or together with CD81 and/or occludin.

The recombinant E1 and E2 proteins prepared in this study were the ectodomain of the viral envelope proteins. They are truncated immediately upstream its transmembrane region, and soluble. The recombinant E1 and E2 proteins have been suggested to adopt a native conformation based on the interaction with conformation-sensitive monoclonal antibodies as well as inhibition of the infection

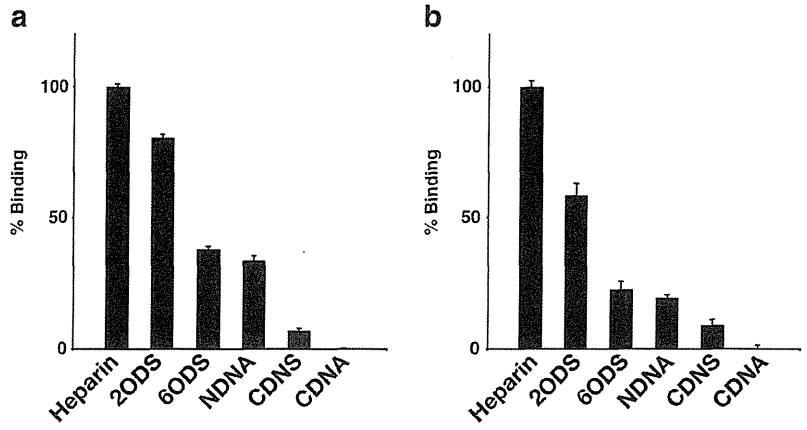

Fig. 3 Interaction of the E1 or E2 protein with chemically modified heparin preparations. ELISA plates were coated with biotinylated heparin derivatives, *CDNS*, *CDNA*, *NDNA*, *2ODS*, *6ODS*, or unmodified heparin (1 μg each per well). The recombinant E1 (**a**) or E2 (**b**) protein (0.75 or 4.5 μg, respectively) was added and incubated for 1 h at 37 °C. The bound E1 or E2 protein was detected using monoclonal anti-V5 or anti-myc antibody. Data are shown as a percentage of the binding of the E1 or E2 protein to unmodified heparin. Values represent the mean±SD (n=2)

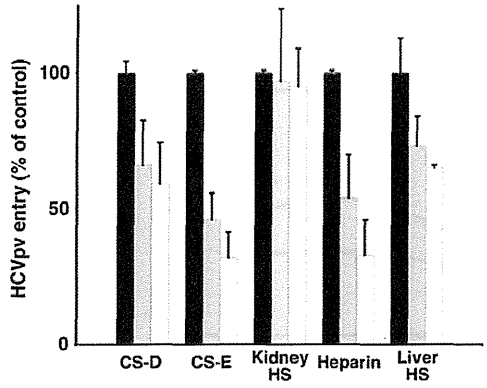

Fig. 4 Effect of highly sulfated CS on the infectivity of pesudotype HCV (HCVpv) to Huh7 cells. HCVpv was preincubated with CS-D, CS-E, HS, or heparin at a concentration of 0 μg/ml (closed columns), 5 μg/ml (hatched columns), and 50 μg/ml (open columns) for 1 h at 37 °C, and the mixture was added to the culture medium of Huh7 cells. After 2 h of incubation at 37 °C, the cells were washed three times with DMEM containing 10 % FBS, and the luciferase activity was measured after 24 h. Data are shown as a percentage of the infectivity of HCVpv to Huh7 cells in the absence of GAGs. Values represent the mean±SD (n=3)

of Huh 7.5 cells by infectious HCV particles [48]. However, the conformation of the recombinant soluble proteins might be different to some extent from that of the native proteins, because liver HS, which shows higher affinity to the recombinant proteins (Fig. 1), was less effective in the inhibition of the infection by HCVpv (Fig. 4) than heparin.

The binding of GAGs to viral proteins is mediated by simple effects of charge and/or highly specific interactions as described for HS [20]. The interaction of E1 and E2 with bovine liver GAGs or chemically modified heparin derivatives provides evidence that the binding of the viral proteins to heparin/HS is not only mediated by simple interactions, but most likely includes a specific interaction with a defined structure present in HS. *2ODS* bound strongly to both E1 and E2, whereas neither *6ODS* nor *NDNA* heparin derivatives did in spite of their similar degree of sulfation (approximately 1.6 sulfate groups per disaccharide unit), indicating the importance of 6-*O*-sulfation and *N*-sulfation in the binding of the proteins. Bovine liver HS (1.80 sulfate groups per disaccharide unit), which is not as highly sulfated as heparin (2.40 sulfate groups per disaccharide unit), bound at least as strongly to the viral proteins as heparin. Barth *et al.* [40] observed that de-*N*-sulfated heparin lost its inhibitory effect on the binding of the E1 and E2 proteins to heparin, while neither de-2-*O*- nor de-6-*O*-sulfated heparin did, suggesting that *N*-sulfation but neither 2-*O*- nor 6-*O*-sulfations is important for the interaction with the viral proteins. This difference in the specificity with which heparin binds to the viral proteins may be due to the experimental design (assays of inhibition or direct interaction). Different methods of preparing the chemically modified heparin derivatives may also be a cause.

The size of the saccharides also seemed to be important in the binding of E1 and E2 to heparin. No significant inhibition of the binding of the proteins to immobilized heparin was shown by heparin oligosaccharides shorter than 10-mer, indicating that a length of at least 10-mer is required for the binding. However, even long oligosaccharides (up to 20-mer) did not exhibit as much inhibitory effect as heparin polysaccharides (data not shown). The longer their chains become, the more effectively oligosaccharides appear to inhibit the binding of E1 or E2 to the immobilized heparin.

CS/DS derived from bovine liver, which is more highly sulfated than CS/DS from other organs [17], bound to neither E1 nor E2. Although both highly sulfated CS/DS and HS are expressed in liver, only the latter specifically bound to E1 and E2 (Fig. 1). It has been demonstrated that treatment of the host cells with heparinase but not with chondroitinase

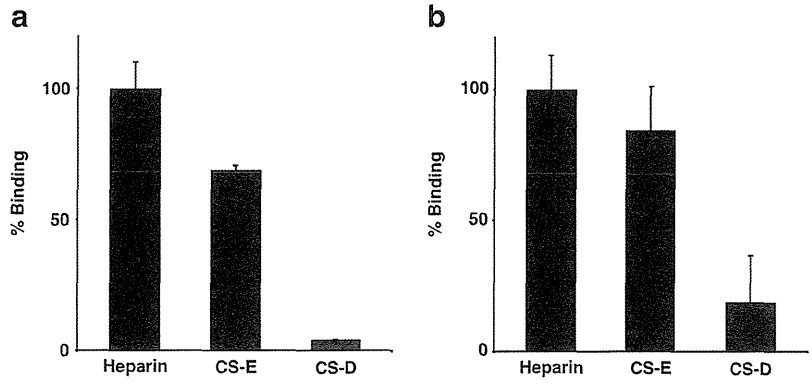

Fig. 5 Interaction of the recombinant E1 or E2 protein with highly sulfated CS preparations. ELISA plates were coated with 0.5 μg/well of biotinylated heparin, CS-E, or CS-D as described under "Materials and methods". The recombinant E1 (**a**) or E2 (**b**) protein (0.75 or 4.5 μg, respectively) was added and incubated for 1 h at 37 °C. The bound E1 and E2 proteins were detected using monoclonal anti-V5 and anti-myc antibodies, respectively. Data are shown as a percentage of the binding of the E1 or E2 protein to heparin. Values represent the mean±SD (n=2)

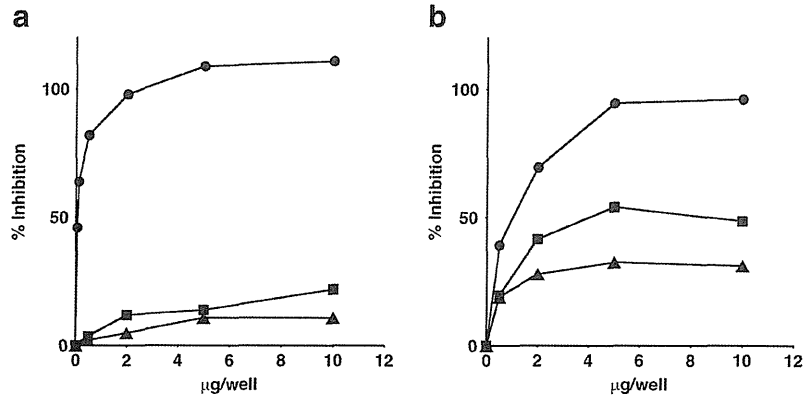

Fig. 6 Inhibition of the binding of biotinylated heparin to the recombinant E1 or E2 protein by CS-E, CS-D, and heparin. The recombinant E1 (**a**) or E2 (**b**) protein (1.2 or 3.6 μg, respectively) was preincubated with CS-D (triangles), CS-E (squares), or heparin (circles) (0, 0.05, 0.1, 0.5, 2, 5, or 10 μg per well) for 30 min at 37 °C and then added to an ELISA plate coated with biotinylated heparin (0.5 μg per well). The bound E1 or E2 protein was detected using monoclonal anti-V5 or anti-myc antibody, respectively. Data are shown as percent inhibition of the binding of the E1 or E2 protein to biotinylated heparin in the absence of inhibitors. Values represent the mean (n=2)

ABC reduced the infectivity of pseudotype HCV, suggesting the importance of HS on the host cell surface for the infection [49]. In contrast to HS, CS/DS seems to contribute little to the cellular attachment of HCV at least in liver tissue. It, however, has not been clarified whether CS/DS from human liver tissue contains a highly sulfated CS-E-like structure that can bind to the HCV envelope proteins.

Highly sulfated CS from marine animals, CS-E and CS-D (1.6 and 1.2 sulfate groups per disaccharide unit, respectively), interacted with both viral proteins (Fig. 5). Since highly sulfated heparin derivatives (*6ODS* and *NDNA*; 1.6 sulfate groups per disaccharide unit) bound only weakly to the viral proteins (Fig. 3), the interaction of the two viral proteins with GAGs may be mediated by not only simple charge effects but also by defined sulfated structures. CS-D and CS-E also inhibited the entry of pseudotype HCV into the target cells (Fig. 4), suggesting the potential medical application of these highly sulfated CS as an anti-HCV drug.

HS-PG in the extracellular matrix is known to function as a reservoir for various growth factors including fibroblast growth factors (FGFs) and prevent proteases from degrading the growth factors [50, 51]. Liver HS may also hold HCV particles and protect them from attack by the immune system of the host. HCV may be concentrated at the liver cell surface through interaction with membrane-bound HS-PG, leading to an acceleration of the infection. The FGF receptor has been revealed to be involved in infections by HCV (Matsuura, Y. *et al.*, unpublished data). The ternary complex formed by HS, FGF, and the FGF receptor has been well investigated, and demonstrated to be required for the mitogenic effects of FGFs [18, 52, 53]. HCV may also be able to form a ternary complex with HS and the FGF receptor. To investigate this possibility, we performed competition ELISA experiments. The binding of E1 and E2 proteins with heparin was inhibited by the addition of basic FGF in a dose-dependent manner (results not shown), indicating that basic FGF and the viral proteins recognize the same or partially overlaping saccharide sequences in heparin. Determination of the sequences in GAGs specific for the binding to E1 and E2 proteins is required for characterization of their interaction to develop anti-HCV drugs.

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Upregulation of nuclear PA28γ expression in cirrhosis and hepatocellular carcinoma

MOTOI KONDO[1], KOHJI MORIISHI[2], HIROSHI WADA[3], TAKEHIRO NODA[3], SHIGERU MARUBASHI[3], KENICHI WAKASA[4], YOSHIHARU MATSUURA[2], YUICHIRO DOKI[3], MASAKI MORI[3] and HIROAKI NAGANO[3]

[1]Evidence Based Medical Research Center, Osaka; [2]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka; [3]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka; [4]Department of Diagnostic Pathology, Graduate School of Medicine, Osaka City University, Osaka, Japan

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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.

Correspondence to: Dr Hiroaki Nagano, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, Osaka 565-0871, Japan
E-mail: hnagano@gesurg.med.osaka-u.ac.jp

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

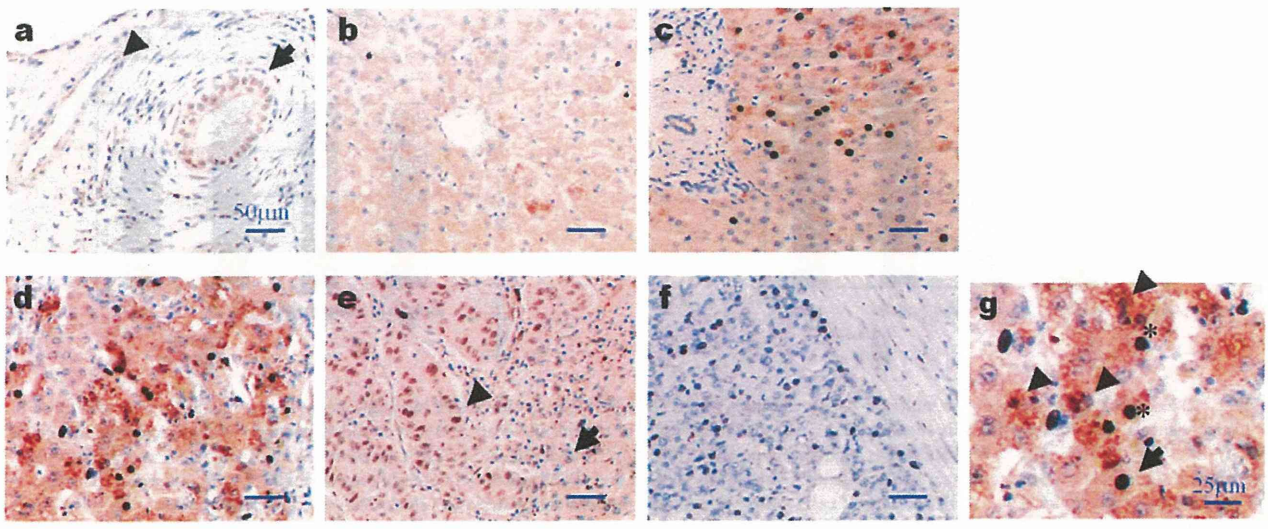

Figure 1. Immunohistochemical staining for PA28γ. (a-f) Representative samples for bile duct (inner control), vascular epithelium and various liver pathologies; (a) bile duct (arrow), vascular epithelium (arrowhead); (b) normal liver; (c) chronic hepatitis; (d) cirrhotic liver; (e) HCC with high nuclear 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)

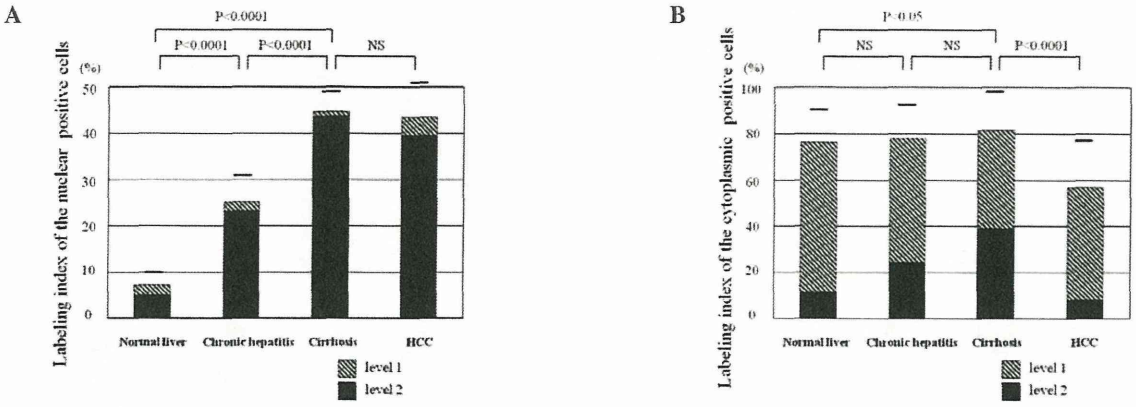

Figure 2. (A) Nuclear PA28γ expression in multistep hepatocarcinogenesis. The labeling index increased in a stepwise manner with the severity of liver damage and carcinogenesis. Quantitative analysis showed that 25, 10 and 1% of cells of the normal liver, CH and cirrhosis, respectively, were moderately 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.

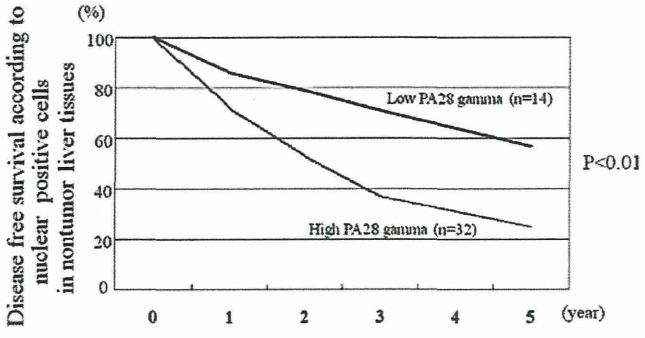

Figure 3. Disease-free survival based on nuclear PA28γ expression in non-tumor tissues. The disease-free survival was significantly different between patients with high nuclear PA28γ expression (levels 1 and 2) and those with low nuclear PA28γ expression (level 0) (P<0.01). PA28γ, proteasome activator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

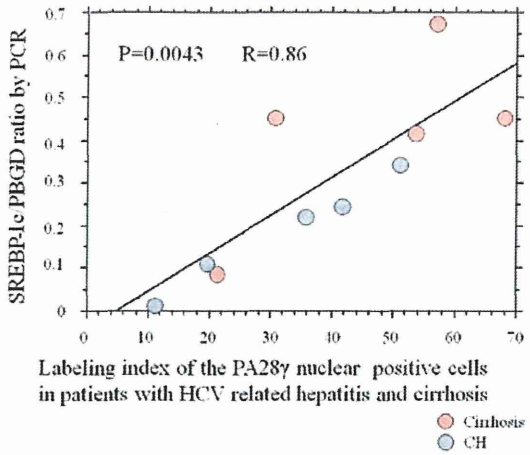

Figure 4. Linear correlation analysis of nuclear PA28γ expression and SREBP-1c gene expression in patients with cirrhosis and chronic hepatitis (CH) (P=0.0043). PA28γ, proteasome activator 28γ; HCV, hepatitis C virus. SREBP-1c, sterol regulatory element binding protein-1c.

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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Human BDCA3^{+} dendritic cells are a potent producer of IFN-λ in response to hepatitis C virus

Sachiyo Yoshio[1], Tatsuya Kanto[1]*, Shoko Kuroda[1], Tokuhiro Matsubara[1], Koyo Higashitani[1], Naruyasu Kakita[1], Hisashi Ishida[1], Naoki Hiramatsu[1], Hiroaki Nagano[2], Masaya Sugiyama[3], Kazumoto Murata[3], Takasuke Fukuhara[4], Yoshiharu Matsuura[4] , Norio Hayashi[5], Masashi Mizokami[3], and Tetsuo Takehara[1]

1 Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

2 Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan

3 The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1, Kohnodai, Ichikawa 272-8516, Japan.

4 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University

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5 Kansai Rosai Hospital, 3-1-69 Amagasaki, Hyogo, Japan

*Corresponding author: Tatsuya Kanto, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, 565-0871 Japan

E-mail: kantot@gh.med.osaka-u.ac.jp

Telephone: +81-6-6879-3621, Fax: +81-6-6879-3629

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