TABLE 5. K_A/K_S ratios of paired samples among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	0.343[1]	—						
Bovine	0.284	0.374	—					
Porcine	0.289	0.355	0.386	—				
Marmoset	0.228	0.411	0.335	0.367	—			
Cebus	0.220	0.414	0.298	0.354	0.273	—		
Orangutan	0.306	0.517	0.445	0.510	0.780	0.808	—	
Human	0.304	0.516	0.411	0.485	0.694	0.763	0.786	—

[1]K_A/K_S ratios calculated using MEGA 2.1 software (Kumar et al., 2001).

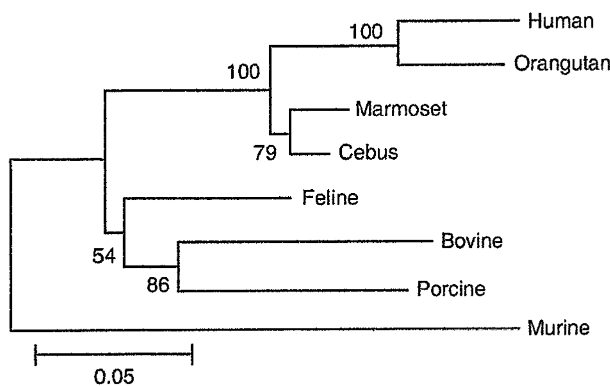

Fig. 4. A phylogenetic tree of the α1,3-GT enzyme. A phylogenetic tree of the amino acid sequences deduced from the nucleotide sequences for murine, feline, porcine, borine, marmoset and cebus α1,3-GT genes and the human and orangutan α1,3-GT pseudogenes was constructed by the N–J method using the MEGA 2.1 software. The phylogenetic relationships of the amino acid sequences are represented as unrooted cladograms. The numbers at branch nodes indicate the bootstrap support level (BSL), which is the percentage of how often each branch presents exactly the same topology in all the resampled trees. The scale bars indicate the number of substitutions per site.

even when α1,3-GT mRNA of human or orangutan is translated.

In our sequence alignment, it appeared that the amino acid residues 61–83 in the feline α1,3-GT gene form a highly variable region and the residues between 84 and 371 form a highly conserved region. This finding also suggests that the catalytic domain of the feline α1,3-GT gene is located in the highly conserved 84–370 domain. In the corresponding domains of animal sequences, including the human and orangutan pseudogenes, the functional constraints appear to have restricted mutations throughout evolution by Wilson et al. ('77). Since most of the amino acid gaps were located between positions 45 and 84 of the feline α1,3-GT sequence, this portion is unlikely to contain catalytic activity.

The α1,3-GT gene is transcribed in a small amount but the enzyme encoded is not active in higher primates (humans, apes and OWM) (Koike et al., 2002): the reason for loss of this enzyme activity in higher primates is unknown. There are several reports on production of α1,3-GT gene-deficient mice (knock-out mice). These mice can grow, live and age normally (Tearle et al., '96; Pearse et al., '98), indicating that the α1,3-GT gene can be dispensable for rodents, although this gene has been evolutionarily conserved as shown in Table 2. On the contrary, the expression of the α1,3-GT gene in human cells did not affect the growth and morphology of human cells, as we could isolate HOS/HTLV-I cell lines highly expressing α1,3-GT (Table 1 and Fig. 1). Thus, its expression did not exert adverse effects on human cells at least in tissue culture. It is intriguing for us that the α1,3-GT genes of marmoset and cebus and the pseudogenes of orangutan and human show similar degrees of substitution rates in their nucleotide sequences as well as in their amino acid sequences to the murine, bovine and porcine genes.

The rate of synonymous substitution (K_S) is usually much higher than that of non-synonymous substitution (K_A) for a normally functioning gene. Synonymous substitution may be used as a molecular clock for dating the evolutionary time of closely related species (Kafatos et al., '77; Kimura, '77; Miyata and Yasunaga, '80; Perler et al., '80). K_A/K_S ratios less than 1.0 are generally taken as negative or purifying selection, and, conversely, K_A/K_S ratios significantly greater than 1.0 are considered to be proper evidence of

directional or positive selection for amino acid replacement (Li et al., '85; Li, '93). That is, when the K_A/K_S value for a given gene is less than 1.0, the encoded protein sequence has been conserved during evolution. For example, the histone H4 gene family protein sequences are under purifying selection (Piontkivska et al., 2002) and some lineages of the primate lysozyme protein sequences are under directional selection (Messier and Stewart, '97). Among the α1,3-GT genes, the synonymous substitution rate is significantly higher than the non-synonymous substitution rate between the paired sequences ($P<0.0005$). Generally, the values of possible non-synonymous substitutions are much higher than the values of possible synonymous substitutions. Among the α1,3-GT genes we examined, the ranges of possible non-synonymous and synonymous substitution sites were 795–810 and 287–296, respectively.

Pairwise K_A/K_S ratios are high (0.7–0.8) in the species where the enzyme had lost its activity (human or orangutan), but are much lower (0.2–0.4) in the species where the enzyme is active. Although the α1,3-GT gene in marmoset and cebus is active and the K_A/K_S value between them is 0.273, their pairwise K_A/K_S values with human and orangutan pseudogenes are as high as 0.7–0.8. High K_A/K_S values (among human, orangutan and cebus) may indicate that these genes have evolved rapidly at the protein level or tend to evolve free of constraint, although their pairwise K_A/K_S ratios are not greater than 1. It is noteworthy that most of the non-synonymous substitutions are concentrated in a region that locates outside of the catalytic domain of the enzyme (Fig. 3).

Humans are known to carry natural antibodies against α1,3-galactosyl epitope at high titers and these antibodies are also known to markedly affect transplantation of xenografts derived from porcine organs and will lead to rejection (Sandrin and McKenzie, '94). A pig strain with knock-out of this gene has already been made (Dor et al., 2004). These antibodies should, however, be beneficial for survival of higher primates. These natural antibodies have been thought to exert prophylactic effects on development of cancer or infection with certain pathogens bearing α-galactosyl epitope (Gollogly and Castronovo, '96; Welsh et al., '98). It remains to be elucidated why α1,3-GT genes should have been inactivated in humans, apes and OWM and why the inactivated gene sequences still have a high degree of similarity to those of the active genes.

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Editor-Communicated Paper

Tandem Repeats of Lactoferrin-Derived Anti-Hepatitis C Virus Peptide Enhance Antiviral Activity in Cultured Human Hepatocytes

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Abstract: **Previously, we found that bovine and human lactoferrin (LF) specifically inhibited hepatitis C virus (HCV) infection in cultured non-neoplastic human hepatocyte-derived PH5CH8 cells, and we identified 33 amino acid residues (termed C-s3-33; amino acid 600–632) from human LF that were primarily responsible for the binding activity to the HCV E2 envelope protein and for the inhibiting activity against HCV infection. Since the anti-HCV activity of C-s3-33 was weaker than that of human LF, we speculated that an increase of E2 protein-binding activity might contribute to the enhancement of anti-HCV activity. To test this possibility, we made two repeats [$(C\text{-}s3\text{-}33)_2$] and three repeats [$(C\text{-}s3\text{-}33)_3$] of C-s3-33 and characterized them. Far-Western blot analysis revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and that the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$. Using an HCV infection system in PH5CH8 cells, we demonstrated that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33. Furthermore, using a recently developed infection system with a VSV pseudotype harboring the green fluorescent protein gene and the native E1 and E2 genes, we demonstrated that the antiviral activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33. These results suggest that tandem repeats of LF-derived anti-HCV peptide are useful as anti-HCV reagents.**

Key words: **Hepatitis C virus, Lactoferrin, Anti-HCV peptide, E2 protein-binding activity**

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (28). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae*. The HCV genome encodes a large polyprotein precursor of about 3,000 amino acids (aa), which is cleaved by the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (7, 8, 18). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (1, 17).

Approximately 170 million people worldwide are infected with HCV (32). The combination of a pegylated interferon with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (6). This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma.

Although the entry mechanism of HCV remains unclear, to date, several candidates for HCV receptors

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Abbreviations: aa, amino acids; DMEM, Dulbecco's modified Eagle's medium; E2, envelope 2; GFP, green fluorescent protein; HCV, hepatitis C virus; LF, lactoferrin; MBP, maltose-binding protein; NS2, non-structural 2; TF, transferrin; VSV, vesicular stomatitis virus.

have been reported: CD81, the scavenger receptor class B type I, the mannose-binding lectins DC-SIGN and L-SIGN, low-density lipoprotein receptors, etc. (4). Most of them have been identified as interacting materials with a soluble and truncated form of the HCV E2 protein, because of the lack of efficient HCV proliferation in cell cultures, although several culture systems using PCR for detection of HCV infection have been reported (20). However, a major advance in investigating HCV entry has been achieved by the development of pseudotype viruses bearing HCV E1 and E2 proteins assembled onto retrovirus particles (2, 9) or vesicular stomatitis virus (VSV) particles (3, 23, 30). Extensive characterization of the pseudotype viruses has shown that these mimic the early steps of the HCV life cycle. This system has allowed the study of the role of candidate receptors in the early steps of HCV infection (4).

We previously found that bovine and human lactoferrins (LFs) specifically prevented HCV infection in cultured human non-neoplastic hepatocyte PH5CH8 cells using the PCR method for detection of HCV infection (10, 12). Regarding these findings, some clinical studies have demonstrated that monotherapy with bovine LF improves the serum HCV RNA and/or alanine aminotransferase levels in patients with chronic hepatitis C (15, 16, 27, 31).

LF is an 80-kDa mammalian iron-binding glycoprotein and consists of two homologous globular lobes (an N-lobe and a C-lobe), each with a single iron (Fe^{3+}) binding site. It is structurally related to the plasma iron-transport protein transferrin (TF). LF's biological roles include activities in the host defense mechanism as well as in iron metabolism (21, 22). Unlike TF, LF is a primary defense protein against microbial infection. LF possesses strong bacteriostatic and bactericidal activities against pathogenic bacteria, as well as inhibitory activity against pathogenic viruses (5, 21, 22, 33).

LF's preventive mechanism against HCV infection has been thought to be the direct interaction between LF and HCV; indeed, by Far-Western blot analysis using thioredoxin-fused LF fragments expressed in *Escherichia coli* (*E. coli*) and the soluble E2 protein expressed in Chinese hamster ovary cells, we demonstrated that the 93 carboxyl aa of LF (human, bovine, and horse), termed C-s3, specifically bound to the E2 protein (25). On the other hand, Yi et al. (34) independently reported that the E1 and E2 proteins could bind to human and bovine LFs, although the binding region of LF was not identified. Furthermore, we identified the 33 aa of human LF (termed C-s3-33; aa 600–632), which was primarily responsible for the E2 protein-binding activity, and demonstrated that maltose-binding protein (MBP)-fused C-s3-33 prevented HCV infection in PH5CH8 hepatocyte cells (25). However, the E2 protein-binding activity and the anti-HCV activity of C-s3-33 were obviously weaker than those of human LF. Therefore, we presumed that the increase of the E2 protein-binding activity would lead to the enhancement of anti-HCV activity.

To evaluate this idea, we made tandem repeats of C-s3-33, and compared their E2 protein-binding activities and anti-HCV activities with those of the C-s3-33. Here, we report our findings that the anti-HCV activity of the tandem repeats were stronger than that of the monomer when accompanied by the enhancement of the E2 protein-binding activity, by analyses using not only the HCV infection system but also the infection system of a VSV pseudotype bearing the native forms of HCV E1 and E2 proteins.

Materials and Methods

Cell cultures. Simian virus 40 large T antigen-immortalized non-neoplastic human PH5CH8 hepatocytes were maintained as described previously (11, 24). Human hepatoblastoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of expression plasmids for E. coli. The pMAL-c2X (hLF600–632) (25) expression plasmid for the MBP-fused C-s3-33 LF fragment, was used as a template for the PCR using a primer set of hLFB6 5'-TGATAGGATCCGTGGTGTCTCGGATGATAAGG-3' containing the *Bam*HI recognition site (underlined) (25) and 632R6A 5'-ATCCATCCGAGACACCA-CAAACTTGTCCGGGCAGTCAGATCC-3' containing an extra 18 nts (underlined) encoding the amino-terminal 6 aa of the C-s3-33 LF fragment. After PCR (20 cycles) using KOD-plus DNA polymerase (Toyobo, Osaka, Japan), the amplified PCR product was used as a template for a second PCR using the primer set of hLFB6 and 632R 5'-TAATAAAGCTTT-TAAAACTTGTCCGGGCAGTCAGATCC-3' containing the *Hin*dIII recognition site (underlined) (25). After PCR (35 cycles) using KOD-plus DNA polymerase, amplified PCR products (approximately 200 bp for the two-repeat form and approximately 300 bp for the three-repeat form) were subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and were used as expression plasmids for the production of the MBP-fused $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$.

To prepare an expression plasmid for the production of the MBP-fused C-s3-33-relevant fragment (aa 587–619) of human TF, pCXbsr/huTF (29) encoding full-length human TF was used as a template for the PCR using a primer set of hTF587F 5'-TGATAG-

GATCCGTGGTCACACGG-3' containing the *Bam*HI recognition site (underlined) and hTF619R 5'-TAATAAAGCTTTTAAAAGTTGCCCG-3' containing the *Hin*dIII recognition site (underlined). After PCR (35 cycles) using KOD-plus DNA polymerase, the amplified PCR product was subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and was used as the expression plasmid.

Expression and purification of the MBP-fused protein. Expression and purification of the MBP-fused LF fragment [C-s3-33, $(\text{C-s3-33})_2$, or $(\text{C-s3-33})_3$] or the MBP-fused C-s3-33-relevant fragment of human TF were carried out as described previously (25). Briefly, the expression plasmid for MBP-fused protein was transformed into the *E. coli* strain JM109. The transformants were cultured at 37 C for several hours, and the harvested cells were sonicated. After removal of insoluble cellular debris by centrifugation, the supernatant obtained as the soluble fraction was applied onto an amylose resin affinity column (New England Biolabs) to obtain the MBP-fused protein. The purity of the obtained MBP-fused protein was evaluated to be more than 95% by electrophoresis on 10% SDS-PAGE gels. The concentration of the purified MBP-fused protein was determined by using Coomassie protein assay reagent (Pierce). The MBP2 (43 kDa) produced from the pMAL-c2X with a stop codon inserted into the *Xmn*I site was used as a control protein.

Far-Western blot analysis. Far-Western blot analysis was carried out as described previously (25). Briefly, 0.5 μg of human LF, MBP2, and MBP-fused LF fragments were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with N-buffer (25), a binding reaction was carried out using the secreted form of the E2 protein (E2-681) consisting of aa 384–681 expressed in Chinese hamster ovary cells as a probe (14), and then rat monoclonal antibody, MO-12 (13), against E2 protein was used for the detection of E2 protein-bound MBP-fused LF fragments.

Assay for anti-HCV activity of MBP-fused protein. An assay for anti-HCV activity of the MBP-fused LF fragment was carried out by the method described previously (25). Briefly, 2 μl (2×10^4 HCV) of the HCV-positive serum HCV-O (previously described as 1B-2 (19)) (genotype 1b) and the MBP-fused LF fragment (final concentration, 0.5, 1.0, and 2.0 mg/ml) were preincubated for 60 min at 4 C and then inoculated onto to the PH5CH8 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed three times with PBS and further cultured for 1 day at 32 C. Cellular RNA (0.5 μg) prepared by ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) was used for the quantitative analysis of HCV RNA using LightCycler PCR as described previously (26). As the positive and negative controls for anti-HCV activity, human LF and MBP2, respectively, were used.

Assay for anti-VSV pseudotype activity of MBP-fused protein. For this assay, the VSV pseudotype VSVΔG*(HCV), bearing the native forms of HCV E1 and E2 proteins from the O strain (19), was used. VSVΔG*(HCV) was prepared by introducing the native form of E1 and E2 proteins into recombinant VSV, VSVΔG*, which harbors the green fluorescent protein (GFP) gene instead of the VSV G envelope protein gene (30). An assay for the anti-VSV pseudotype activity of the MBP-fused LF fragment was carried out by a method described previously (30). Briefly, VSVΔG*(HCV) (Approximately 100 IU/assay) was pre-incubated with the MBP-fused LF fragment (final concentration, 0.1–1.0 mg/ml) at 37 C for 60 min and inoculated onto PH5CH8 or HepG2 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed with DMEM three times and incubated with fresh culture medium. VSVΔG*G was used as a control in this assay. After 24 hr of incubation, each infectious titer was determined by counting the number of GFP-expressing cells under a fluorescence microscope. As the positive and negative controls for the assay, human LF and MBP2 were used, respectively. Human TF and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay.

Results

Two and Three Repeats of the Human LF Fragment (C-s3-33) Strengthened the E2 Protein-Binding Activity

Previously we found that bovine and human LFs prevented HCV infection in PH5CH8 cells via direct interaction between LF and HCV (10, 12), and we further identified 33 aa residues (C-s3-33; aa 600–632 of human LF) as an essential and minimum domain possessing binding activity for the HCV E2 protein (secreted form consisting of aa 384–681) and inhibiting activity against HCV infection (25). This result suggested that the E2 protein-binding activity contributes to the anti-HCV activity. However, the E2 protein-binding activity of C-s3-33 was somewhat weaker than that of human LF (25), and the anti-HCV activity of C-s3-33 (IC_{50}=20 μM) in the infection system using PH5CH8 cells was also weaker than that of human LF (IC_{50}=5 μM) (25). To improve these points, we first tried to

enhance the E2 protein-binding activity of C-s3-33 by the multiplication of C-s3-33. Initially, we made pMAL-c2X-based expression vectors encoding two, three, and four repeats of C-s3-33 as MBP-fused proteins, and then expressed them in *E. coli.* We successfully purified two repeats $(C\text{-}s3\text{-}33)_2$ and three repeats $(C\text{-}s3\text{-}33)_3$ of C-s3-33 as soluble forms of the MBP-fused protein; the purification of the four repeats of C-s3-33 failed due to problems with solubility. Using the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, we performed Far-Western blot analysis to compare their E2 protein-binding activities. The result revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$ (Fig. 1). Although the E2 protein-binding activity of C-s3-33 was weaker than that of human LF, the binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$

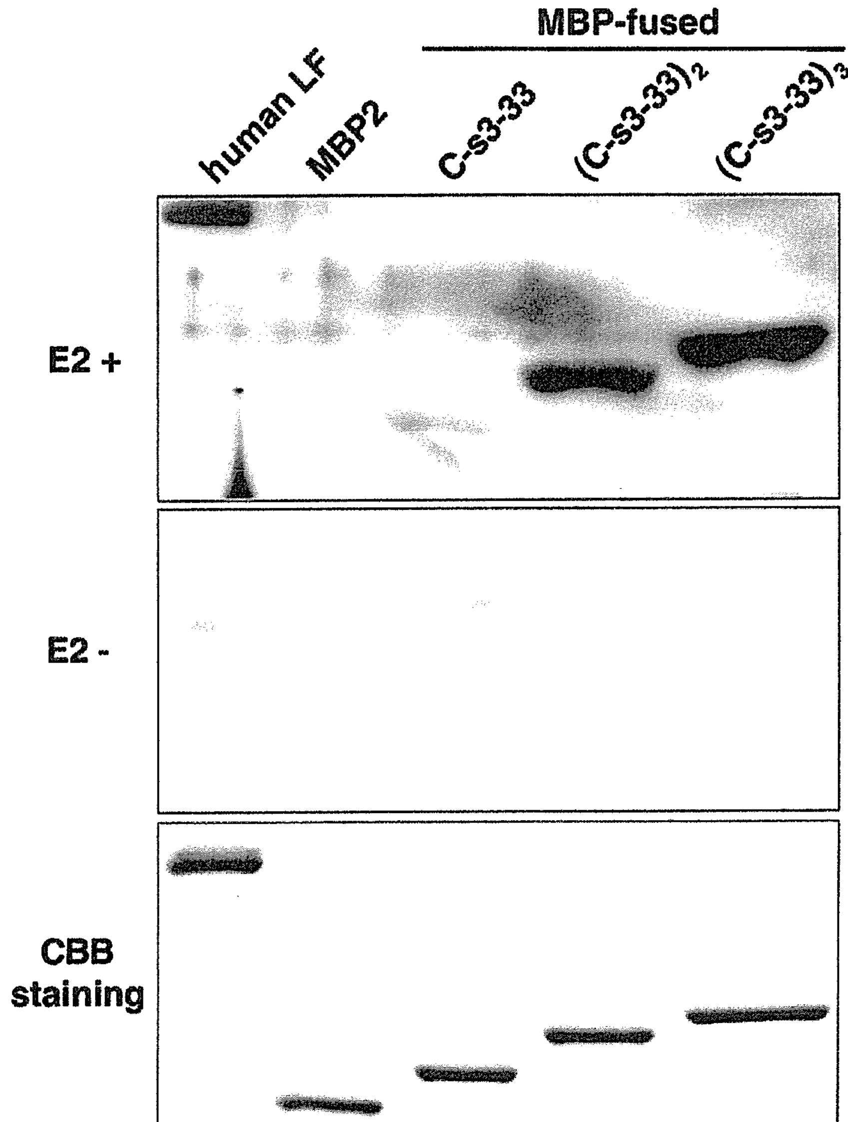

Fig. 1. Comparison of the E2 protein-binding activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$. MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ (0.5 μg each) were resolved by 10% SDS-PAGE. Human LF and MBP2 (0.5 μg each) were also used for the assay as control materials. Far-Western blot analysis using the E2 protein expressed in Chinese hamster ovary cells (14) as a probe was performed as described under "Materials and Methods." Rat monoclonal antibody MO-12 (13) against the E2 protein was used for the detection of the E2 protein bound to MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ as well as human LF. Far-Western blot analysis in the absence of the E2 protein was also performed. The bottom panel shows the results for human LF, MBP2, and MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ detected by staining with Coomassie Brilliant Blue.

became comparable with that of human LF (Fig. 1). To exclude the possibility of cross-reactions between C-s3-33 and the anti-E2 antibody, we performed a Far-Western blot analysis in the absence of the E2 protein. No significant bands were obtained in this control experiment (Fig. 1). The Far-Western blot analysis using normal rat serum instead of anti-E2 antibody also detected no significant bands (data not shown). These results suggest that the specific E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ increase with the degree of multiplication of C-s3-33.

$(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ Efficiently Prevented HCV Infection in PH5CH8 Cells

Since we obtained the expected results that the E2-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33, we next compared their anti-HCV activities in our HCV infection system using PH5CH8 cells (10, 25). The obtained result (Fig. 2) revealed that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ (IC_{50}=10 μM in both) became stronger than that of the C-s3-33 (IC_{50}=23 μM), although their activities were somewhat weaker than that of human LF (IC_{50}=5 μM). These results support the previous suggestion that the E2 protein-binding activity of C-s3-33 contributes to the inhibition of HCV infection (inoculum HCV-O) in human hepatocyte cells (25). However, in our HCV infection system, we failed to clearly show a difference in inhibiting activities between $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, because each standard deviation became somewhat large value due to the low level of cell culture-based HCV infection (20, 25, 31). In order to improve this point, we developed an infection system with VSVΔG*(HCV), a VSV pseudotype bearing the native E1 and E2 proteins derived from HCV-O (30), and this VSV pseudotype was used for further analysis as described below.

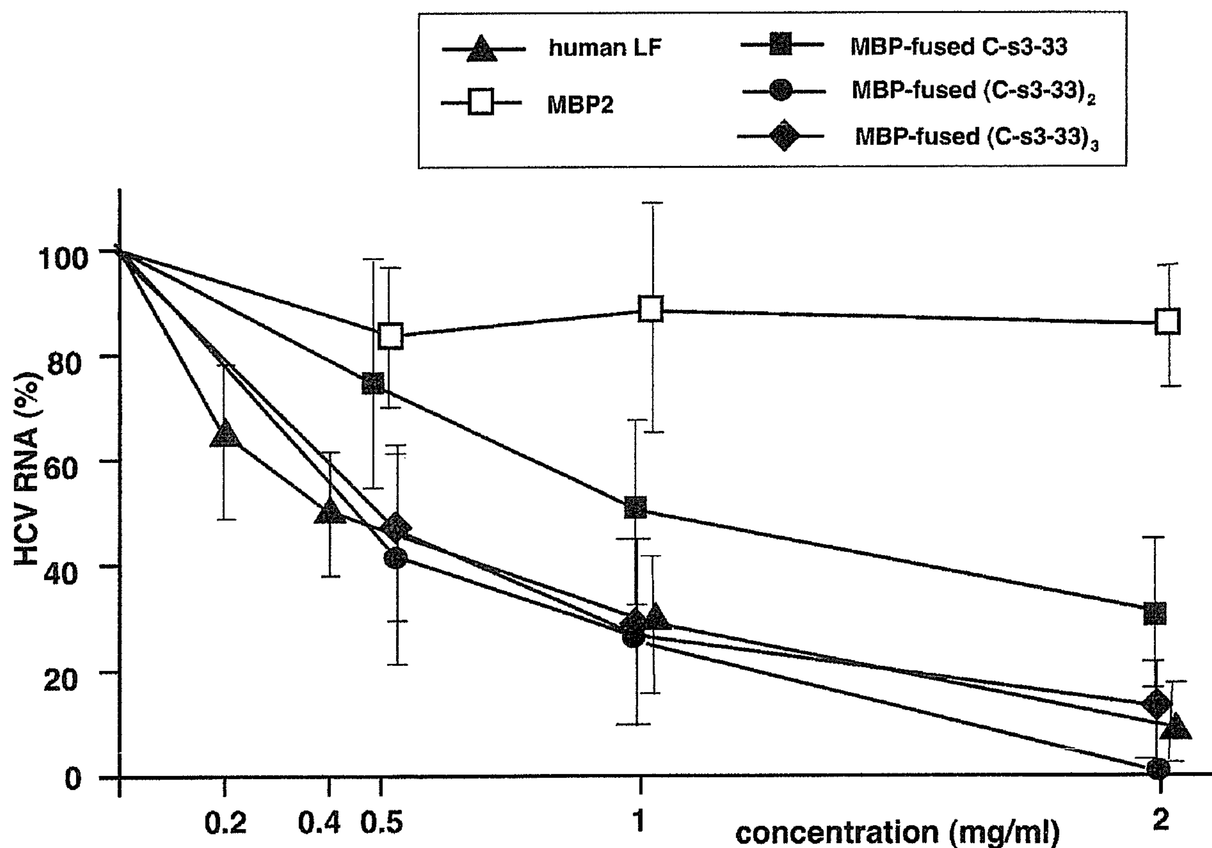

Fig. 2. Anti-HCV activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in an HCV infection system using PH5CH8 cells. PH5CH8 cells and the inoculum HCV-O were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the percent of HCV RNA determined by real-time LightCycler PCR (26). Approximately 2,000 copies of HCV RNA per μg of cellular RNA were reproducibly obtained using this HCV infection system (10, 26). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF and MBP2 were also used for the assay as control materials. The data are means ± SD of triplicates from three independent experiments.

Antiviral Effects of (C-s3-33)$_2$ and (C-s3-33)$_3$ against VSVΔG(HCV) Infection in PH5CH8 Cells*

Since PH5CH8 cells showed good susceptibility to our developed VSV pseudotype, VSVΔG*(HCV) (30), we examined the antiviral effects of (C-s3-33)$_2$ and (C-s3-33)$_3$ against VSVΔG*(HCV) infection in PH5CH8 cells, and compared them with those of the C-s3-33 and human LF. In this experiment, the antiviral effects of human TF and a C-s3-33-relevant fragment of human TF were also examined. The results (Fig. 3) clearly showed that human LF (IC_{50}=0.6 μM) strongly inhibited VSVΔG*(HCV) infection, but that human TF and the C-s3-33-relevant fragment of human TF did not, nor did MBP2, suggesting that inhibition against VSVΔG*(HCV) infection also occurred in an LF-specific manner as observed previously in the HCV infection system (25, 31). These results support previous findings (23, 30) using the VSV pseudotype infection system. Furthermore, we obtained clear results that C-s3-33 showed inhibiting activity against VSVΔG*(HCV) infection, and that its inhibiting activity was increased with multiplication of C-s3-33. The IC_{50} doses of C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ were 17 μM, 5.0 μM, and 3.0 μM, respectively. This result indicates that antiviral activity of C-s3-33 is improved by the duplication and triplication of C-s3-33, although the antiviral activity of (C-s3-33)$_3$ is still weaker than that of human LF. We confirmed that these LF fragments did not inhibit VSVΔG*G (control virus without E1 and E2 proteins) infection in PH5CH8 cells (data not shown). In summary, our results suggest that direct interaction of the C-s3-33 fragment with the E2 protein in VSVΔG*(HCV) prevents the virus infection in PH5CH8 cells.

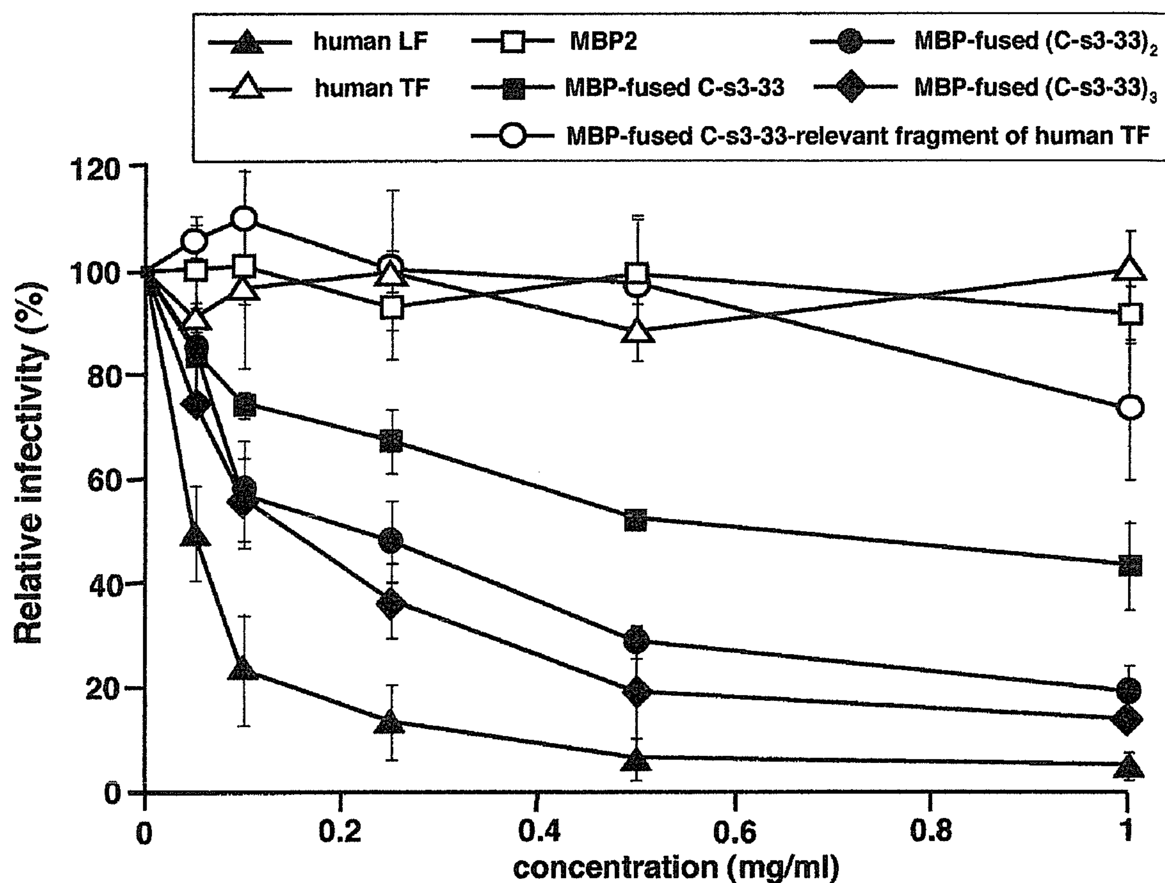

Fig. 3. Antiviral activity of the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ in the infection system of pseudotype virus using PH5CH8 cells. PH5CH8 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$, human LF, human TF, MBP2, and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay as controls. The data are means ± SD of three independent experiments.

Antiviral Effects of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ against VSVΔG(HCV) Infection in HepG2 Cells*

We have shown the inhibiting activities of LF fragments against HCV infection or VSV pseudotype infection in PH5CH8 cells; however, it is not clear whether or not the LF fragments used in this study show inhibiting activities against virus infection in cells other than PH5CH8 cells. To clarify this point, HepG2 cells were used for the analysis, because HepG2 cells showed the highest susceptibility to VSVΔG*(HCV) among 25 cell lines examined (30). As a consequence, we obtained similar results (Fig. 4) with those obtained in the infection system using PH5CH8 cells. The IC_{50} doses of C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ were >12 μM, 7.6 μM, and 3.9 μM, respectively, indicating that, again, the inhibiting activity was increased with multiplication of C-s3-33, although antiviral activity of $(C\text{-}s3\text{-}33)_3$ was still weaker than that of human LF (IC_{50}=1.2 μM). In conclusion, our results indicated that tandem repeats of C-s3-33 enhanced the inhibiting activity in cell culture-based HCV infection.

Discussion

In our previous (30) and present studies, we showed that pretreatment of VSV pseudotypes with bovine and human LFs reduced the infectivity of VSVΔG*(HCV) and VSVΔG*(E2) bearing only the E2 protein in a dose-dependent manner, whereas pretreatment with TF did not. In contrast, LFs partially inhibited the infectivity of VSVΔG*(E1) bearing only the E1 protein (30). These results suggested that the interaction of LF and the E2 protein is the main contributing factor to the prevention of HCV infection. This idea has been strongly supported by the results obtained in this study. We demonstrated that tandem repeats of C-s3-33, an anti-HCV peptide derived from human LF, enhanced the E2 protein-binding activity and the inhibiting activity

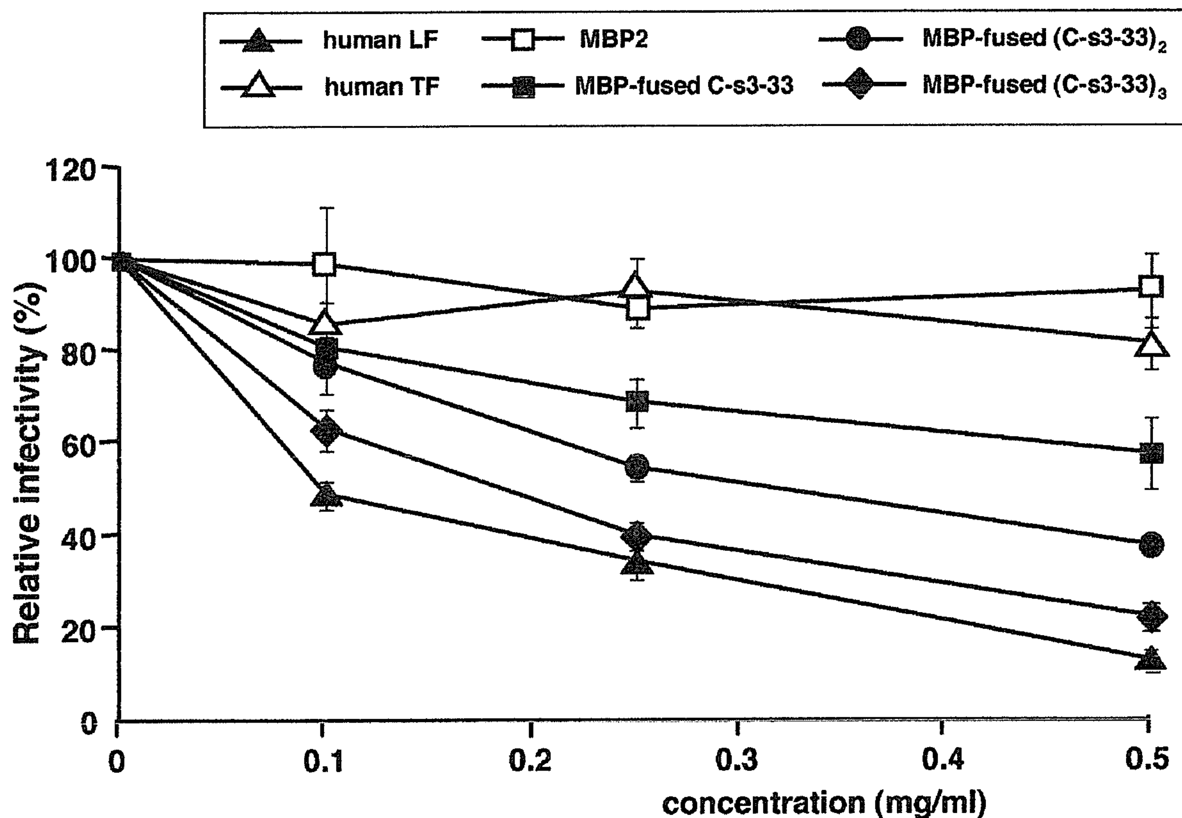

Fig. 4. Antiviral activity of the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in the infection system of the pseudotype virus using HepG2 cells. HepG2 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described in "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF, human TF, and MBP2 were used for the assay as controls. The data are means ± SD of three independent experiments.

against infection by HCV or the VSV pseudotype, VSVΔG*(HCV), in human hepatic cell lines. These results strongly suggest that the direct interaction between C-s3-33 and the E2 protein plays a central role in the inhibition of HCV infection by LF.

Since C-s3-33 or repeated forms of C-s3-33 could prevent HCV and VSVΔG*(HCV) infection, C-s3-33 must bind to a region other than the region (aa 441–500 of E2 protein) required for heteromeric complex formation between E1 and E2 proteins. Our preliminary results suggested that the C-s3-33 bound to aa 411–500 and aa 600–661 of the E2 protein, indicating that the target sites of C-s3-33 may be plural. This result suggests a rather complex interaction between C-s3-33 and the E2 protein. To clarify this point, further comprehensive analysis will be needed.

Although tandem repeats of C-s3-33 enhanced the anti-HCV activity compared with that of the C-s3-33, the fact that their antiviral activities were still several-fold weaker than that of original human LF remains a subject to be resolved. As one approach to increase anti-HCV activity, tandem repeats of C-s3-33-relevant fragment of bovine LF may be useful, because we previously observed that the anti-HCV activity of bovine LF (IC_{50}=1.5 μM) was stronger than that of human LF (IC_{50}=5.0 μM) (26), and that the E2 protein-binding activity of the C-s3 (93 aa)-relevant fragment of bovine LF was stronger than that of C-s3 (25). Since 10 aa out of 33 aa differ between C-s3-33 and its relevant fragment of bovine LF, some aa substitutions between both fragments may help to further increase the anti-HCV activity of LF-derived peptides. Alternatively, some spacer between the C-s3-33 repeats may be needed. Therefore, further trials will be needed to achieve the maximum anti-HCV activity of C-s3-33.

In conclusion, the results of the present study demonstrated that tandem repeats of human LF-derived 33 aa prevented HCV infection more strongly than the 33 aa, and suggest that this repeated form will be useful as a novel anti-HCV reagent.

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