compared with the previous results for the combination treatment of IFN-α with ribavirin.[10]

Discussion

In this study, we found that different statins have different anti-HCV profiles. FLV, ATV, and SMV each exerted a stronger inhibitory effect on HCV RNA replication than did that of LOV reported previously.[11,12] However, PRV exhibited no anti-HCV activity. We also demonstrated that anti-HCV activity was drastically increased when these statins except PRV were used in combination with IFN-α. Because these statins are currently used for the clinical treatment of patients with hypercholesterolemia without inducing severe side effects, our findings suggest that these statins might be useful in combination therapy with IFN-α or IFN-α plus ribavirin.

That PRV exhibited no anti-HCV activity is interesting. From the information on LOV only[10,11] to date, the mechanism underlying statins' inhibition of HCV RNA replication has not been considered their cholesterol-lowering activity but rather their inhibition of geranylgeranylation of cellular proteins. In other words, statins' inhibition of HMG-CoA reductase leads to the reduction of intracellular mevalonate and consequently to a reduction in geranylgeranyl pyrophosphate. In fact, in OR6 cells we observed that mevalonate and geranylgeraniol restored HCV RNA replication in the FLV- or LOV-treated cells. However, we found unexpectedly that PRV did not inhibit HCV RNA replication, whereas PRV inhibited HMG-CoA reductase as effectively as other statins possessing anti-HCV activity. Although PRV is a water-soluble reagent (others are lipophilic), we confirmed PRV did induce expression of HMG-CoA reductase by a positive feedback mechanism[18] and LST-1 was expressed in our cell culture system. These findings suggest the presence of a mechanism in which PRV's inhibition of HMG-CoA reductase does not cause the depletion of geranylgeranyl pyrophosphate. Interestingly, it has been reported that PRV has a unique effect among statins on the induction of p450.[18] Therefore, further studies are needed to explain why PRV exhibits no anti-HCV activity.

We minutely examined the effect of FLV, the statin exhibiting the strongest inhibition of HCV replication of those tested in this study, in combination with IFN-α. We found that a combination treatment of IFN-α and FLV had a synergistic inhibitory effect on HCV RNA replication. Although high doses of IFN-α are more effective than low doses for eliminating HCV from a patient, the side effects increase in a dose-dependent manner. Because ribavirin enhances the effect of IFN-α slightly in a cotreatment, it is the only reagent currently used with IFN-α to treat patients with CH C. In our previous study of anti-HCV activity using the OR6 assay system, we found the IC_{50} of ribavirin to be 76 μmol/L.[10] This concentration is much higher than the clinically achievable ribavirin concentration (10-14 μmol/L) previously reported.[25] Furthermore, when administered in combination with IFN-α (2 IU/mL) and ribavirin (50 μmol/L), HCV RNA replication was reduced by only approximately 50%, compared with the effect of treatment with IFN-α alone.[10] It has been reported that the maximum blood concentration of FLV after 40 mg/day being administered orally for 4 weeks is approximately 0.6 μmol/L.[26] This concentration is rather low for the inhibition of HCV replication *in vivo*, because the IC_{90} of FLV was assigned as 6.7 μmol/L in our assay system (Fig. 5B). In addition, our study showed reatment of OR6 cells with 5 μmol/L FLV alone was almost equal to the effect of 10 IU/mL IFN-α. Although statins are known to concentrate in the liver, FLV monotherapy will not be effective for patients with CH C. However, we demonstrated that the combination of IFN-α and FLV exhibited synergistic effects on HCV RNA replication. For example, when administered in combination with IFN-α (2-8 IU/mL) and FLV (5 μmol/L), HCV RNA replication fell remarkably, to approximately 3%, compared with the effects of treatment with IFN-α alone (Fig. 4A). From these results, we propose that therapy combining FLV with IFN-α may be effective for the treatment of patients with CH C. Furthermore, additional treatment with reagents in combination (e.g., IFN-α, ribavirin, and FLV) will help to improve the SVR rate.

In conclusion, the results of the present study suggest that statins other than PRV are good reagents for combination therapy with IFN-α in patients with CH C. Although the mechanism by which PRV lacks anti-HCV activity has not been clarified in the present study, a better understanding of this mechanism may lead to the discovery of statin-related anti-HCV reagents possessing no cholesterol-lowering activity. Furthermore, our developed OR6 assay system will be useful for the time-saving screening of new anti-HCV reagents.

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References

1. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967-972.
2. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
3. Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the

HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol 2002;76:2997-3006.
4. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008-4021.
5. Yi M, Bodola F, Lemon SM. Subgenomic hepatitis C virus replicons inducing expression of a secreted enzymatic reporter protein. Virology 2002;304:197-210.
6. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
7. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
8. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
9. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 2005;329:1350-1359.
10. Naka K, Ikeda M, Abe K, Dansako H, Kato N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. Biochem Biophys Res Commun 2005;330:871-879.
11. Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc Natl Acad Sci U S A 2003;100:15865-15870.
12. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci U S A 2005; 102:2561-2566.
13. Wang C, Gale M Jr, Keller BC, Huang H, Brown MS, Goldstein JL, et al. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol Cell 2005;18:425-434.
14. Naganuma A, Nozaki A, Tanaka T, Sugiyama K, Takagi H, Mori M, et al. Activation of the interferon-inducible 2′-5′-oligoadenylate synthetase gene by hepatitis C virus core protein. J Virol 2000;74:8744-8750.
15. Noguchi M, Hirohashi S. Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In Vitro Cell Dev Biol Anim 1996;32:135-137.
16. Dansako H, Naganuma A, Nakamura T, Ikeda F, Nozaki A, Kato N. Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element. Virus Res 2003;97:17-30.
17. Kato N, Sugiyama K, Namba K, Dansako H, Nakamura T, Takami M, et al. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem Biophys Res Commun 2003;306:756-766.
18. Kocarek TA, Dahn MS, Cai H, Strom SC, Mercer-Haines NA. Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme A inhibitors in primary cultured human hepatocytes. Drug Metab Dispos 2002;30:1400-1405.
19. Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, Nakai D, et al. Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. J Biol Chem 1999;274:17159-17163.
20. Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, et al. Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. J Pharmacol Exp Ther 2001;297:861-867.
21. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 2001;75:1252-1264.
22. Sutter AP, Maaser K, Hopfner M, Huether A, Schuppan D, Scherubl H. Cell cycle arrest and apoptosis induction in hepatocellular carcinoma cells by HMG-CoA reductase inhibitors. Synergistic antiproliferative action with ligands of the peripheral benzodiazepine receptor. J Hepatol 2005; 43:808-816.
23. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27-55.
24. Hwang DR, Tsai YC, Lee JC, Huang KK, Lin RK, Ho CH, et al. Inhibition of hepatitis C virus replication by arsenic trioxide. Antimicrob Agents Chemother 2004;48:2876-2882.
25. Pawlotsky JM, Dahari H, Neumann AU, Hezode C, Germanidis G, Lonjon I, et al. Antiviral action of ribavirin in chronic hepatitis C. Gastroenterology 2004;126:703-714.
26. Park JW, Siekmeier R, Lattke P, Merz M, Mix C, Schuler S, et al. Pharmacokinetics and pharmacodynamics of fluvastatin in heart transplant recipients taking cyclosporine A. J Cardiovasc Pharmacol Ther 2001;6: 351-361.

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Clinical Studies

Zinc is a negative regulator of hepatitis C virus RNA replication

Yuasa K, Naganuma A, Sato K, Ikeda M, Kato N, Takagi H, Mori M. Zinc is a negative regulator of hepatitis C virus RNA replication.
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Abstract: *Background/Aims:* Hepatitis C virus (HCV) infection is a significant global public health problem. In clinical studies, zinc has been closely related to the pathogenesis of chronic hepatitis C. However, the role of zinc in both viral replication and the expression of viral proteins remains unclear. We aimed to clarify the effect of zinc on the replication of HCV *in vitro*. *Methods:* We incubated subgenomic HCV replicon cells (sO) and genome-length HCV RNA-replicating cells (O) treated with several chemicals including trace elements. Total RNAs were collected and subjected to real-time reverse-transcriptase polymerase chain reaction in order to examine the level of HCV RNA replication, and Western blotting was performed to confirm the expression of viral proteins. *Results:* Iron salts and interferon-α suppressed HCV RNA replication and protein expression in both sO and O cells. Zinc salts effectively reduced the viral replication in the genome-length HCV RNA replication system but not in the subgenomic HCV replicon system. *Conclusions:* We demonstrated that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation thus appears to offer a novel approach to the development of future strategies for the treatment of intractable chronic hepatitis C.

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Key words: genome-length HCV RNA – hepatitis C virus – replication – subgenomic HCV replicon – zinc

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Hepatitis C virus (HCV) infection is a significant global public health problem. Persistent HCV infection eventually develops into liver cirrhosis or hepatocellular carcinoma (1). A sustained viral response (SVR) to anti-HCV therapy has been demonstrated to prevent the progression of liver disease and even to promote the regression of pathologic changes (2). Peginterferon plus oral ribavirin, currently the most powerful therapy for chronic hepatitis C, has successfully induced SVR in about half of treated patients of genotype 1b with high viral load (3, 4). However, there are still a number of non-responders to interferon (IFN)-based therapy. As a result, the treatment efficacy still needs to be improved.

HCV is a positive-polarity, single-stranded RNA virus, a member of the *Hepacivirus* genus of the Flaviviridae family (5). The HCV genome consists of an ~9.6 kb RNA molecule containing a large open reading frame flanked by structured 5′- and 3′-non-translated regions (NTR). Located within the 5′-NTR is an internal ribosome entry site (IRES) directing the translation of an approximately 3000-amino-acid polyprotein that is co- and posttranslationally cleaved by cellular and viral proteases into the following 10 products (listed from the N to the C termini): core, envelope protein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. The NS2–NS3 cleavage is performed by NS2, and the remaining processing of the NS3–NS4A–NS4B–NS5A–NS5B fragment depends on the NS3/NS4A protease, which is similar to chymotrypsin-like serine protease (6).

Zinc is an essential nutrient for a broad range of biological activities and for cell proliferation (7) and it also functions as an antioxidant (8). It also plays an important role in the function and maintenance of the crystal structures of such HCV proteins as NS2–NS3 (9–11) and NS5A (12, 13). The virus-encoded NS2–NS3 protease that is responsible for autocatalytic cleavage at the NS2–NS3 site is stimulated by $ZnCl_2$ (9, 10). The NS3 protease domain contains a zinc atom (11). These observations have led researchers to propose that zinc plays an important role in the NS2–NS3 protease activity. Several studies have examined the direct inhibitory effects of zinc on viruses, such as human immunodeficiency virus (14), rhinovirus (15), herpes simplex virus (16),

and respiratory syncytial virus (17) *in vitro*. However, the direct effect of zinc on the replication of HCV *in vitro* has never been previously reported.

Despite the clinical significance of HCV, molecular investigations of the virus have been hampered due to the lack of cell culture systems that efficiently support HCV replication, although a reproducible HCV proliferation system in cell culture has very recently been reported (18). In 1999, the situation changed for the better when a subgenomic HCV replicon cell culture system was introduced (19). The replicon RNA is composed of the HCV 5′-NTR containing an HCV IRES, a neomycin phosphotransferase (Neo) gene, and the HCV NS3 through NS5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′-NTR. The Neo gene is expressed under the control of the HCV IRES, thereby inducing G-418 resistance to cells that contain replicon RNA. As the replicon RNA proliferates autonomously in cultured cells, this replicon system is thus considered to be a powerful tool for the analysis of molecular mechanisms underlying HCV replication and also for the screening of anti-HCV reagents (20). However, the subgenomic HCV replicon system may be insufficient because it lacks HCV structural proteins. A genome-length HCV RNA replication system may reflect the phenomenon that the HCV-infected human liver undergoes. To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have so far been reported (21–24).

Clinical data suggest that the trace element metabolism is tightly linked to the pathogenesis of chronic hepatitis C (25, 26). We previously showed zinc supplementation to increase the therapeutic response of IFN-α for intractable chronic hepatitis C with genotype 1b (27, 28). However, it remains unclear as to whether or not zinc interferes with viral replication or the expression of viral proteins. We therefore examined the effect of zinc supplement on viral replication using HuH-7 cells harboring subgenomic HCV replicons (29) or genome-length HCV RNAs (24) derived from the HCV-O strain. We herein showed that zinc effectively suppressed the replication of genome-length HCV RNA but not that of the subgenomic HCV replicon.

Materials and methods

Cell culture systems

We incubated sO (previously described as 1B-2R1) cells (29) replicating the subgenomic HCV replicon and O cells (24) replicating the genome-length HCV RNA in a real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. The sequences of HCV RNA replicating in sO and O cells are derived from HCV RNA in non-neoplastic human hepatocytes PH5CH8 inoculated with HCV-O, and the basal replication levels of both O and sO cells were almost the same as those described previously (24, 29). In a luciferase reporter assay system, we incubated ORN/3-5B/KE cells supporting the subgenomic HCV replicon encoding the luciferase reporter gene, and ORN/C-5B/KE cells supporting genome-length HCV-RNA encoding the luciferase reporter gene (24). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM) and maintained in the presence of G418 (300 μg/ml; Geneticin, Invitrogen). We passaged these cells twice a week at a 5:1 split ratio and used them within six to 10 passages for the experiments in this study.

Reagents

Iron sulfate ($FeSO_4$), iron chloride ($FeCl_3$), zinc sulfate ($ZnSO_4$), and zinc chloride ($ZnCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The purities of both reagents exceeded 99%. Purified human lymphoblast IFN-α (OIF) was kindly provided by the Otsuka Pharmaceutical Co. (Tokushima, Japan).

Cell viability

As it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth (30), we examined the cytotoxicities of $ZnSO_4$ and $ZnCl_2$ to sO or O cells. In brief, the cells were seeded at a density of 4×10^5 cells per dish onto dishes with a diameter of 95 mm. After a 24-h culture, the cells were treated with or without zinc salts at final concentrations of 50, 100, and 150 μM for 72 h in the absence of G418. Next, the number of viable cells was counted using an improved Neubauer-type hematocytometer after trypan blue dye (Invitrogen) treatment. The effect of zinc salts was calculated as a percentage of the number of control cells to which no reagent was added. All assays were conducted more than three times.

Quantification of HCV RNA by real-time RT-PCR

The subgenomic HCV replicon (29) and replicable genome-length HCV RNA (24) are both well known to be highly sensitive to IFN-α and

recently iron has been reported to suppress the subgenomic HCV replicon (31). To confirm that our subgenomic HCV replicon and genome-length HCV RNA replication system are useful for evaluating antiviral reagents, we examined the established inhibitory effects of IFN-α and iron on the replication of the subgenomic HCV replicon and genome-length HCV RNA using sO and O cells. Next, the effect of zinc salts on the replication of subgenomic HCV replicon and the genome-length HCV RNA was observed by real-time RT-PCR. In brief, sO or O cells seeded on six-well plates (1×10^5 cells per well) were treated with IFN-α, $FeSO_4$, $FeCl_3$, $ZnSO_4$, or $ZnCl_2$ at several concentrations. The total RNAs from cells were harvested at different time points using ISOGEN extraction kits (Nippon Gene Co., Tokyo, Japan) and subjected to a real-time RT-PCR analysis. The 5′-NTR of HCV genomic RNA was quantified using the ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA) as described previously (32), using the 5′-CGGGAG-AGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTT CG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCG-GAACCGGTGAGTACAC-3′. As an internal control, the level of human GAPDH mRNA was quantified using TaqMan hGAPDH reagents (Applied Biosystems). All experiments were conducted more than three times.

Western blot analysis

The cell lysates and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared, and an immunoblotting analysis with a polyvinylidene difluoride membrane was performed as described previously (33). The antibodies used in this study were those against NS3 (Novocastra Laboratories, Newcastle, UK) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (Sigma-Aldrich, Tokyo, Japan) was also used to detect β-actin as the internal control. The immunocomplexes on the membranes were detected by an enhanced chemiluminescence assay (Amersham Co., Tokyo, Japan). Image scanning was analyzed using the Scion Image software program (Beta 4.0.2., Scion Corporation, NIH, Frederick, MD).

Luciferase reporter assay

To confirm the effect of zinc salts on the replication of HCV RNA by the different assay system, we performed the experiment while utilizing the luciferase reporter assay system using ORN/3-5B/KE cells and ORN/C-5B/KE cells with or without zinc salt. In brief, the cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Next, the cells were treated with $ZnSO_4$ or $ZnCl_2$ at several concentrations for 24 h, and then the cells were subjected to the luciferase reporter assay using the *Renilla* luciferase assay system (Promega, Madison, MI) (24). Briefly, after removing the medium, the cells were washed twice with phosphate-buffered saline. The cells were extracted with 100 µl of *Renilla* lysis reagent, and the relative luciferase unit value in 10 µl of lysates was measured by adding 50 µl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. Flash'n Glow LB 955 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect the luciferase activity.

Statistical analysis

All data were expressed as the mean ± standard deviation. The differences between groups were evaluated with Student's *t*-test or one-way analysis of variance $P<0.01$ was considered to be significant.

Results

Inhibitory effects of IFN-α on HCV RNA replication in sO and O cells

IFN-α efficiently inhibited the replication of the subgenomic HCV replicon and genome-length HCV RNA in a dose-dependent manner (Fig. 1). Based on the dose–response curve, the

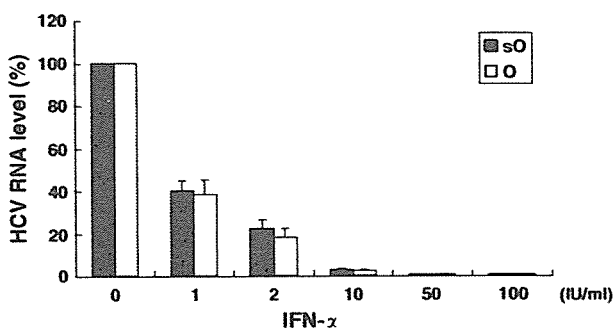

Fig. 1. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with interferon-α (IFN-α). IFN-α sensitivity of HCV RNA replication in sO (black bars) and O cells (white bars). Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. sO and O cells were treated for 48 h with IFN-α (0, 1, 2, 10, 50, and 100 IU/ml). The replication level of HCV RNA of the respective non-treated cells was assigned as 100%. The replication level of HCV RNA was normalized to the respective GAPDH mRNA expression levels. The data indicate the mean ± SD of triplicates from three independent experiments.

concentrations of IFN-α required for a 50% reduction (IC_{50}) of the subgenomic HCV replicon and genome-length HCV RNA were calculated to be almost equal (0.7 IU/ml). These values were comparable to the previous findings obtained using another HCV-strain-derived subgenomic HCV replicon system (34) or an O-strain-derived HCV RNA replication system (35).

Inhibitory effects of iron salts on HCV RNA replication in sO and O cells

$FeSO_4$ or $FeCl_2$ significantly suppressed the replication of genome-length HCV RNA to the same extent as the subgenomic HCV replicon in a dose-dependent manner (Fig. 2). We demonstrated for the first time the inhibitory effect of iron via a genome-length HCV replication system. Both IFN-α and iron salts inhibited HCV RNA replication in sO and O cells in a dose-dependent manner, thus suggesting that our subgenomic HCV replicon and genome-length HCV RNA replication systems are useful for the evaluation of anti-HCV reagents.

Cytotoxicity of zinc salts to sO and O cells

Although 150 μM and higher of $ZnSO_4$ or $ZnCl_2$ was cytotoxic to sO and O cells, $ZnSO_4$ or $ZnCl_2$ at a concentration of 100 μM or lower had no significant cytotoxic effect on both cells in this assay (Fig. 3A and B). We therefore examined the inhibitory effects of zinc salts at a concentration of 100 μM or lower.

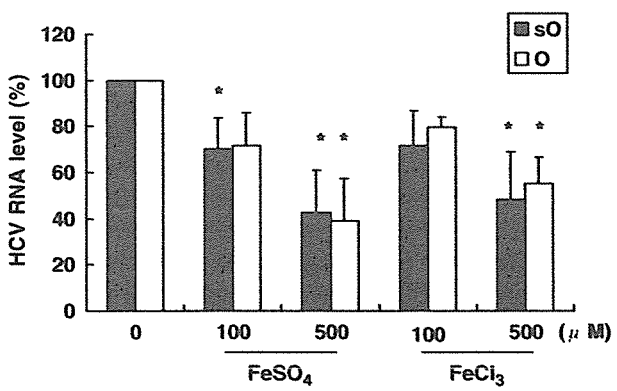

Fig. 2. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with iron. Iron inhibition of HCV RNA replication in sO (black bars) and O cells (white bars). sO and O cells were treated for 48 h with iron sulfate (100 and 500 μM) or iron chloride (100 and 500 μM). The control cells without iron salts (0 μM) were treated similarly. The quantification of HCV RNA was performed as described in Fig. 1. The data indicate the mean ± SD of triplicates from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by iron sulfate or iron chloride ($P<0.01$).

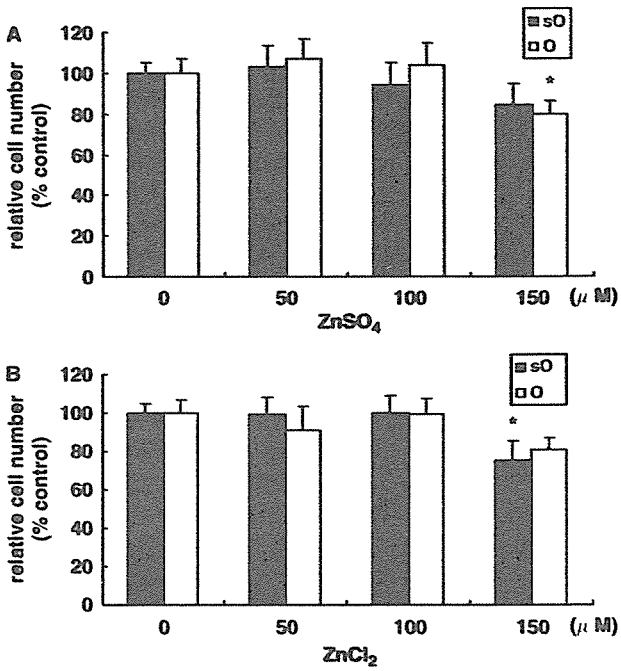

Fig. 3. Cytotoxicity of zinc salts to sO and O cells. (A) sO and O cells were cultured in the absence or presence of zinc sulfate (50, 100, and 150 μM each) for 72 h, and then the cell number was determined as described under the Materials and methods. The relative cell number (% control) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented herein. The data indicate the mean ± SD of three independent experiments. (B) sO and O cells were cultured in the absence or presence of zinc chloride as described in (A). The asterisk (*) indicates significant cytotoxicity by zinc sulfate or zinc chloride ($P<0.01$).

Different effects of zinc salts on the HCV RNA replication between sO and O cells

$ZnSO_4$ or $ZnCl_2$ significantly suppressed the genome-length HCV RNA replication in a dose-dependent manner. The IC_{50} values of $ZnSO_4$ and $ZnCl_2$ were calculated to be 76 and 89 μM, respectively. In contrast, only slight inhibitory effects on the subgenomic HCV replicon were observed in sO cells by 100 μM $ZnSO_4$ and $ZnCl_2$ (Fig. 4A and B). Zinc salts reduced the replication of the genome-length HCV RNA more markedly than that of the subgenomic HCV replicon. To determine whether the inhibitory effect of zinc on the genome-length HCV RNA replication is time dependent or not, O cells were incubated with 100 μM $ZnSO_4$ or $ZnCl_2$ and harvested at three different time points (24, 48, and 72 h) after treatment. The maximum inhibitory effect of zinc salts in O cells occurred at 48 h after treatment (Fig. 4C).

Effects of zinc salts on NS3 and NS5B protein expression

The expression levels of NS3 and NS5B proteins, which are the essential proteins for HCV RNA

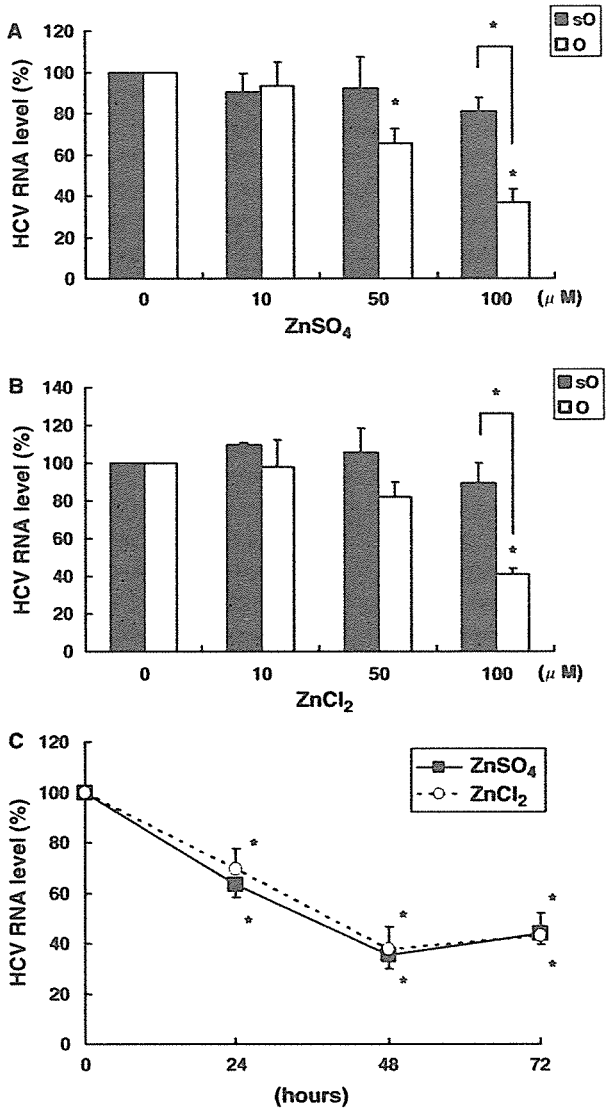

Fig. 4. Different effect of zinc salts between subgenomic hepatitis C virus (HCV) replicon and genome-length HCV RNA replication systems. (A) The sO and O cells were treated for 48 h with zinc sulfate (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (B) sO and O cells were treated for 48 h with zinc chloride (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (C) Time–response curve of zinc salts. O cells were treated with a fixed concentration (100 μM) of zinc sulfate or zinc chloride for 24, 48, and 72 h. Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. We herein show the replication level of HCV RNA (%) calculated at each point, when the replication level of HCV RNA of the respective non-treated cells at 0 h was assigned as 100%. The replication level of HCV RNA was normalized by the level of GAPDH mRNA. The data indicate the mean ± SD of triplicates findings from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by zinc sulfate or zinc chloride and a significant difference of the inhibitory effect of zinc salts between sO and O cells ($P<0.01$).

replication, did not decrease in the sO cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM), whereas the expression levels of NS3 and NS5B proteins were clearly decreased in the sO cells treated with $FeSO_4$, $FeCl_3$ (100 or 500 μM), or IFN-α (Fig. 5A and B). However, the expression levels of the NS3 and NS5B proteins both significantly decreased in the O cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM) as well as $FeSO_4$ or $FeCl_3$ (100 μM) (Fig. 5C and D). These results were consistent with those of the quantification analysis of HCV RNA as described above.

Anti-HCV activity of zinc salts on luciferase reporter assay system

Zinc salts significantly inhibited the *Renilla* luciferase activity in a dose-dependent manner but the extents of the suppressive effects were found to be rather weak depending on real-time RT-PCR (Fig. 6A and B). Zinc salts tend to reduce the replication of genome-length HCV RNA more markedly than that of the subgenomic HCV replicon even though the difference in chemical sensitivity to zinc salts was not significant.

Discussion

We demonstrated that zinc supplementation inhibited the replication of genome-length HCV RNA in O cells without causing cell toxicity, and the effects of zinc supplementation on HCV replication were significantly different between the genome-length HCV RNA replication system and the subgenomic HCV replicon system. On the other hand, IFN-α and iron supplementation suppressed the replication of HCV RNA almost equally between the subgenomic HCV replicon and genome-length HCV RNA replication system. However, other divalent cations, such as magnesium salts, did not suppress the replication of genome-length HCV RNA (data not shown). Therefore, the inhibition of the replication of HCV RNA is not an ubiquitous phenomenon caused by the divalent cations, but a specific phenomenon caused by certain divalent cations such as zinc and iron.

We showed the inhibitory effect of zinc salts in real-time RT-PCR and Western blotting on genome-length HCV RNA systems. In real-time RT-PCR, zinc inhibited the replication of HCV RNA as strongly as that of iron salts, whereas in Western blotting, the inhibitory effect of zinc salts was weaker than that of iron salts. There was a discrepancy in the inhibitory effects of zinc salts on RNA replication and protein expression in both systems. One possible reason is that zinc may affect the function of NS3 proteins of HCV through structural or NS2 proteins and consequently inhibit the replication of genome-length

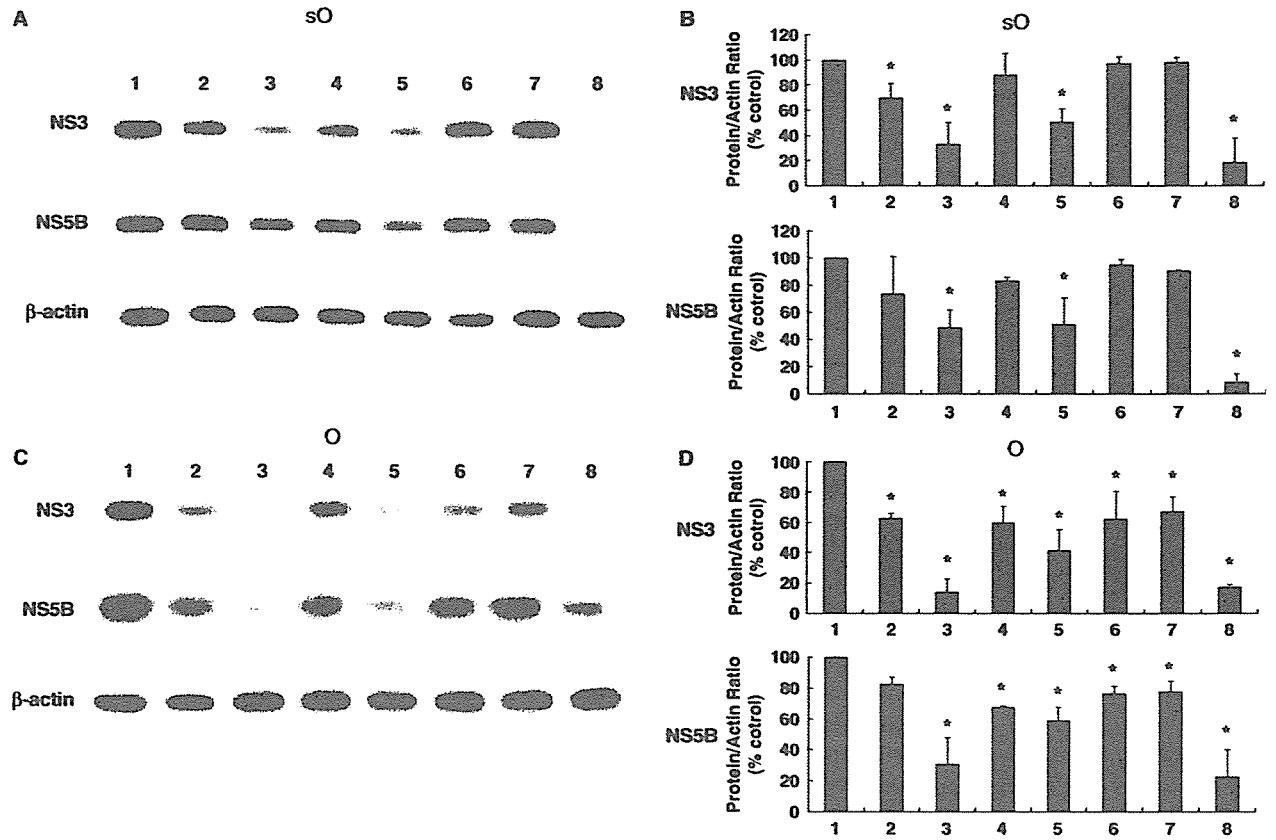

Fig. 5. Inhibitory effect of the expression of hepatitis C virus (HCV) proteins. (A) The sO cells were treated for 48 h without reagents (0 μM) as control wells (lane 1), or treated with 100 and 500 μM of iron sulfate (lanes 2 and 3), 100 and 500 μM of iron chloride (lanes 4 and 5), 100 μM zinc sulfate (lane 6), 100 μM zinc chloride (lane 7), or 20 IU/ml interferon-α (IFN-α) (lane 8). The production of NS3 and NS5B protein in sO cells was analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-actin was used as an internal control for the amount of protein loaded per lane. (B) The quantification of NS3 and NS5B production by densitometry using Scion Image software in sO normalized for the respective β-actin and the density of non-treated cells was assigned as 100%. These results were based on three separate experiments performed using three separate sets of cells and expressed as the mean ± SD for Western blotting. The asterisk (*) indicates a significant inhibition of NS3 and NS5B production by reagents ($P<0.01$). (C) The treatment of O cells with reagents and a Western blot analysis for NS3 and NS5B production were performed as described in (A). (D) The quantification of NS3 and NS5B in O cells was performed as described in (B).

HCV RNA, because sO cells replicate the subgenomic HCV replicon RNA lacking the HCV core to the NS2 region. This hypothesis is supported by the findings that the expression levels of NS3 proteins seemed to decrease more than that of NS5B expression in the O cells-treated zinc salts as shown in Fig. 5C and D.

We showed that iron supplementation inhibited the HCV RNA replication in both systems almost equally. It has recently been reported that iron directly inactivates the RNA-dependent RNA polymerase activity of HCV, which is mediated by the viral NS5B, thus impairing the HCV replication using the subgenomic HCV replicon system (31). The iron compound-induced inhibitory effect of HCV RNA replication on genome-length HCV RNA system may be caused by NS5B, which is a common structure in both systems.

We could not confirm the inhibitory effect of zinc on other genome-length HCV RNA replication systems because we could not obtain any other cell lines. However, the previous reports that describe iron and not zinc to inhibit significantly the HCV RNA replication in another subgenomic HCV replicon system is consistent with the result of our subgenomic HCV replicon (31), and it is also consistent with our results. In the luciferase reporter system, we confirmed that the inhibitory effect of zinc salts in the genome-length HCV replication system was also observed in a dose-dependent manner. However, 100 μM zinc salts significantly inhibited the luciferase activity in the subgenomic HCV replicon, but less than that in the genome-length HCV RNA replication systems, in contrast to the results of real-time RT-PCR. The luciferase reporter assay system showed reproducible results but the extent of the inhibitory effect between the replication system of subgenomic HCV and that of genome-length HCV RNA was slightly different. In our results of real-time RT-PCR as shown in Fig. 4A

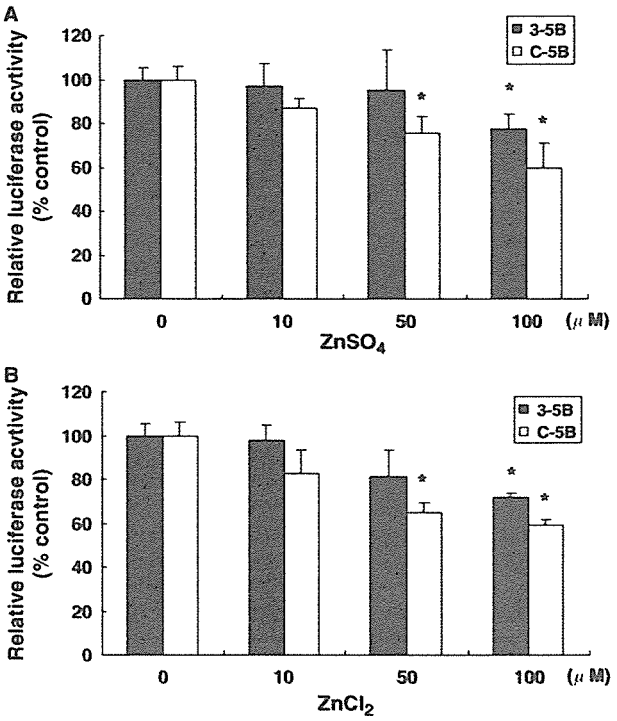

Fig. 6. Inhibitory effect of zinc of hepatitis C virus (HCV) RNA replication in ORN/3-5B/KE and ORN/C-5B/KE cells treated with zinc salts on a luciferase reporter assay system. (A) Inhibitory effect of zinc sulfate against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in Materials and methods. ORN/3-5B/KE and ORN/C-5B/KE cells were treated for 24 h with zinc sulfate (0, 10, 50, and 100 μM), and the relative luciferase activity was calculated. The relative luciferase activity of respective non-treated cells was assigned to be 100%. The data indicate the mean ± SD of triplicate findings from three independent experiments. (B) The inhibitory effect of zinc chloride against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in (A). The asterisk (*) indicates a significant inhibition of the *Renilla* luciferase activity by zinc sulfate or zinc chloride ($P<0.01$).

and B, the HCV replication level in 100 μM zinc salts tended to be slightly lower than in either the control or 50 μM zinc salts. Therefore, this is probably due to a difference in the detection sensitivity of the real-time RT-PCR and luciferase reporter assay systems.

The subgenomic HCV replicon and the replicable genome-length HCV RNA in this study were highly sensitive to IFN-α as described previously (33, 34). Moreover, clinically, zinc supplementation increased the therapeutic response of IFN-α for intractable chronic hepatitis C (27, 28). However, zinc supplementation did not show the additional or synergistic inhibitory effect of IFN-α in a genome-length HCV RNA replication system (data not shown). The inhibitory effect of zinc on the replication of both replicon cells may be masked with that of IFN-α, because the inhibitory effect of IFN-α on the replication of both replicon cells is much more effective than that of zinc.

To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have been reported (21–24). Genome-length HCV RNA replication, including the structural region of HCV RNA, closely mimics the *in vivo* situation within an HCV-infected hepatocyte. In this study, different degrees of chemical sensitivity were observed between the subgenomic HCV replicon system and genome-length HCV RNA replication system. This significant difference suggests that a useful investigation may have been overlooked in the subgenomic HCV replicon systems. Previous studies using subgenomic HCV replicon systems should therefore be re-examined using genome-length HCV RNA replication systems.

In conclusion, our study suggests that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation appears to be a novel approach in the development of future strategies for the treatment of chronic hepatitis C. The mechanisms underlying the inhibitory effect of zinc on virus replication are presently being investigated in our laboratory.

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References

1. Seeff L B. Natural history of chronic hepatitis C. Hepatology 2002; 36: S35–46.
2. Yoshida H, Arakawa Y, Sata M, et al. Interferon therapy prolonged life expectancy among chronic hepatitis C patients. Gastroenterology 2002; 123: 483–91.
3. Fried M W, Shiffman M L, Reddy K R, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002; 347: 975–82.
4. Manns M P, McHutchison J G, Gordon S C, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001; 358: 958–65.
5. Shi S T, Lai M M. Hepatitis C viral RNA: challenges and promises. Cell Mol Life Sci 2001; 58: 1276–95.
6. Bartenschlager R, Lohmann V. Replication of hepatitis C virus. J Gen Virol 2000; 81: 1631–48.
7. MacDonald R S. The role of zinc in growth and cell proliferation. J Nutr 2000; 130: 1500S–8S.
8. Powell S R. The antioxidant properties of zinc. J Nutr 2000; 130: 1447S–54S.
9. Hijikata M, Mizushima H, Tanji Y, et al. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc Natl Acad Sci 1993; 90: 10773–7.

10. Thibeault D, Maurice R, Pilote L, Lamarre D, Pause A. In vitro characterization of a purified NS2/3 protease variant of hepatitis virus. J Biol Chem 2001; 276: 46678–84.
11. Stempniak M, Hostomska Z, Nodes B R, Hostomsky Z. The NS3 proteinase domain of hepatitis C virus is a zinc-containing enzyme. J Biol Chem 1997; 71: 2881–6.
12. Tellinghuisen T L, Marcotrigiano J, Gorbalenya A E, Rice C M. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem 2004; 279: 48576–87.
13. Tellinghuisen T L, Marcotrigiano J, Rice C M. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature 2005; 435: 374–9.
14. Haraguchi Y, Sakurai H, Hussain S, Anner B M, Hoshino H. Inhibition of HIV-1 infection by zinc group metal compounds. Antiviral Res 1999; 43: 123–33.
15. Novick S G, Godfrey J C, Pollack R L, Wilder H R. Zinc-induced suppression of inflammation in the respiratory tract, caused by infection with human rhinovirus and other irritants. Med Hypotheses 1997; 49: 347–57.
16. Kumel G, Schrader S, Zentgraf H, Brendel M. Therapy of banal HSV lesions: molecular mechanisms of the antiviral activity of zinc sulfate. Hautarzt 1991; 42: 439–45.
17. Suara R O, Crowe J E Jr. Effect of zinc salts on respiratory syncytial virus replication. Antimicrob Agents Chemother 2004; 48: 783–90.
18. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005; 11: 791–6.
19. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999; 285: 110–3.
20. Bartenschlager R. The hepatitis C virus replicon system: from basic research to clinical application. J Hepatol 2005; 43: 210–6.
21. Ikeda M, Yi M, Li K, Lemon S M. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol 2002; 76: 2997–3006.
22. Blight K J, McKeating J A, Marcotrigiano J, Rice C M. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol 2003; 77: 3181–90.
23. Pietschmann T, Lohmann V, Kaul A, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002; 76: 4008–21.
24. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 2005; 329: 1350–9.
25. Ebara M, Fukuda H, Hatano R, et al. Metal contents in the liver of patients with chronic liver disease caused by hepatitis C virus. Oncology 2003; 65: 323–30.
26. Chapoutot C, Esslimani M, Joomaye Z, et al. Liver iron excess in patients with hepatocellular carcinoma developed on viral C cirrhosis. Gut 2000; 46: 711–4.
27. Nagamine T, Takagi H, Takayama H, et al. Preliminary study of combination therapy with IFN-alpha and zinc in chronic hepatitis C patients with genotype Ib. Biol Trace Element Res 2000; 75: 53–63.
28. Takagi H, Nagamine T, Abe T, et al. Zinc supplementation enhances the response to interferon therapy in patients with hepatitis C. J Viral Hepat 2001; 8: 367–71.
29. Kato N, Sugiyama K, Namba K, et al. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem Biophys Res Commun 2003; 306: 756–66.
30. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 2001; 75: 1252–64.
31. Fillebeen C, Rivas-Estilla A M, Bisaillon M, et al. Iron inactivates the RNA polymerase NS5B and suppresses subgenomic replication of hepatitis C virus. J Biol Chem 2005; 280: 9049–57.
32. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotono K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 2003; 38: 1282–8.
33. Naganuma A, Nozaki A, Tanaka T, et al. Activation of the interferon-inducible 2′-5′-oligoadenylate synthetase gene by hepatitis C virus core protein. J Virol 2000; 74: 8744–50.
34. Tanabe Y, Sakamoto N, Enomoto N, et al. Synergistic inhibition of intercellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis 2004; 189: 1129–39.
35. Naka K, Ikeda M, Abe K, Dansako H, Kato N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. Biochem Biophys Res Commun 2005; 330: 871–9.

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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'-ATCCATCCGAGACACCACAAACTTGTCCGGGCAGTCAGATCC-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'-TAATAAAGCTTTTAAAACTTGTCCGGGCAGTCAGATCC-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, $(C\text{-}s3\text{-}33)_2$, or $(C\text{-}s3\text{-}33)_3$] 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 $(C\text{-}s3\text{-}33)_3$

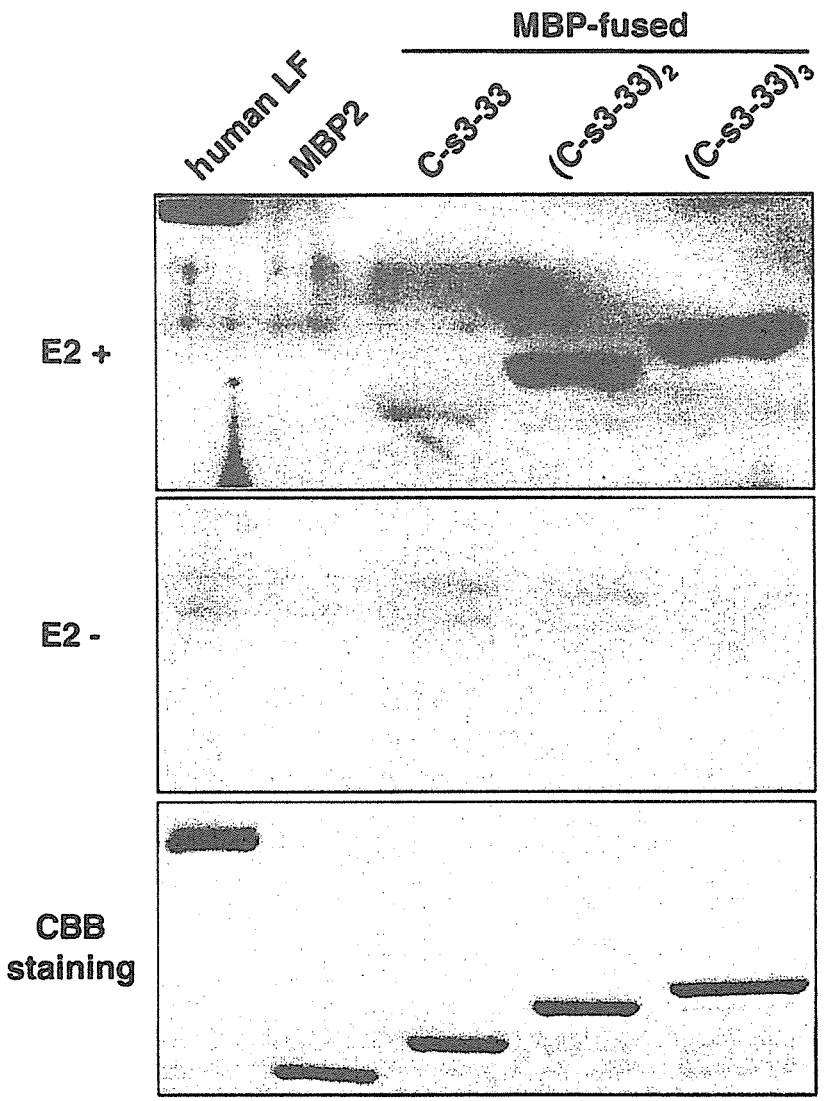

Fig. 1. Comparison of the E2 protein-binding activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$. MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ (0.5 μg each) were resolved by 10% SDS-PAGE. Human LF and MBP2 (0.5 μg each) were 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.

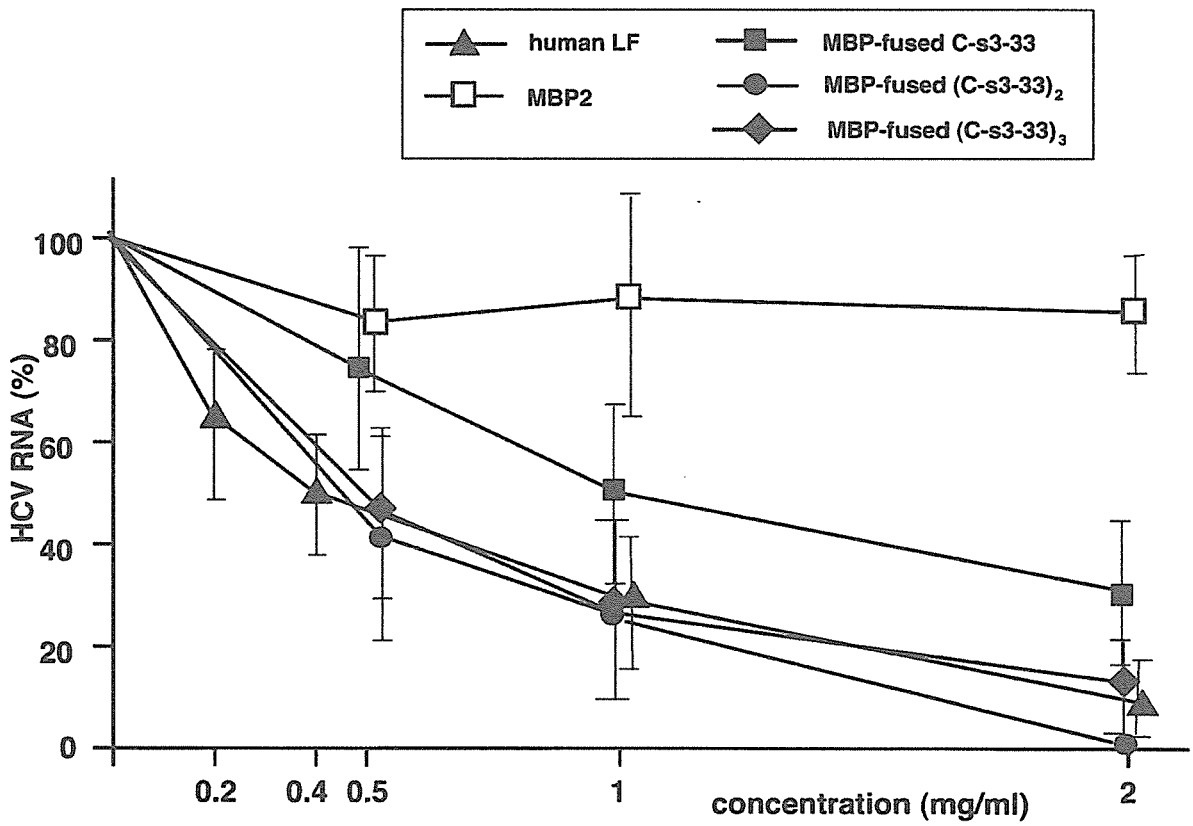

Fig. 2. Anti-HCV activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in an HCV infection system using PH5CH8 cells. PH5CH8 cells and the inoculum HCV-O were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate 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 $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ 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 $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ 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, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ 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 $(C\text{-}s3\text{-}33)_3$ 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.

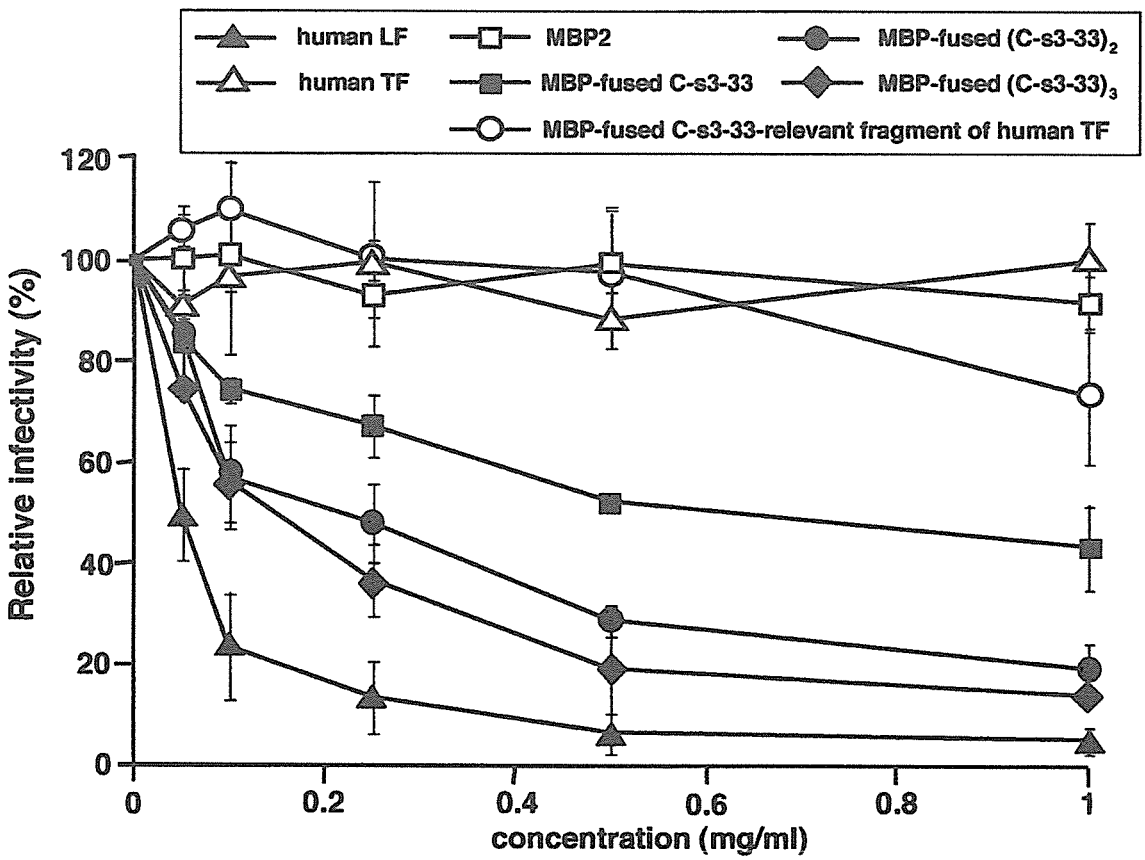

Fig. 3. Antiviral activity of the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in the infection system of pseudotype virus using PH5CH8 cells. PH5CH8 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described under "Materials and 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, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, 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

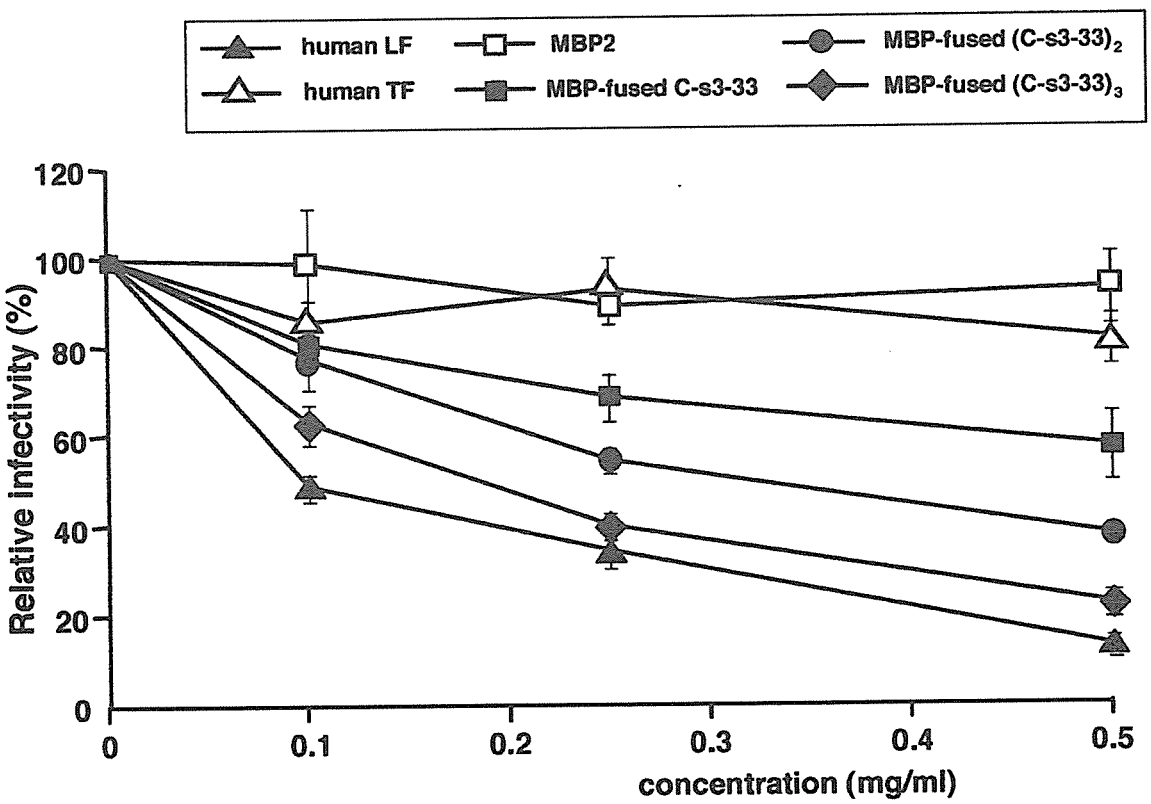

Fig. 4. Antiviral activity of the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in the infection system of the pseudotype virus using HepG2 cells. HepG2 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described in "Materials and 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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References

1) Bartenschlager, R., and Lohmann, V. 2000. Replication of hepatitis C virus. J. Gen. Virol. **81**: 1631–1648.
2) Bartosch, B., Dubuisson, J., and Cosset, F.L. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. **197**: 633–642.
3) Buonocore, L., Blight, K.J., Rice, C.M., and Rose, J.K. 2002. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. J. Virol. **76**: 6865–6872.
4) Cocquerel, L., Voisset, C., and Dubuisson, J. 2006. Hepatitis C virus entry: potential receptors and their biological functions. J. Gen. Virol. **87**: 1075–1084.
5) Farnaud, S., and Evans, R.W. 2003. Lactoferrin—a multifunctional protein with antimicrobial properties. Mol. Immunol. **40**: 395–405.
6) Feld, J.J., and Hoofnagle, J.H. 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature **436**: 967–972.
7) Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. Proc. Natl. Acad. Sci. U.S.A. **88**: 5547–5551.
8) Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K., and Shimotohno, K. 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. **90**: 10773–10777.
9) Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C.M., and McKeating, J.A. 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. U.S.A. **100**: 7271–7276.
10) Ikeda, M., Nozaki, A., Sugiyama, K., Tanaka, T., Naganuma, A., Tanaka, K., Sekihara, H., Shimotohno, K., Saito, M., and Kato, N. 2000. Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells. Virus Res. **66**: 51–63.
11) Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., and Kato, N. 1998. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res. **56**: 157–167.
12) Ikeda, M., Sugiyama, K., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., and Kato, N. 1998. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. Biochem. Biophys. Res. Commun. **245**: 549–553.
13) Inudoh, M., Kato, N., and Tanaka, Y. 1998. New monoclonal antibodies against a recombinant second envelope protein of hepatitis C virus. Microbiol. Immunol. **42**: 875–877.
14) Inudoh, M., Nyunoya, H., Tanaka, T., Hijikata, M., Kato, N., and Shimotohno, K. 1996. Antigenicity of hepatitis C virus envelope proteins expressed in Chinese hamster ovary cells. Vaccine **14**: 1590–1596.
15) Ishii, K., Takamura, N., Shinohara, M., Wakui, N., Shin, H.,

Sumino, Y., Ohmoto, Y., Teraguchi, S., and Yamauchi, K. 2003. Long-term follow-up of chronic hepatitis C patients treated with oral lactoferrin for 12 months. Hepatol. Res. **25**: 226–233.

16) Iwasa, M., Kaito, M., Ikoma, J., Takeo, M., Imoto, I., Adachi, Y., Yamauchi, K., Koizumi, R., and Teraguchi, S. 2002. Lactoferrin inhibits hepatitis C virus viremia in chronic hepatitis C patients with high viral loads and HCV genotype 1b. Am. J. Gastroenterol. **97**: 766–767.

17) Kato, N. 2001. Molecular virology of hepatitis C virus. Acta Med. Okayama **55**: 133–159.

18) Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. **87**: 9524–9528.

19) Kato, N., Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., and Shimotohno, K. 1998. Hepatitis C virus population dynamics in human lymphocytes and hepatocytes infected *in vitro*. J. Gen. Virol. **79**: 1859–1869.

20) Kato, N., and Shimotohno, K. 2000. Systems to culture hepatitis C virus. Curr. Top. Microbiol. Immunol. **242**: 261–278.

21) Levay, P.F., and Viljoen, M. 1995. Lactoferrin: a general review. Haematologica **80**: 252–267.

22) Lonnerdal, B., and Iyer, S. 1995. Lactoferrin: molecular structure and biological function. Annu. Rev. Nutr. **15**: 93–110.

23) Matsuura, Y., Tani, H., Suzuki, K., Kimura-Someya, T., Suzuki, R., Aizaki, H., Ishii, K., Moriishi, K., Robison, C.S., Whitt, M.A., and Miyamura, T. 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology **286**: 263–275.

24) Noguchi, M., and Hirohashi, S. 1996. Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In Vitro Cell. Dev. Biol. Anim. **32**: 135–137.

25) Nozaki, A., Ikeda, M., Naganuma, A., Nakamura, T., Inudoh, M., Tanaka, K., and Kato, N. 2003. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. J. Biol. Chem. **278**: 10162–10173.

26) Nozaki, A., and Kato, N. 2002. Quantitative method of intracellular hepatitis C virus RNA using LightCycler PCR. Acta Med. Okayama **56**: 107–110.

27) Okada, S., Tanaka, K., Sato, T., Ueno, H., Saito, S., Okusaka, T., Sato, K., Yamamoto, S., and Kakizoe, T. 2002. Dose-response trial of lactoferrin in patients with chronic hepatitis C. Jpn. J. Cancer Res. **93**: 1063–1069.

28) Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.L., Houghton, M., and Kuo, G. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. **87**: 6547–6549.

29) Tamura, T., Nozaki, A., Abe, K., Dansako, H., Naka, K., Ikeda, M., Tanaka, K., and Kato, N. 2005. cDNA microarray analysis of lactoferrin expression in non-neoplastic human hepatocyte PH5CH8 cells. Biochim. Biophys. Acta **1721**: 73–80.

30) Tamura, K., Oue, A., Tanaka, A., Shimizu, N., Takagi, H., Kato, N., Morikawa, A., and Hoshino, H. 2005. Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication. Microbes Infect. **7**: 29–40.

31) Tanaka, K., Ikeda, M., Nozaki, A., Kato, N., Tsuda, H., Saito, S., and Sekihara, H. 1999. Lactoferrin inhibits hepatitis C virus viremia in patients with chronic hepatitis C: a pilot study. Jpn. J. Cancer Res. **90**: 367–371.

32) Thomas, D.L. 2000. Hepatitis C epidemiology. Curr. Top. Microbiol. Immunol. **242**: 25–41.

33) van der Strate, B.W., Beljaars, L., Molema, G., Harmsen, M.C., and Meijer, D.K. 2001. Antiviral activities of lactoferrin. Antiviral Res. **52**: 225–239.

34) Yi, M., Kaneko, S., Yu, D.Y., and Murakami, S. 1997. Hepatitis C virus envelope proteins bind lactoferrin. J. Virol. **71**: 5997–6002.

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Protein kinase Cδ binds TIRAP/Mal to participate in TLR signaling

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Abstract

Toll-like receptor (TLR) family members recognize specific molecular patterns within pathogens. Signaling through TLRs results in a proximal event that involves direct binding of adaptor proteins to the receptors. We observed that TIRAP/Mal, an adaptor protein for TLR2 and TLR4, binds protein kinase Cδ (PKCδ). TIRAP/Mal GST-fusion protein and a TIRAP/Mal antibody were able to precipitate PKCδ from rat peritoneal macrophage and THP1 cell lysates. Truncation mutants of TIRAP/Mal showed that the TIR domain of TIRAP/Mal is responsible for binding. TLR2- and TLR4-mediated phosphorylation of p38 MAPK, IKK, and IκB in RAW264.7 cells were abolished by depletion of PKCδ. These results suggest that PKCδ binding to TIRAP/Mal promotes TLR signaling events.

Keywords: PKCδ; TLR; Macrophage; Signal transduction

1. Introduction

The toll-like receptor (TLR) family plays a central role in regulating innate immunity. TLRs recognize conserved microbial products such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and unmethylated CpG motifs in bacterial DNA (**Kaisho and Akira, 2001**). TLR signaling is initiated from the toll-interleukin-1 receptor (TIR)-domain, a ~200 amino acid motif found in the cytosolic domain of all TLRs and the IL-1 receptor. Some adaptor molecules also contain TIR domains and bind to the TIR domains of TLR through stimulation-induced TIR/TIR interactions (**Akira, 2003**; **Yamamoto et al., 2003**). The TLR adaptor family has four known members, MyD88, TIRAP/Mal, TICAM-1/TRIF, and TICAM-2/TRAM. Studies with mice that lack the individual adaptors have defined a specific role for each one. MyD88 is shared by all TLRs except TLR3, while TIRAP/Mal only binds to TLR2 and TLR4, TICAM-1 binds exclusively to TLR3 and TLR4, and TICAM-2 only binds to TLR4 (**Yamamoto et al., 2004**; **Oshiumi et al., 2003a**; **Oshiumi et al., 2003b**). The diversity of adaptor molecules may partially account for the variety of inflammatory responses to different ligands.

MyD88 was the first adaptor protein to be identified, and its signaling pathway is now well characterized (**Akira, 2003**). Once MyD88 is recruited to the TLR, it associates with the serine/threonine kinases, IRAK-1 (IL-1R associated kinase-1) and IRAK-4. This association leads to IRAK-1 phosphorylation by IRAK-4 or other IRAK-1 molecules, a key event that allows IRAK-1 to bind TRAF6. This complex activates TAK-1, a MAPKKK family member, which activates SAPK/JNK and p38MAPK. TAK1 acts at a divergence point in the TLR signaling pathway because it can also activate IKK, a protein kinase responsible for IκB degradation and subsequent NF-κB activation.

Abbreviations: LPS, lipopolysaccharide; TLR, Toll-like receptor; CBB, coomassie brilliant blue; MAPK, MAP kinase; shRNA, short hairpin RNA

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