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## 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- $\alpha$  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<sub>2</sub> (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- $\alpha$  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  $\mu$ g/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<sub>4</sub>), iron chloride (FeCl<sub>3</sub>), zinc sulfate (ZnSO<sub>4</sub>), and zinc chloride (ZnCl<sub>2</sub>) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The purities of both reagents exceeded 99%. Purified human lymphoblast IFN- $\alpha$  (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<sub>4</sub> and ZnCl<sub>2</sub> to sO or O cells. In brief, the cells were seeded at a density of  $4 \times 10^5$  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  $\mu$ M for 72 h in the absence of G418. Next, the number of viable cells was counted using an improved Neubauer-type hemacytometer 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- $\alpha$  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- $\alpha$  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 ( $1 \times 10^5$  cells per well) were treated with IFN- $\alpha$ , FeSO<sub>4</sub>, FeCl<sub>3</sub>, ZnSO<sub>4</sub>, or ZnCl<sub>2</sub> 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- $\beta$ -actin antibody (Sigma-Aldrich, Tokyo, Japan) was also used to detect  $\beta$ -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 ( $1.5 \times 10^4$  cells per well) and cultured for 24 h. Next, the cells were treated with ZnSO<sub>4</sub> or ZnCl<sub>2</sub> 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  $\mu$ l of *Renilla* lysis reagent, and the relative luciferase unit value in 10  $\mu$ l of lysates was measured by adding 50  $\mu$ l 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  $\pm$  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- $\alpha$ on HCV RNA replication in sO and O cells

IFN- $\alpha$  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

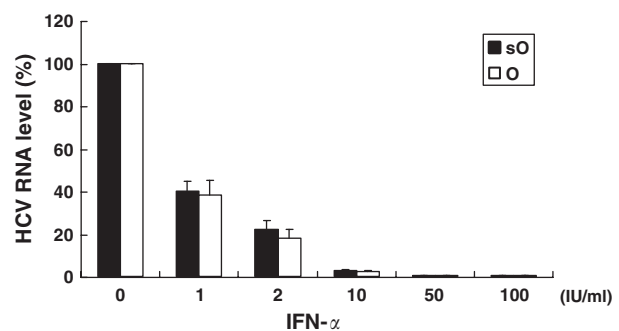


Fig. 1. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with interferon- $\alpha$  (IFN- $\alpha$ ). IFN- $\alpha$  sensitivity of HCV RNA replication in sO (black bars) and O cells (white bars). Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. sO and O cells were treated for 48 h with IFN- $\alpha$  (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  $\pm$  SD of triplicates from three independent experiments.

concentrations of IFN- $\alpha$  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<sub>4</sub> or FeCl<sub>2</sub> 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- $\alpha$  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  $\mu$ M and higher of ZnSO<sub>4</sub> or ZnCl<sub>2</sub> was cytotoxic to sO and O cells, ZnSO<sub>4</sub> or ZnCl<sub>2</sub> at a concentration of 100  $\mu$ M 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  $\mu$ M or lower.

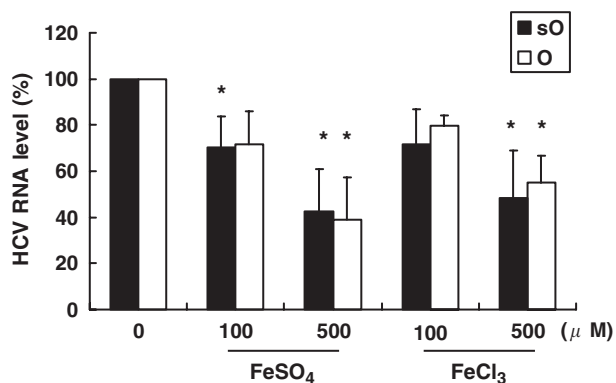


Fig. 2. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with iron. Iron inhibition of HCV RNA replication in sO (black bars) and O cells (white bars). sO and O cells were treated for 48 h with iron sulfate (100 and 500  $\mu$ M) or iron chloride (100 and 500  $\mu$ M).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

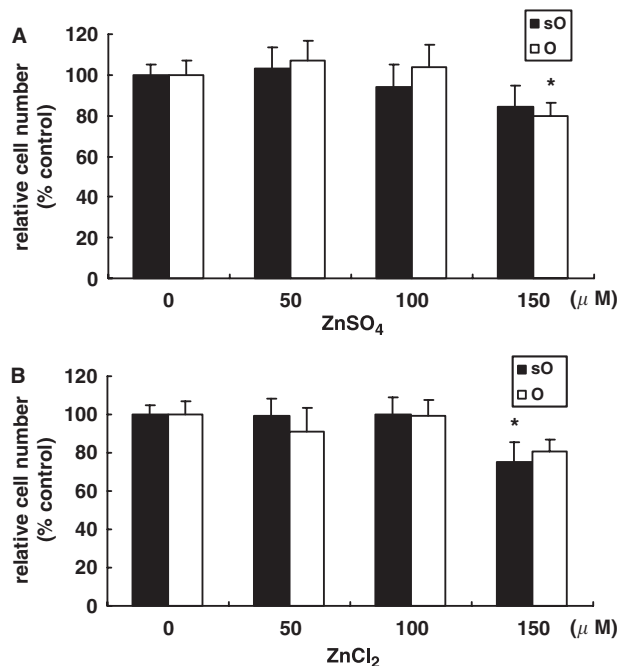


Fig. 3. Cytotoxicity of zinc salts to sO and O cells. (A) sO and O cells were cultured in the absence or presence of zinc sulfate (50, 100, and 150  $\mu$ M each) for 72 h, and then the cell number was determined as described under the Materials and methods. The relative cell number (% control) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented herein. The data indicate the mean  $\pm$  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 ( $P < 0.01$ ).

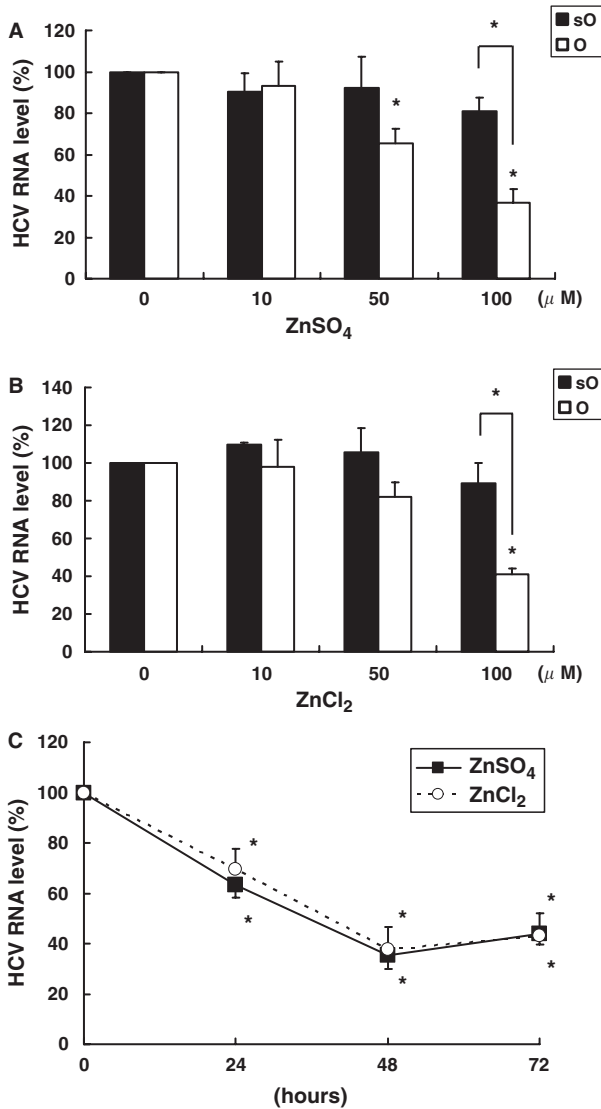
**Different effects of zinc salts on the HCV RNA replication between sO and O cells**

ZnSO<sub>4</sub> or ZnCl<sub>2</sub> significantly suppressed the genome-length HCV RNA replication in a dose-dependent manner. The  $IC_{50}$  values of ZnSO<sub>4</sub> and ZnCl<sub>2</sub> were calculated to be 76 and 89  $\mu$ M, respectively. In contrast, only slight inhibitory effects on the subgenomic HCV replicon were observed in sO cells by 100  $\mu$ M ZnSO<sub>4</sub> and ZnCl<sub>2</sub> (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  $\mu$ M ZnSO<sub>4</sub> or ZnCl<sub>2</sub> 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

## Zinc regulates HCV RNA replication



**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  $\mu$ M). 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  $\mu$ M). 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  $\mu$ M) 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  $\pm$  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<sub>4</sub> or ZnCl<sub>2</sub> (100  $\mu$ M), whereas the expression levels of NS3 and NS5B proteins were clearly decreased in the sO cells treated with

FeSO<sub>4</sub>, FeCl<sub>3</sub> (100 or 500  $\mu$ M), or IFN- $\alpha$  (Fig. 5A and B). However, the expression levels of the NS3 and NS5B proteins both significantly decreased in the O cells treated with ZnSO<sub>4</sub> or ZnCl<sub>2</sub> (100  $\mu$ M) as well as FeSO<sub>4</sub> or FeCl<sub>3</sub> (100  $\mu$ M) (Fig. 5C and D). These results were consistent with those of the quantification analysis of HCV RNA as described above.

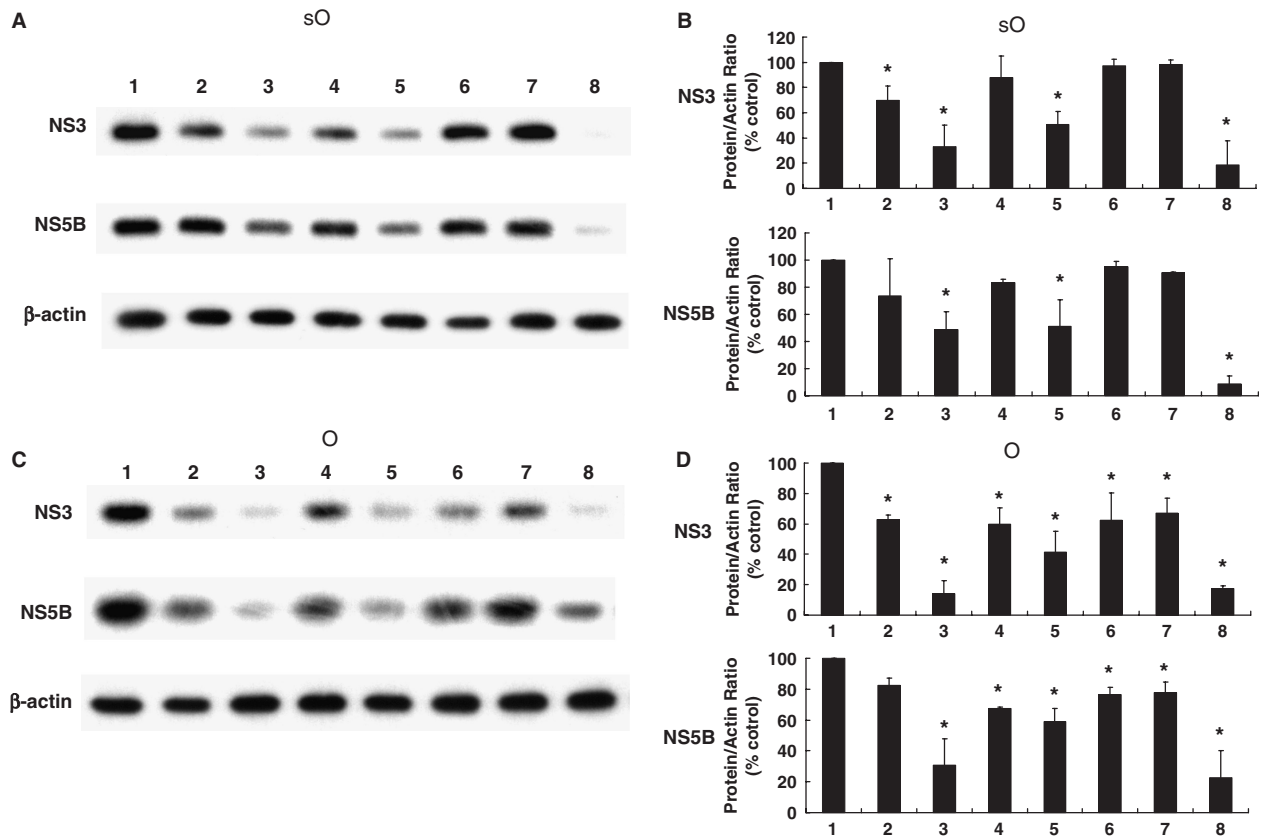
### Anti-HCV activity of zinc salts on luciferase reporter assay system

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- $\alpha$  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



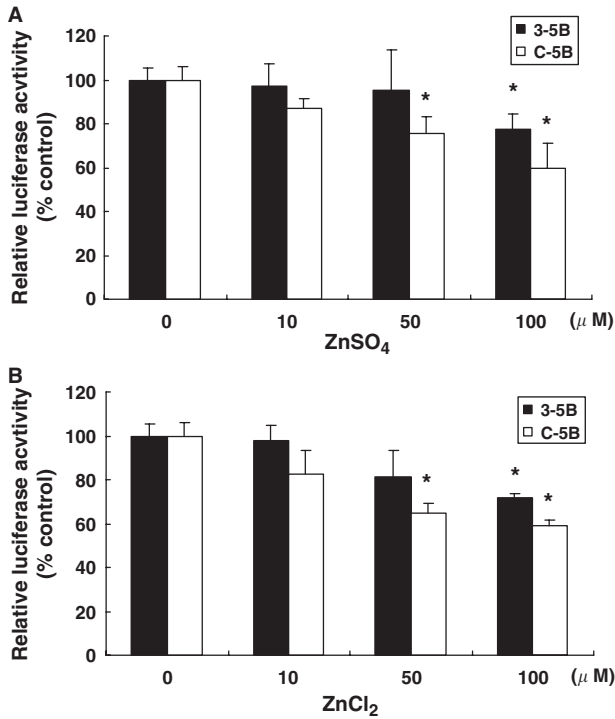
**Fig. 5.** Inhibitory effect of the expression of hepatitis C virus (HCV) proteins. (A) The sO cells were treated for 48 h without reagents (0 μM) as control wells (lane 1), or treated with 100 and 500 μM of iron sulfate (lanes 2 and 3), 100 and 500 μM of iron chloride (lanes 4 and 5), 100 μM zinc sulfate (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 replica-

tion 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 alpha-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 alpha-2b plus ribavirin compared with interferon alpha-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 NSSA 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, BRENDEN 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, DANKO 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 1b. *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, DANKO H, KATO N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. *Biochem Biophys Res Commun* 2005; 330: 871–9.