0·3 mg ml^{-1}, and the medium was changed twice per week. After 3 weeks, the colonies obtained on the culture dish were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004).

RESULTS

Efficient replication of HCV replicons is maintained in long-term cell culture

In order to prepare the specimens for the genetic analysis of 50-1 and 1B-2R1 replicons, three people independently cultured 50-1 cells; one person cultured for 18 months (M) (K cell culture line; MK) and the two people cultured for 12 months (D and N cell culture lines; MD and MN), and one person cultured 1B-2R1 cells for 12 months. Using the specimens obtained at several time points (after 0, 4, 6, 12 and 18 months in culture), the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 1(a), replicon RNAs approximately 8 kb long were detected in all specimens except those from the cured cells, from which the replicons had been eliminated from the replicon cells by treatment with interferon-α. The number of copies of replicon RNAs in total RNA (each 3 μg) extracted from the replicon cells was estimated to be in the range of 10^7 to 10^8 by comparing these replicon RNAs with replicon RNA synthesized *in vitro*. The NS3 and NS5B were also detected in all specimens except those from the cured cells (Fig. 1b). The expression levels of replicon RNAs and HCV proteins differed somewhat among these specimens, and no strong quantitative relationship between replicon RNA and HCV proteins was observed (Fig. 1). These results suggest that the stability of replicon RNA or HCV proteins produced from the replicon RNA, or the efficiency of translation, changes during the periods of cell culture. In summary, we demonstrated that the replication efficiencies of the 50-1 and 1B-2R1 replicons remained high under the G418 selection pressure.

Sequence analysis of the 50-1 and 1B-2R1 replicon RNAs

To clarify the genetic variations and diversities of the replicons during the period of cell culture, we carried out sequence analysis of 50-1 and 1B-2R1 replicon RNAs obtained at several time points in the cultures of both replicon cells. Two separate RNA fragments (one was 2·0 kb in length, containing the 5′ UTR to the amino-terminal of the NS3 region; the other was 6·1 kb in length, containing the NS3 to NS5B regions) were amplified by RT-PCR, and three independent clones of each were sequenced after subcloning into pBR322MC, as described previously (Kato *et al.*, 2003b).

Genetic variations of 50-1 and 1B-2R1 replicons during long-term cell culture

The determined nucleotide sequences of the 50-1 and 1B-2R1 replicon RNAs were compared with those of the

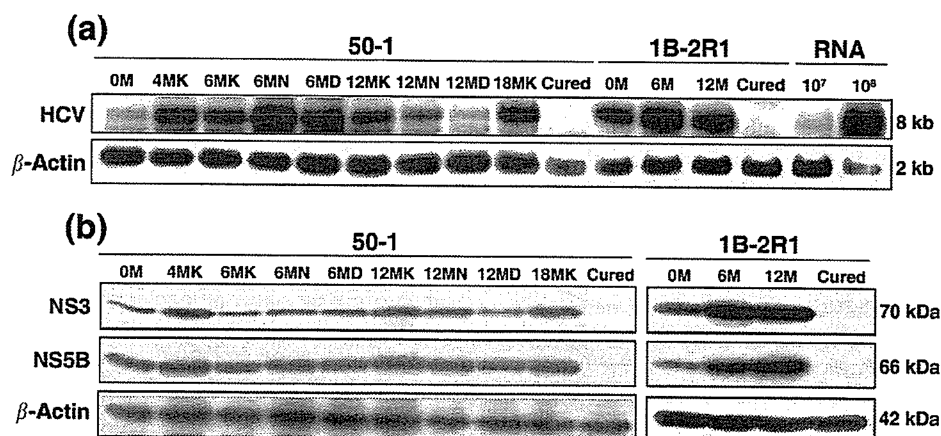

Fig. 1. Characterization of replicon cells in long-term cell culture. (a) Northern blot analysis. Total RNAs from 50-1 cells after 4 months (4MK), 6 months (6MK, 6MN and 6MD), 12 months (12MK, 12MN and 12MD) and 18 months (18MK) in culture, as well as total RNA from the parental 50-1 cells (0M) were used for the analysis. Total RNAs from 1B-2R1 cells after 6 months (6M) and 12 months (12M) in culture, as well as total RNA from the parental 1B-2R1 cells (0M) were used for the analysis. Total RNAs from each cured cells obtained from 50-1 and 1B-2R1 cells by interferon treatment were also used as a negative control. Northern blot analysis was performed using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific probe (lower panel). Synthetic RNA transcribed from pNSS1RZ2RU (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (b) Western blot analysis. The orders of specimens were the same as in (a). Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analysed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane.

original 50-1 (Kishine *et al.*, 2002; GenBank accession no. AB041927) and 1B-2R1 replicons (Kato *et al.*, 2003b; AB109543), respectively. The results revealed that the numbers of base substitutions in the first 2·0 kb region and in the NS region (6·1 kb) of both replicon RNAs were time-dependently increased with linearity (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of replicon RNA. Based on the results after 12 months in culture, the apparent mutation rates in 50-1 replicon RNA were calculated to be $3{\cdot}1 \times 10^{-3}$ and $3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region and NS region, respectively, indicating that there was no difference in mutation rate between the two regions of 50-1 replicon RNA. Interestingly, almost the same mutation rates ($3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region; $3{\cdot}1 \times 10^{-3}$ base substitutions/site/year in NS region) were obtained for the 1B-2R1 replicon RNA, suggesting that the replication efficiency of the 1B-2R1 replicon was almost equal to that of the 50-1 replicon.

Fig. 3(a) shows the schematic presentation of mutations detected in the first 2 kb region by comparison with the original sequences (NNRZ2RU) of 50-1 and 1B-2R1 replicon RNAs (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The results revealed that there were no common mutations among the four cell culture lines (three for 50-1 and one for 1B-2R1) over at least 12 months of cell culture. However, genetic mutations in both replicons were time-dependently increased and accumulated, and several mutations became abundant during the subsequent cell culture (Fig. 3a).

The NS regions (6·1 kb) of the 50-1 and 1B-2R1 replicon RNAs were also analysed in addition to the first 2 kb region. The mutation sites that showed amino acid substitutions are schematically presented in Fig. 3(b). Regarding the 50-1 replicon, 2 aa substitutions (P1115L and E1966A) were newly detected after 6 months in culture in all three cell culture lines, in addition to 2 aa substitutions (K1609E and V1896F) already observed when the replicon was first established. These four substituted amino acids were stably maintained over at least 12 months of cell culture. However, such amino acid substitutions were not observed in the 1B-2R1 replicon even after 12 months of culture. After more than 12 months in culture, several culture line-specific amino acid substitutions (*1–5 for the K culture line; *6–8 for the D culture line; and *9–12 for the N culture line in Fig. 3b) were observed in the 50-1 replicon. Also in the 1B-2R1 replicon, 1 aa substitution (*13 in Fig. 3b) was detected after 12 months in culture; however, no common amino acid substitutions were observed between the 50-1 and 1B-2R1 replicons. The mean numbers of amino acid substitutions occurring after 6 and 12 months in culture were 4·2 and 8·9, respectively, for the 50-1 replicon, and 4·7 and 10·0, respectively, for the 1B-2R1 replicon. These values indicate a steady genetic evolution of 50-1 and 1B-2R1 replicons during the cell culture.

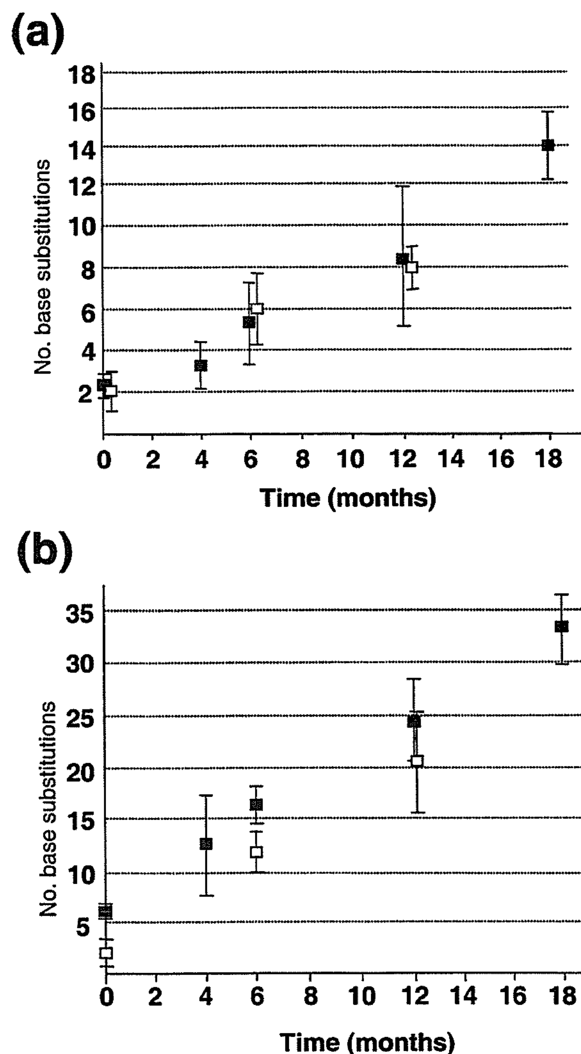

Fig. 2. Genetic variations of 50-1 and 1B-2R1 replicon RNAs. (a) First 2·0 kb region of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the first 2·0 kb region of 50-1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the first 2·0 kb region of 1B-2R1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). (b) NS region (6·1 kb) of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the NS region of 50-1 replicon RNA, by comparison with its original sequences (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the NS region of 1B-2R1 replicon RNA, by comparison with its original sequences (Kato *et al.*, 2003b).

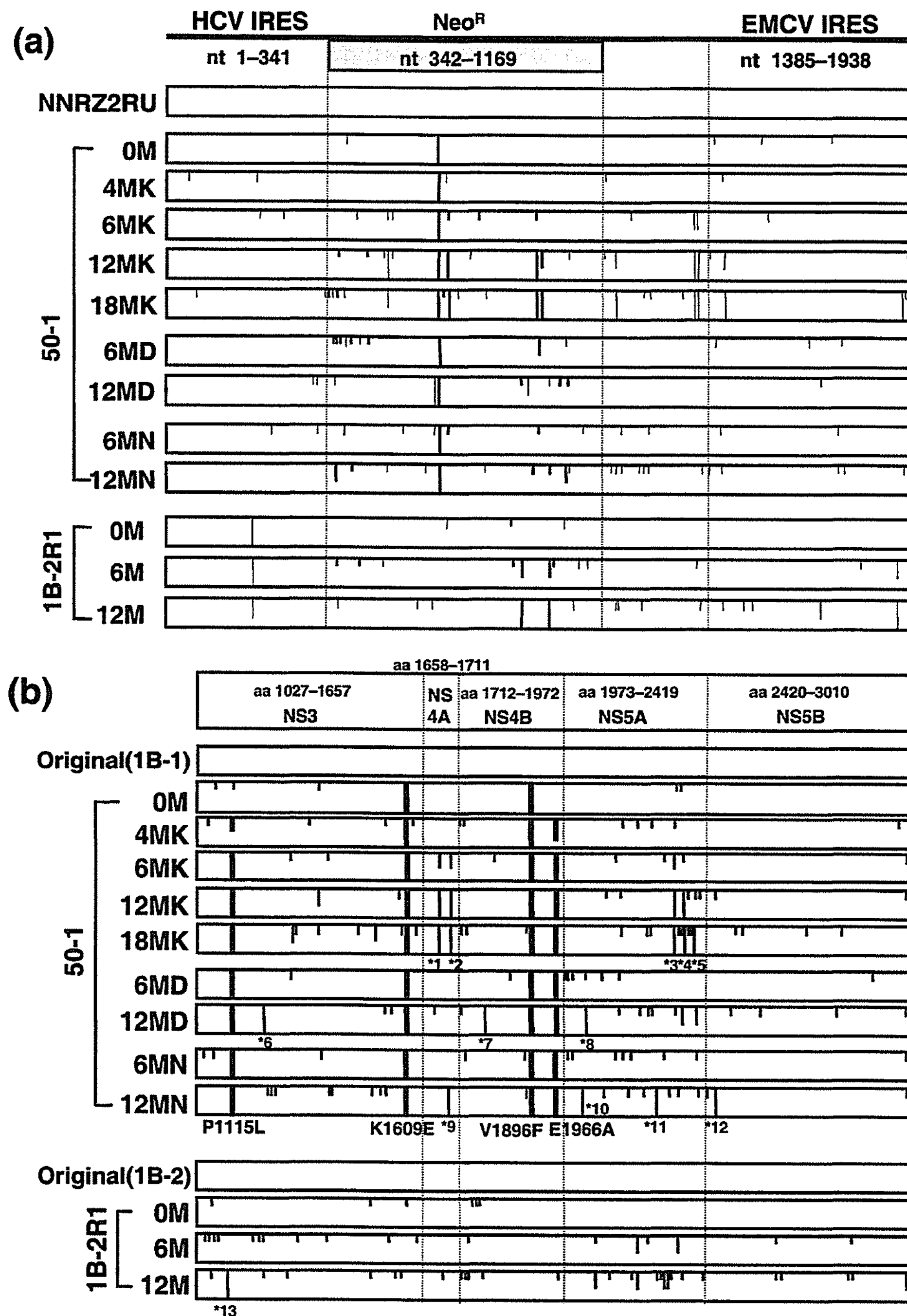

Fig. 3. Genetic variations of 50-1 and 1B-2R1 replicons in long-term cell culture. (a) Schematic presentation of mutations detected in first 2·0 kb regions of the replicon RNAs. Compared with the nucleotide sequences of the first 2·0 kb region of the original replicon RNA (NNRZ2RU), nucleotide positions mutated in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Non-synonymous substitutions in the Neo^{R} region are indicated by heavy vertical lines. (b) Schematic presentation of amino acid substitutions detected in the NS regions of the replicons. Compared with the amino acid sequences of NS region of the original 50-1 (Kishine *et al.*, 2002) and 1B-2R1 replicons (Kato *et al.*, 2003b), amino acid positions substituted in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Four amino acid substitutions (P1115L, K1609E, V1896F and E1966A) are indicated by heavy vertical lines. Culture line-specific amino acid substitutions (indicated by the numbers with asterisks) are as follows: *1, I1686V; *2, L1701R; *3, T2332A; *4, G2336E; *5, A2372T; *6, A1243G; *7, I1797V; *8, S2053G; *9, L1701R; *10, T2051N; *11, R2279G; *12, L2476M; *13, I1097V.

Classification of mutations occurring in 50-1 and 1B-2R1 replicon RNAs during the long-term cell culture

To understand the mutation mode of the replicons in long-term cell culture, we examined the numbers of synonymous and non-synonymous mutations with transition or transversion. The results are summarized in Table 1. The ratio of synonymous to non-synonymous mutations in 50-1 replicon RNA was 0·81 to 1·50 (1·38±0·14 after 6 months in culture and 1·03±0·20 after 12 months in culture), and the ratio in 1B-2R1 replicon RNA was 0·63 after 6 months in culture and 0·59 after 12 months in culture. These values indicate that amino acid substitutions in the replicons occur frequently during the cell culture. The rate of mutations with transition in the 50-1 replicon was 1·82–4·06-fold (2·00±0·18 after 6 months in culture and 2·85±1·07 after 12 months in culture) greater than the rate of mutations with transversion. Similarly, the 1B-2R1 replicon showed a transition-to-transversion ratio of 2·69 (after 6 months in culture) or 2·86 (after 12 months in culture).

Regarding the mutation patterns over more than 12 months of culture, we observed that A→G and U→C mutations were the most and second-most common mutations, and these mutations were approximately two to three times more common than G→A and C→U mutations (Supplementary Table A, which is available as Supplementary material in JGV Online). The rarest mutation was G→U (Supplementary Table A).

Genetic diversity of the 50-1 and 1B-2R1 replicons arising during long-term cell culture

To clarify whether or not the replicons acquire a quasispecies nature during long-term cell culture, we estimated the genetic diversities of the 50-1 and 1B-2R1 replicon populations. First, based on the sequence data of all clones obtained in this study, we constructed phylogenetic trees for the first 2 kb region and the NS region. The results revealed that the genetic diversity of 50-1 replicon populations was expanded in a time-dependent manner (Fig. 4). Similar phylogenetic trees were obtained for the 1B-2R1 replicon populations as well (data not shown). Next, as another index of genetic diversity, we calculated the mean number of nucleotide differences among three independent clones at each time point. The schematic presentation of such analysis on the NS regions of 50-1 and 1B-2R1 replicon RNAs was shown in Supplementary Fig. A, which is available as Supplementary material in JGV Online. The results also showed a time-dependent expansion of genetic diversity. After 12 months in culture, 0·32 % (mean of three cell culture lines) and 0·55 % diversities in nucleotide sequences were observed in the NS region of 50-1 and 1B-2R1 replicon RNAs. A similar time-dependent expansion of genetic diversity was also observed in the first 2 kb regions of both replicon RNAs (data not shown). These results indicate that the quasispecies nature of replicon RNA was easily acquired during the replication of the replicons.

Table 1. Base substitutions occurring in 50-1 and 1B-2R1 replicon RNAs during long-term cell culture

The counting of base substitutions was performed by comparison with the consensus sequence obtained from the 0M series of 50-1 or 1B-2R1 replicon.

Replicon	series	No. base substitutions										Synonymous/ non-synonymous	Transition/ transversion
		Transition					Transversion						
		Synonymous		Non-synonymous		Non-coding region	Synonymous		Non-synonymous		Non-coding region		
		Neo^R	NS	Neo^R	NS		Neo^R	NS	Neo^R	NS			
50-1	4MK	1	13	0	8	4	0	5	0	6	2	1·36	2·00
	6MK	0	20	2	10	8	3	8	1	9	1	1·41	1·82
	12MK	3	29	6	19	13	4	9	4	9	2	1·18	2·50
	18MK	5	43	8	26	16	3	10	4	14	5	1·17	2·72
	6MD	3	20	3	9	2	0	5	4	7	1	1·22	2·18
	12MD	5	29	2	26	3	2	5	1	8	0	1·11	4·06
	6MN	2	19	2	8	3	2	4	0	8	3	1·50	2·00
	12MN	3	25	2	21	9	1	6	5	15	3	0·81	2·00
1B-2R1	6M	1	14	5	14	1	1	1	3	5	3	0·63	2·69
	12M	2	22	4	29	6	1	2	3	10	6	0·59	2·86

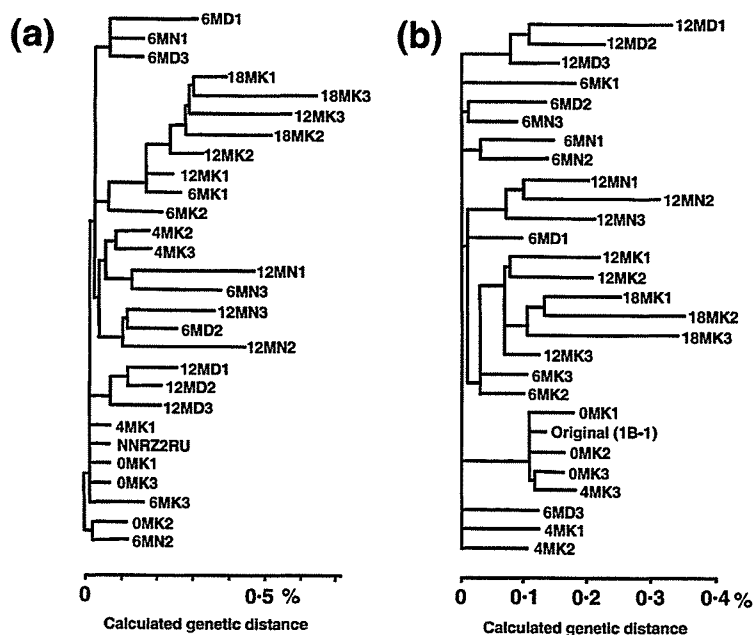

Fig. 4. Phylogenetic trees of 50-1 replicon populations obtained in long-term cell culture. The phylogenetic tree is depicted on the basis of nucleotide sequences of all replicon clones obtained by long-term culture of 50-1 cells. (a) The first 2·0 kb region of replicon RNA. NNRZ2RU indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones. (b) The NS region of replicon RNA. Original (1B-1) indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones.

Enhancement of HCV replication is associated with the expansion of the replicons' genetic diversity

To assess whether or not the mutations accumulating in the replicons increase the replication efficiencies of the replicons, the efficiency of colony formation (ECF) of the replicon was examined at each time point of the culture. An ECF assay was performed by transfection of total RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points into naïve HuH-7 cells. After 3 weeks of G418 selection, only a few colonies were obtained when RNAs from 50-1 replicon cells cultured less than 4 month were used (Fig. 5). However, ECF was apparently increased when RNAs from cells cultured 6 months, in particular the D and N cell culture lines, were used, and much higher numbers of colonies were obtained when RNAs from cells cultured 12 months were used (Fig. 5). Interestingly, ECFs of RNAs from D and N cell lines cultured more than 6 months were higher than those in the K cell culture line. These results indicated that ECF of the replicon was increased with the cultured periods of the replicon cells and suggested that ECF enhancement is associated with the expansion of the 50-1 replicon's genetic diversity.

In contrast to the case with 50-1 replicon cells, a number of colonies were obtained even when RNA from the initial culture of 1B-2R1 replicon cells was used (Fig. 5). In this replicon also, the ECF of RNA from cells cultured 12 months was apparently higher than those of RNA from the initial culture or 6 months of culture (Fig. 5). These results suggest that S2200R substitution, which was detected when the 1B-2R1 replicon was established (Kato *et al.*, 2003b), function as an adaptive mutation, and that the expansion of genetic diversity in the 1B-2R1 replicon also contributes to the enhancement of ECF, as was the case with the 50-1 replicon.

Effect of ribavirin and mizoribine on the genetic evolution and dynamics of the 50-1 replicon

Combined treatment of interferon plus ribavirin for patients with chronic hepatitis C has been shown to be more effective than treatment with interferon alone (McHutchison *et al.*, 1998), although it has been shown that ribavirin alone does not cause a decrease of HCV level in patients with chronic hepatitis C. Recently, several groups have reported that ribavirin might cause 'error catastrophe' of HCV genome (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003), however, controversial results have also been reported (Schinkel *et al.*, 2003). Therefore, to clarify whether or not ribavirin affects the genetic alterations of HCV, we cultured parent 50-1 cells (corresponding to 0M in Fig. 1) for 6 months in the presence of ribavirin (5 or 25 μM) or its derivative molecule, mizoribine (25 μM). As a control, the parent 50-1 cells were also cultured for 6 months in the absence of ribavirin or mizoribine. After 6 months in culture, the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 6(a), the level of replicon RNA in the cells treated with ribavirin or mizoribine was almost the same as that in the cells without ribavirin or mizoribine treatment. The NS3 and NS5B were also expressed at similar levels in the cells irrespective of ribavirin or mizoribine treatment (Fig. 6b). These results indicate that even 6 months of treatment with ribavirin or mizoribine did not prevent the replication of replicon RNA under the G418 selection pressure. Using the 50-1 cells cultured for 6 months with or without ribavirin or mizoribine, we performed sequence analysis of replicon

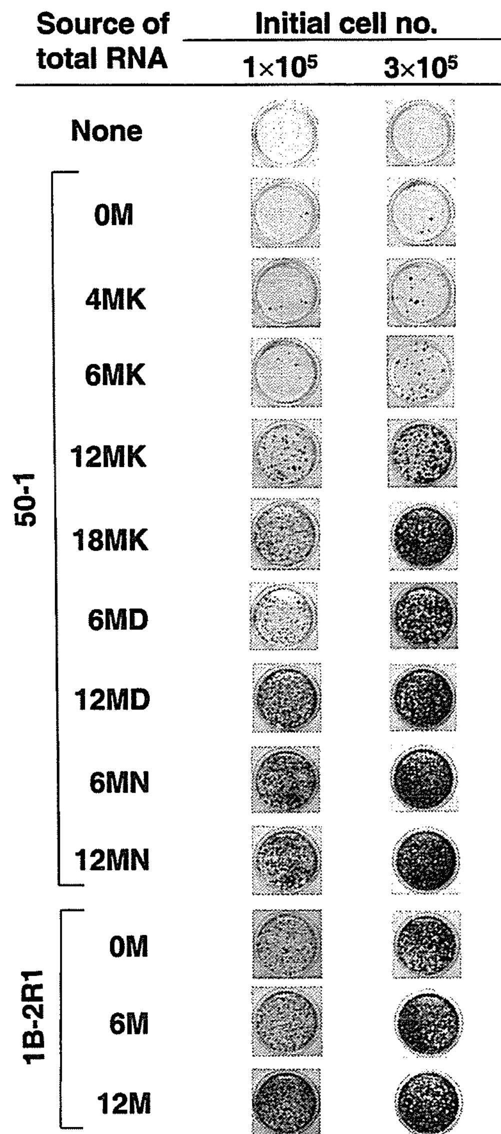

Fig. 5. ECF of the RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points in the culture. Total RNAs obtained from the replicon cells were transfected into HuH-7 cells as described in Methods. The panels show the cell colonies that were recovered after 3 weeks of G418 selection.

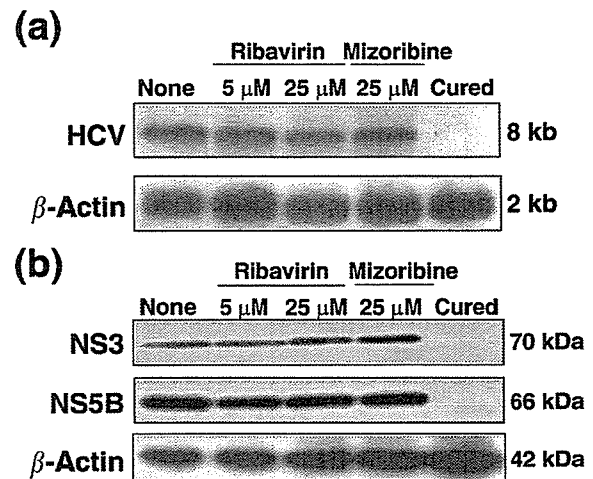

Fig. 6. Characterization of 50-1 cells cultured for 6 months in the presence of ribavirin or mizoribine. (a) Northern blot analysis. Total RNAs from 50-1 cells cultured for 6 months in the presence of ribavirin (5 and 25 μM) or mizoribine (25 μM), as well as total RNA from 50-1 cells cultured for 6 months in the absence of ribavirin and mizoribine were used for the analysis. Northern blot analysis was performed as indicated in Fig. 1(a). (b) Western blot analysis. The orders of specimens were the same as in (a). Western blot analysis was performed as indicated in Fig. 1(b).

RNAs as described above. As shown in Table 2, the results revealed that the numbers of mutations in the first 2·0 kb and NS regions of the replicon RNAs sequenced were not significantly different among the specimens, although the number in the NS region derived from the cells treated with 25 μM of ribavirin was a little lower than those of the other specimens. These results suggest that the treatment of replicon cells with either ribavirin or mizoribine does not increase the mutation rate of replicon RNA. The ratio of synonymous and non-synonymous mutations, and the ratio of transition and transversion mutations were also not altered by ribavirin or mizoribine treatment (data not shown). In addition, we did not observe any ribavirin- or mizoribine-specific common amino acid substitutions in either the first 2 kb or NS regions of the replicon RNA, although P1115L and E1966A were detected after 6 months in culture in all cell culture lines. The above-described analysis of genetic diversity among the replicon RNAs did

Table 2. Base substitutions occurred in 50-1 replicon RNA during 6 months culture in the presence of ribavirin or mizoribine

6MR5 and 6MR25 indicate the series treated with 5 and 25 μM of ribavirin, respectively. 6MM25 indicates the series treated with 25 μM of mizoribine. The counting of base substitutions was performed by the comparison with the original sequence of 50-1 replicon (Kishine *et al.*, 2002).

Series	First 2-kb region	NS region
6M (Fig. 2)	5·4±1·9*	16·4±1·8
6M	5·7±2·5	16·0±0·0
6MR5	5·7±1·5	16·3±1·5
6MR25	5·7±1·5	10·7±1·2
6MM25	3·7±0·6	18·7±4·0

*Numbers of base substitutions±SD.

not reveal any significant differences between the specimens derived from the replicon cells with and those without ribavirin or mizoribine treatment (data not shown). Taken together, these results suggest that neither ribavirin nor mizoribine accelerated the mutation rate of HCV replicons or the development of their quasispecies nature.

DISCUSSION

In this study, we analysed the genetic evolution and dynamics of HCV replicons, and time-dependent genetic mutations of HCV replicons were observed. Time-dependent expansions of their genetic diversities were also revealed. Our results should provide useful fundamental information for understanding the remarkable genetic diversity and variation among the HCV genomes observed in patients with chronic hepatitis C.

Although RT-PCR techniques were used to amplify the replicon RNAs in this study, it is unlikely that the detected mutations were due to errors related to the use of the KOD-plus DNA polymerase in the PCR reaction, because we previously showed that KOD-plus DNA polymerase possessed a high proofreading activity (Alam *et al.*, 2002; Naganuma *et al.*, 2004). Furthermore, in the present study, we sequenced several clones (containing a 2·0 or 6·1 kb fragment) obtained by PCR using KOD-plus DNA polymerase and a single sequenced clone as a template, but no mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity. However, we are not able to completely exclude the possibility that some substitutions resulted from the erroneous use of KOD-plus DNA polymerase during the PCR. Even if such errors occurred, the error frequency is estimated to be less than one nucleotide per sequenced clone. This is explained as follows. Fig. 2 shows that the numbers of substitutions time-dependently increased with linearity in both HCV replicons. Interestingly, when these linear lines are extrapolated to zero base substitutions, the crossing points show approximately −2–−3 months in the time axis. These range of months is in accord with the time of initial electroporation of HCV replicon RNA to HuH-7 cells. Therefore, PCR-induced mutations are considered to be very rare and such mutations would have very little effect on the results shown in Fig. 2. In addition, to avoid a sampling effect, we sequenced three independent clones derived from each time point.

We showed that the mutation rates for the 50-1 and 1B-2R1 replicon RNAs were almost the same – about 3×10^{-3} base substitutions/site/year. However, the actual mutation frequency of the replicon RNAs would be higher than this value, because the mutations that occurred in positions that were critical for the replication of replicon RNA should not have been passed on to the progeny. Our observed mutation rates of the replicon RNAs were approximately two times higher than those previously obtained in chimpanzees and clinical patients with chronic hepatitis C (Major *et al.*, 1999; Ogata *et al.*, 1991; Okamoto *et al.*, 1992). Since the selective pressure of the immune system also functions *in vivo* (Kato *et al.*, 1993), the mutation rate in cell culture obtained in this study may be reasonable value as a potential mutation rate of HCV. However, direct comparison of these mutation rates would be difficult, because both the experimental model and analytical method were different in this study compared with the previous studies. It would be interesting to examine whether this mutation rate (3×10^{-3} base substitutions/site/year) would be maintained during longer-term culture of the replicon cells. If so, approximately 3 % of nucleotide sequences of the replicon RNAs might be mutated after 10 years in cell culture. Alternatively, the mutations might become saturated during further long-term culture of the replicon cells. To clarify this point, further long-term culture of replicon cells is in progress.

Although the mutations detected in this study were dispersed throughout the entire length of the replicon RNAs (Fig. 3), the mutation frequencies in the 5′ UTR and NS5B region were lower than those in other regions, and the NS5A region showed the highest mutation frequency. These observations are consistent with the genetic diversities of HCVs in patients with chronic hepatitis C reported to date (Kato, 2001). In addition, the positions in which amino acid substitutions were observed during the cell culture did not appear to be critical for replication of the HCV genome.

Time-dependent expansions of genetic diversities of HCV replicons were also found in this study. However, this finding seems to be different from the previous findings that HCV populations in the cells infected *in vitro* gradually altered with time and converged to the limited populations (Kato *et al.*, 1998; Kato, 2001). This gap may have been due to the differences in the HCV sources used: a patient's inoculum containing a quasispecies of HCV was used for the *in vitro* infection experiment, and a single HCV species was used for the replicon system. Alternatively, the gap may have been due to the overwhelming difference between the replication level of the HCV genome in the cells infected *in vitro* and that in the replicon cells.

To date, a number of amino acid substitutions belonging to adaptive mutations that enhance the frequency with which the replicon is established *in vitro* have been found in established HCV replicons (Bartenschlager, 2002; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Krieger *et al.*, 2001; Lanford *et al.*, 2003; Lohmann *et al.*, 2001, 2003; Pflugheber *et al.*, 2002). Although none of the amino acid substitutions detected in the long-term cultures of the 50-1 and 1B-2R1 replicons were the same as those reported as adaptive mutations, ECF analysis of the replicons using naïve HuH-7 cells suggested that adaptive mutations accumulated in the replicon populations in a time-dependent manner. In particular, drastic enhancement of ECF was observed in the 50-1 replicon after 6 months of culture. However, this result suggests that the four common amino acid substitutions (P1115L, K1609E, V1896F and E1966A) do not contribute much to the drastic enhancement of ECF,

because the ECFs of 4MK and 6MK samples possessing these substitutions did not increase much. Therefore, we estimate that some uncommon amino acid substitutions accumulated as so-called adaptive mutations. The candidates for such adaptive mutations are culture-line-specific amino acid substitutions (Fig. 3b, *1–12), and many amino acid substitutions sporadically appeared in the replicons in the long-term cell cultures. To identify which amino acid substitution is the main contributor to the drastic enhancement of ECF, further transfection experiments using replicon RNAs possessing mutations will be needed. Based on the results of this study, S2200R substitution in the 1B-2R1 replicon is considered an adaptive mutation. This description is supported by the previous result that we were unable to obtain any G418-resistant colonies when the original 1B-2 replicon RNA library, used in the isolation of the 1B-2R1 replicon, was transfected into naïve HuH-7 cells (Kato *et al.*, 2003b). Since the ECF of 1B-2R1 replicon RNA from 12 months of culture was further enhanced, it may be that the I1097V substitution, detected commonly at 12 months of culture, functions as an additional adaptive mutation.

Interestingly, once a new mutation was observed in all three clones at a particular time point, the clones which went back to the original sequences were never obtained in the subsequent cell culture, except for one clone (a mutation in the HCV IRES region) derived from 1B-2R1 replicon cells after 12 months in culture (Fig. 3a). This finding suggests that the genetic evolution of HCV replicons is irreversibly progressing.

Although the mechanism of action of ribavirin for patients with chronic hepatitis C is ambiguous, an 'error catastrophe' theory of ribavirin has been proposed by several groups (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003). However, our results obtained in this study were not able to support this 'error catastrophe' theory, because ribavirin had no effect on the genetic variation and diversity of the 50-1 replicon. The concentration (5 and 25 μM) of ribavirin used in this study was considered to be reasonable, because the growth rate of 50-1 cells decreased at a ribavirin concentration of more than 50 μM, and approximately 10 μM of ribavirin is the maximum plasma concentration in current clinical usage (Tanabe *et al.*, 2004). Higher concentration (more than 50 μM) of ribavirin used in previous studies may be required for causation of the error catastrophe. Recently, a single amino acid substitution (F2834Y) was identified as a ribavirin-resistant NS5B mutation in genotype 1a (Young *et al.*, 2003); however, it is difficult to evaluate that finding in this study, because most of the HCV strains belonging to genotype 1b, including 1B-1 (50-1) and 1B-2 (1B-2R1), already possess a Tyr residue at position 2834. No amino acid substitution at position 2834 in NS5B was observed in the replicon cells treated with ribavirin.

This study provided the fact that the genetic diversity of HCV replicons was enlarged in a time-dependent manner during long-term cell culture. Since all the HCV replicons established to date have been shown to be highly sensitive to interferon-α, -β and -γ (Kato *et al.*, 2003b), and most of the HCV replicons established to date are able to replicate in only HuH-7 cells, the extensive genetic polymorphism of HCV replicon populations obtained by long-term cell culture may change the sensitivity against interferon or the ability of replication in the cells except for HuH-7. In the future, it will be necessary to clarify these points. Thus, HCV replicon populations obtained by long-term cell culture may be useful not only for analysis of the genetic variations and dynamics of HCV but also for analysis of the variable properties of HCV.

ACKNOWLEDGEMENTS

This work was supported by grants-in-aid for research on hepatitis from the Ministry of Health, Labour and Welfare of Japan and by grants-in-aid for scientific research from the Organization for Pharmaceutical Safety and Research (OPSR).

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Liver International 2006: 26: 1111–1118

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Clinical Studies

Liver International
DOI: 10.1111/j.1478-3231.2006.01352.x

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.
Liver International 2006: 26: 1111–1118.
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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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Received 5 December 2005,
accepted 18 June 2006

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

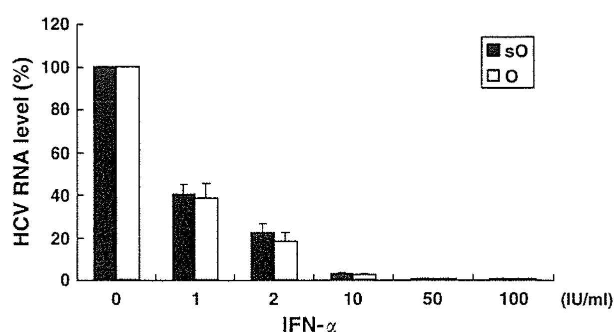

Fig. 1. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with interferon-α (IFN-α). IFN-α sensitivity of HCV RNA replication in sO (black bars) and O cells (white bars). Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials 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.

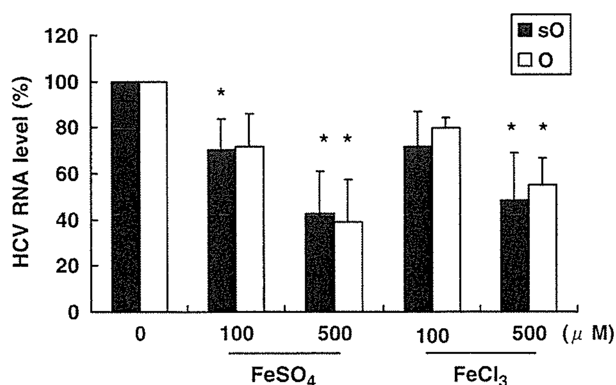

Fig. 2. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with iron. Iron inhibition of HCV RNA replication in sO (black bars) and O cells (white bars). sO and O cells were treated for 48 h with iron sulfate (100 and 500 μM) or iron chloride (100 and 500 μM). The control 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 ($P<0.01$).

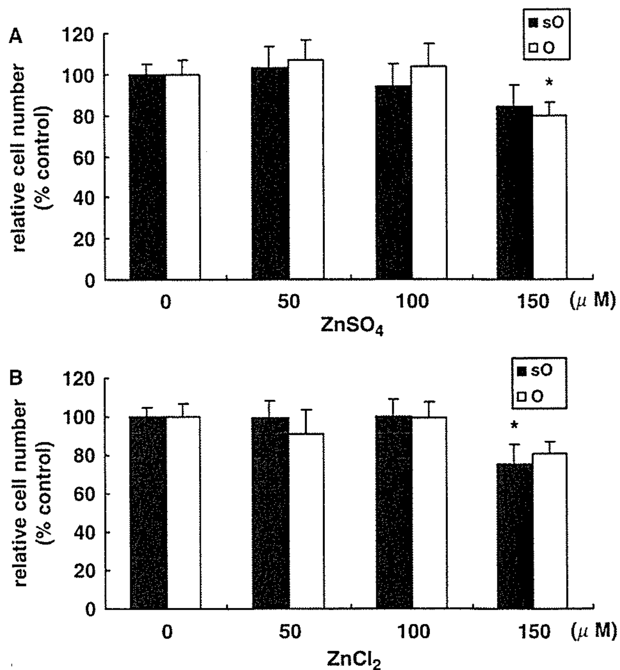

Fig. 3. Cytotoxicity of zinc salts to sO and O cells. (A) sO and O cells were cultured in the absence or presence of zinc sulfate (50, 100, and 150 μM each) for 72 h, and then the cell number was determined as described under the Materials and methods. The relative cell number (% control) calculated 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

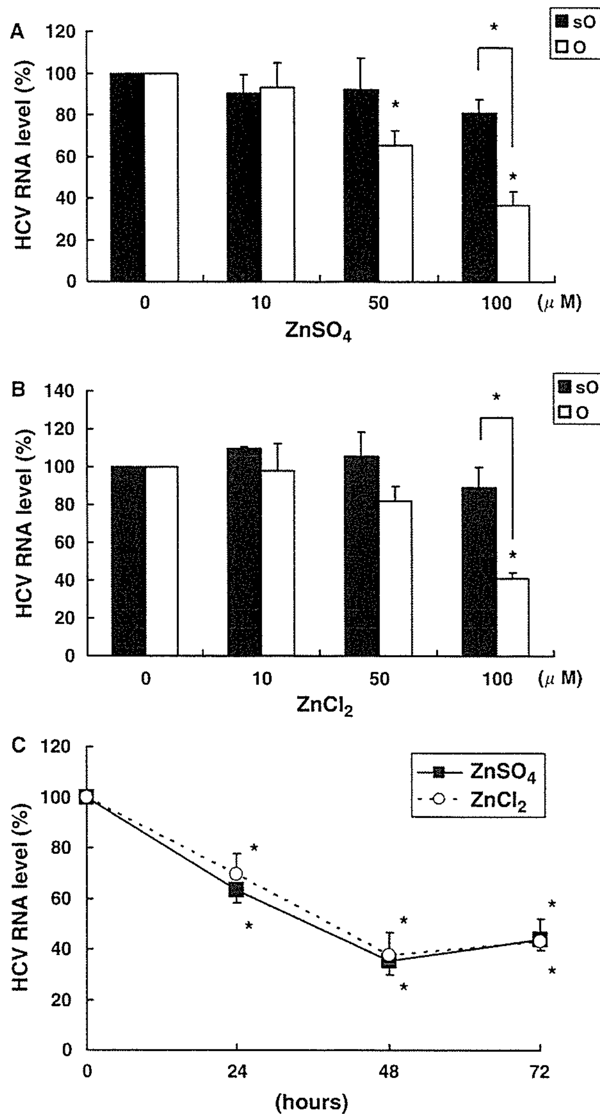

Fig. 4. Different effect of zinc salts between subgenomic hepatitis C virus (HCV) replicon and genome-length HCV RNA replication systems. (A) The sO and O cells were treated for 48 h with zinc sulfate (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (B) sO 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

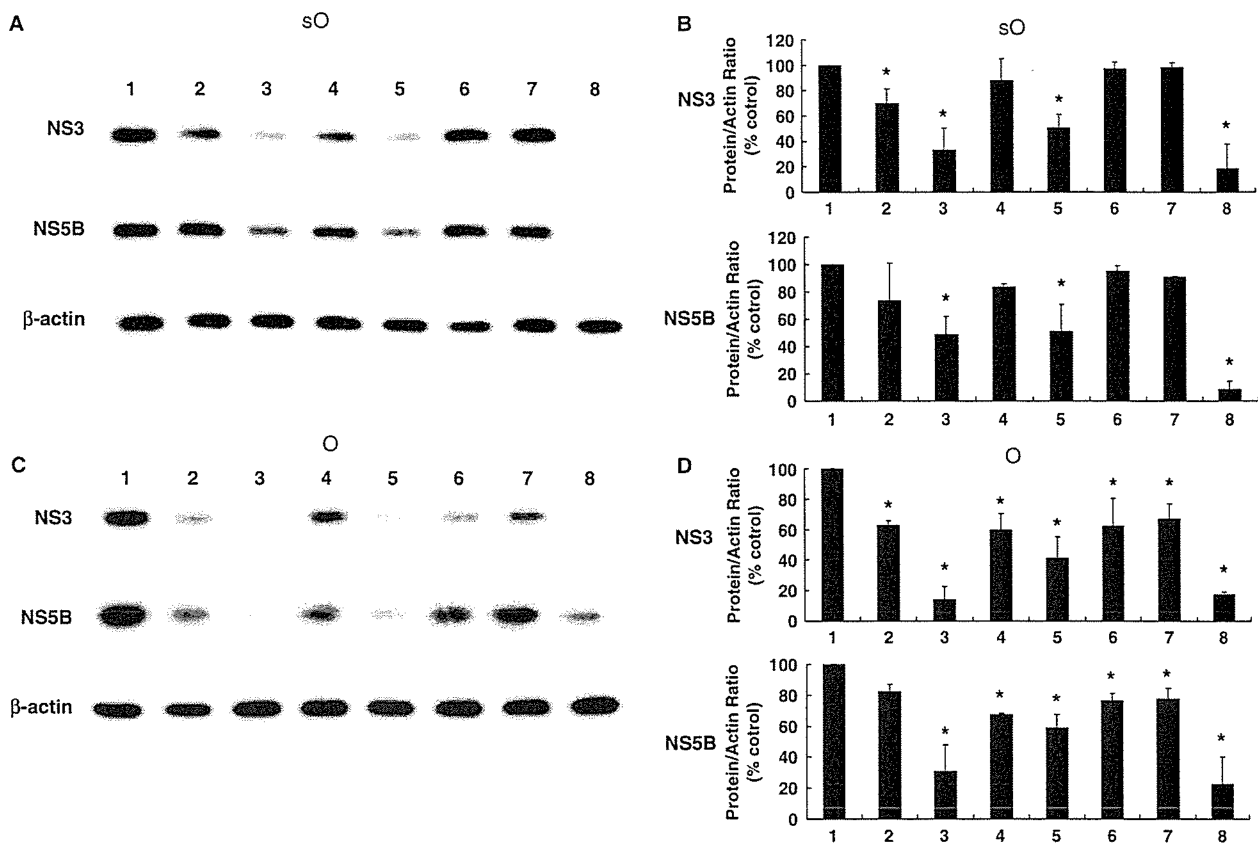

Fig. 5. Inhibitory effect of the expression of hepatitis C virus (HCV) proteins. (A) The sO cells were treated for 48 h without reagents (0 μM) as control wells (lane 1), or treated with 100 and 500 μM of iron sulfate (lanes 2 and 3), 100 and 500 μM of iron chloride (lanes 4 and 5), 100 μM zinc sulfate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

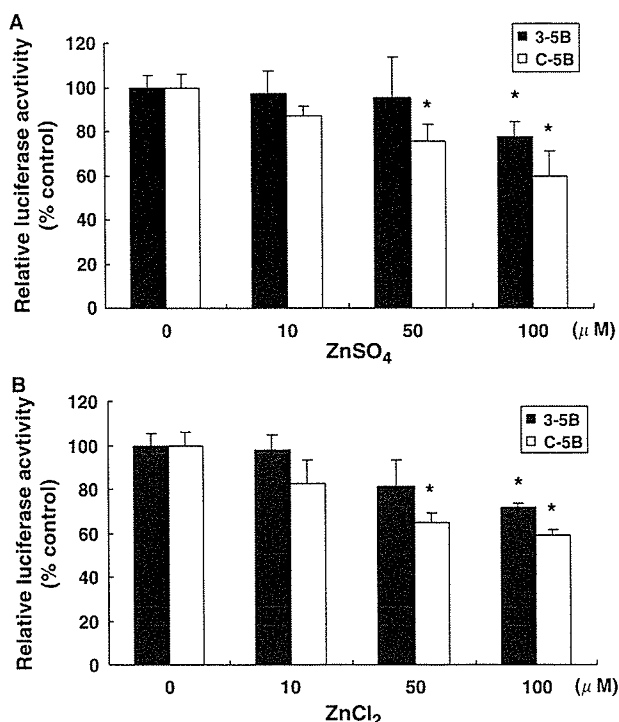

Fig. 6. Inhibitory effect of zinc of hepatitis C virus (HCV) RNA replication in ORN/3-5B/KE and ORN/C-5B/KE cells treated with zinc salts on a luciferase reporter assay system. (A) Inhibitory effect of zinc sulfate against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). 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.

Acknowledgements

This study was supported by a grant from the 21st century Center of Excellence (COE) in Biomedical Research Using Accelerator Technology, Gunma, Japan.

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Different Anti-HCV Profiles of Statins and Their Potential for Combination Therapy With Interferon

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We recently developed a genome-length hepatitis C virus (HCV) RNA replication system (OR6) with luciferase as a reporter. The OR6 assay system has enabled prompt and precise quantification of HCV RNA replication. Pegylated interferon (IFN) and ribavirin combination therapy is the world standard for chronic hepatitis C, but its effectiveness is limited to about 55% of patients. Newer therapeutic approaches are needed. In the present study, we used the OR6 assay system to evaluate the anti-HCV activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, called statins, and their effects in combination with IFN-α. Five types of statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) were examined for their anti-HCV activities. Fluvastatin exhibited the strongest anti-HCV activity (IC_{50}: 0.9 μmol/L), whereas atorvastatin and simvastatin showed moderate inhibitory effects. However, lovastatin, reported recently as an inhibitor of HCV replication, was shown to exhibit the weakest anti-HCV activity. The anti-HCV activities of statins were reversed by the addition of mevalonate or geranylgeraniol. Surprisingly, however, pravastatin exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. The combination of IFN and the statins (except for pravastatin) exhibited strong inhibitory effects on HCV RNA replication. In combination with IFN, fluvastatin also exhibited a synergistic inhibitory effect. In conclusion, statins, especially fluvastatin, could be potentially useful as new anti-HCV reagents in combination with IFN. (HEPATOLOGY 2006;44:117-125.)

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C (CHC) and yields a sustained virological response (SVR) rate of about 55%.[1] This means that about 45% of patients with CH C are still threatened by the progression of the disease to cirrhosis and hepatocellular carcinoma. Until 1999, when Lohmann et al. developed the subgenomic replicon of HCV, it was difficult to screen anti-HCV reagents.[2] Many improvements followed that breakthrough, such as a genome-length HCV RNA replication system[3,4] and a subgenomic replicon with a reporter assay system[5]; more recently, Wakita et al. used a genotype 2a strain, JFH1, to produce the infectious virus in cell culture.[6-8]

Genotype 1 is the major genotype of HCV found in Japan, the United States, and many other countries. Unfortunately, the SVR rate after combination therapy of pegylated IFN with ribavirin is less than 50% for this genotype. To find a more effective therapy especially for CHC patients with genotype 1, we recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6), which has been an effective screening tool.[9,10]

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are in wide use for the treatment of hypercholesterolemia. Recently, it

Abbreviations: HCV, hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; SVR, sustained virological response; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 2'-5'-OAS1, 2'-5'-oligoadenylate synthetase 1; LOV, lovastatin; ATV, atorvastatin; PRV, pravastatin; SMV, simvastatin; FLV, fluvastatin; RL, renilla luciferase; LST-1, human liver-specific organic anion transpoter-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; AMPH-B, amphotericin B; RT-PCR, reverse-transcription polymerase chain reaction.

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Received November 17, 2005; accepted April 17, 2006.

Supported by a grant-in-aid for the third-term comprehensive 10-year strategy for cancer control and by a grant-in-aid for research on hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan, and by the Program for the Promotion of Fundamental Studies in Health Science of the Pharmaceutical and Medical Device Agency (PMAD), Japan.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21232

Potential conflict of interest: Nothing to report.

was reported that lovastatin (LOV) inhibited HCV RNA replication.[11,12] These reports suggested the anti-HCV activity of LOV resulted from inhibition of the geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis.[11,12] More recently, FBL2 has been reported to be a host target protein for geranylgeranylation, which is responsible for HCV replication.[13] Although several types of statins are used clinically to lower cholesterol, thus far only LOV has been tested for anti-HCV activity. In the present study, we used the OR6 assay system to test the anti-HCV activity of five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. We found that ATV, FLV, and SMV exhibited stronger anti-HCV activity than that previously reported for LOV and that PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. Because FLV showed the strongest anti-HCV activity, we also examined the effect of the combination of IFN-α and FLV on HCV RNA replication and found a synergistic inhibitory effect of IFN-α and FLV on HCV RNA replication.

Materials and Methods

Cell Cultures. OR6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and G418 (300 μg/mL; Geneticin, Invitrogen) and passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/mL for 2 weeks) without G418, as previously described.[9] HepG2 and PH5CH8 (human immortalized hepatocytes) cells were cultured as previously described.[14,15]

Luciferase Reporter Assay. For the renilla luciferase (RL) assay, 2×10^4 OR6 cells were plated in 24-well plates at least in triplicate for each assay and were cultured for 24 hours. The cells were treated with statins for 72 hours and were harvested with Renilla lysis reagent (Promega) and subjected to the RL assay according to the manufacturer's protocol.

Reagents. FLV, LOV, and PRV were purchased from Calbiochem. ATV and SMV were purchased from Astellas Pharma Inc. and Banyu Pharmaceutical Co. Ltd., respectively. Mevalonate, geranylgeraniol, and geranylgeranyl pyrophosphate were purchased from Sigma.

Reverse-Transcriptase Polymerase Chain Reaction. Total RNA from the cultured cells was extracted with the RNeasy Mini Kit (Qiagen). Reverse-transcriptase polymerase chain reaction (RT-PCR) for HMG-CoA reductase, human liver-specific organic anion transporter (LST-1), 2′-5′-oligoadenylate synthetase 1 (2′-5′-OAS1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a method described previously.[16] Briefly, using cellular total RNA (2 μg), cDNA was synthesized using Superscript II with oligo dT primer. One-tenth of the synthesized cDNA was subjected to polymerase chain reaction (PCR) with the following primer pairs: HMG-CoA reductase, 5′-ATGCCATCCCTGTTGGAGTG-3′ and 5′-TGTTCATCCCCATGGCATCCC-3′; LST-1, 5′-TGGCACACGTGGGTCATGTAGG-3′ and 5′-CACTATCTGCCCCAGCAGAAGG-3′, 2′-5′-OAS1, 5′-AGTACCTGAGAAGGCAGCTCACGA-3′ and 5′-ACTGGCATTCAGAGGATGGTGCAG-3′; and GAPDH, 5′-GACTCATGACCACAGTCCATGC-3′, and 5′-GAGGAGACCACCTGGTGCTCAG-3′.

Western Blot Analysis. Preparation of the cell lysate, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described.[17] The antibodies used in this study were those against Core (Institute of Immunology, Tokyo), NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), β-actin (Sigma, St. Louis, MO), STAT1 (BD Transduction Laboratories, San Diego, CA), and phospho-STAT1 (Y701; Cell Signaling Technology, Beverly, MA). Immunocomplexes on the membranes were detected by the enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

Plasmid Construction. The plasmids pORN/C-5B/KE (Fig. 1A) and pHCV-O were described previously.[9] To construct the pEMCV-RL, two fragments, the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and the *RL* gene, were amplified by PCR from pORN/C-5B/KE using the following primer pairs: EMCV IRES, 5′-CGGGATCCGCGGGACTCGGGGGTTCG-3′ and 5′-CCGCTCGAGGGTATTATCGTGTTTTTCAAAGG-3′; and RL, 5′-CCGCTCGAGATGGCTTCCAAGGTGTACGACC-3′, and 5′-GCTCTAGACTAGACGTTGATCCTGGCGC-3′. The two fragments were ligated into the *Bam*HI and *Xba*I sites in pcDNA 3.1/Zeo (Invitrogen).

Statistical Analysis. Differences in anti-HCV activity between statins were tested using the Student t test. P values $< .05$ were considered statistically significant.

Results

Inhibition of HCV RNA Replication by Statins. We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter.[9,10] This OR6 reporter assay system has enabled prompt and precise quantification of HCV