HMG CoA Reductase Inhibitors Inhibit HCV RNA Replication of HCV Genotype 1b but Not 2a

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Replication of hepatitis C virus (HCV) is regulated by statin, one of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) inhibitors that block mevalonate pathway and cholesterol biosynthesis, which has been used usefully for health improvement and disease control in clinic. In order to know which statin can be used to inhibit HCV replication, we examined the effects of HCV genotype 1b replication by 6 kinds of statins with different structure. We treated six statins to HCV genotype 1b replicon cell. Atorvastatin, simvastatin, fluvastatin, mevastatin, and lovastatin inhibited HCV RNA replication and HCV protein expression in HCV genotype 1b replicon cells, though pravastatin did not affect HCV replication. In order to know whether inhibition of HCV replication by statin is depended on HCV genotype, we treated the statins to HCV genotype 2a producing cells, and investigated HCV RNA replication and HCV protein expression. HCV RNA replication and protein expression was not affected in HCV genotype 2a producing cells by treatment of statins and cholesterol inhibitor. These results suggest that HMG-CoA reductase and cholesterol inhibitors might be used depending on HCV genotype. In addition, inhibition of HCV genotype 1b replication by statins has been depended on structure of various statins which should be seriously selected for HCV clinic. In future, we will study on inhibition of another HCV genotype replication by HMG-CoA reductase and cholesterol inhibitors.

Key Words: Hepatitis C virus, HMG CoA reductase inhibitor, Statin, Mavalonate pathway, Genotypes

INTRODUCTION

Hepatitis C virus (HCV) is a common cause of viral hepatitis in humans. Chronic HCV infection can cause cirrhosis, hepatocellular carcinoma, and death. It is the leading cause of liver transplantation in the United States (1). Interferon (IFN) and pegylated IFN (PEG-IFN) in combination with ribavirin have been used as optimal therapy to treat HCV infection (2). However, the sustained virologic response (SVR) to IFN-based therapy varied significantly among patients infected with different HCV genotypes (3). In general, HCV genotypes 2 and 3 are sensitive to PEG-IFN and ribavirin combination therapy, with an SVR of more than 80%. By contrast, genotypes 1 and 4 are refractory to IFN-based therapy, with an SVR of only about 40 to 50%. Antiviral effects of IFN against HCV replication have been shown differently according to HCV genotypes, however, the mechanism has not been known yet (2, 3).

Recently, statin (3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor), which is widely used in the treatment to reduce the level of serum cholesterol, is one of new candidate drugs to treat HCV patients (4, 5). Some statins inhibit HCV replication in the HCV RNA 1b
genotype replicon cell line (4, 5). This inhibition is recovered by addition of mevalonate. Six of statins, which are atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, and simvastatin are approved by the FDA for use in humans. Five types of statins (atorvastatin, fluvastatin, lovastatin, rosuvastatin, and simvastatin) exhibited anti-HCV activity whereas pravastatin exhibited no anti-HCV activity. The combination of IFN and the statins exhibited strong inhibitory effects on HCV RNA replication (6, 7).

There are a lot of statins used in clinical trial to reduce cholesterol level in blood and body (4, 5). There have been remained many questions that which statin is more effective to reduce cholesterol and virus infection, which HCV genotype is more sensitive or resistant to statin treatment, and how to apply in clinics. Many studies demonstrated that cholesterol, fatty acids, and lipid rafts have been critical for replication and infection of many RNA and DNA viruses (8–11). Particularly, host protein geranylgeranylation and fatty acids have regulated HCV RNA replication (4, 5). However, the data have shown inhibition of HCV RNA replication by decrease of HMG-CoA reductase only in HCV genotype 1b replicon system (4, 5, 12). Statins do not reduce HCV RNA titers during routine clinical use (12, 13). There is no data whether different HCV genotypes shall be affected for HCV RNA replication by statin treatment.

This study was undertaken to elucidate anti-HCV effects in HCV different genotypes using various HMG-CoA inhibitor. In addition, we studied whether HMG-CoA inhibitor inhibits HCV RNA replication as well as HCV infection. Here, we demonstrated that inhibition of HCV replication was differently affected by a kind of HMG-CoA reductase inhibitor. Then, our results elucidated that HCV 2a genotype was resistant to the inhibitors though HCV 1b genotype was sensitive to treatment of HMG-CoA reductase inhibitors. In addition, HMG-CoA reductase inhibitor did not inhibit the infection of HCV 2a genotype.

MATERIALS AND METHODS

Cell culture and HCV infection

Huh7, a human hepatoma cell line, and Huh7.5, a Huh7 variant cell line that is highly permissive to HCV RNA replication, were kindly provided by Charles M. Rice. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen).

HCV genotype 1b replicon constructs

Synthetic replicon RNA was prepared from PBR322I377 DNA linearized with Scal using the T7 Megascript kit (Ambion, Austin, TX) and was purified by DNase treatment, RNazol (Leedo, Houston, TX, USA) extraction, and ethanol precipitation (14). RNA was quantified by optical density and transfected to Huh7 and Huh7.5 cells by a lipofection method (15). Briefly, 1 μg of RNA was mixed with 5 μl of DMRIE-C reagent in Opti-MEM (Invitrogen) and then transferred onto Huh7 cells. At 24 h posttransfection, cells were split into 100-mm cell culture dishes at various cell densities. Cell colonies were selected by incubation with DMEM containing 10% FBS and 0.5 mg/ml of G418 for approximately 3 weeks. Stable cell lines were picked up and amplified. The expression of HCV proteins was detected by Western blotting using an NS5A-specific monoclonal antibody (Biodesign, MN, USA), while the levels of the positive- and negative-strand HCV RNAs were determined by an RNase protection assay (RPA) using HCV strand-specific and radiolabeled RNA probes. The HCV genotype 1b replicon RNA-harboring Huh7 cells were maintained in DMEM containing 10% FBS and 0.5 μg/ml of G418 sulfate.

HCV genotype 2a producing cell line constructs

The vector pSGR-JFH1, which contains a subgenomic JFH1 cDNA (15), was kindly provided by Wakita, and used as a vector for the construction of a subgenomic HCV cDNA that carries a neomycin resistance gene as a selective marker. The pcDNA6/TR-Tight/JFH1-FL/AR DNA, which contains a full-length JFH1 cDNA that carries a blasticidin resistance gene as a selective marker, was constructed by modification of pSGR-JFH1 (15). Both vectors were transfected into Huh7 cells in a six-well cell culture plate by a
lipofection method (15). Briefly, 1 μg of vector DNA was mixed with 5 μl of DMRIE-C reagent in Opti-MEM (Invitrogen) and then transferred onto Huh7 cells. At 24 h posttransfection, cells were split into 100 mm cell culture dishes at various cell densities. Cell colonies were selected by incubation with DMEM containing 10% FBS and 5 μg/ml of blasticidin for approximately 3 weeks. Stable cell lines were picked up and amplified. The expression of HCV proteins was detected by Western blotting using an NS3-specific monoclonal antibody, while the levels of the positive- and negative-strand HCV RNAs were determined by an RNase protection assay (RPA) using HCV strand-specific and radiolabeled RNA probes. The HCV genotype 2a producing cell lines were maintained in DMEM containing 10% FBS and 5 μg/ml of blasticidin.

Reagents

Atorvastatin, fluvastatin, pravastatin and simvastatin were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Lovastatin, mevastatin, mevalonolactone, geranylgeraniol cholesterol, and methyl beta-cyclodextrin (MβCD) were purchased from Sigma (St. Louis, MO, USA). Sodium mevalonate was prepared as described in the indicated references (4, 5). Reagents were suspended in solvents and added to the medium at a final concentration of 0.1% (vol/vol) dimethyl sulfoxide (atorvastatin, fluvastatin, lovastatin, mevastatin, pravastatin, simvastatin, geranylgeraniol). Cholesterol was suspended in DDW and prepared at a final concentration of 44 mg/g solid (10 μg/μl).

Western blot analysis

The HCV genotype 1b replicon cells and the HCV genotype 2a-infected Huh7.5 cells were lysed in a radioimmuno-precipitation assay buffer (RIPA, 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P40, 0.5% sodium deoxycholate) containing a cocktail of protease inhibitors (Roche). The protein concentration of cell extracts was determined by using a protein assay reagent (Bio-Rad, Hercules, CA, USA). Twenty-five micrograms of total protein for each sample was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked by incubation with 5% skim milk. The HCV genotype 1b NS5A monoclonal antibodies have been described previously (16). The levels of HCV genotype 2a NS3 protein were determined by using monoclonal antibodies specific to HCV genotype 2a NS3 protein. To raise HCV genotype 2a NS3-specific monoclonal antibodies, the HCV NS3 helicase domain with a six-His tag (NS3H) was expressed in Escherichia coli and purified by a nickel column chromatograph method. The purified recombinant NS3H was used as an antigen to immunize mice, and hybridoma cell lines producing NS3 monoclonal antibodies were selected and identified by screening with the recombinant NS3H protein (unpublished data). The HCV genotype 1b NS5A and 2a NS3 proteins were subsequently visualized by using a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce, Rockford, IL, USA) and staining with a chemiluminescence substrate (Pierce). The β-actin protein used as an internal control was detected by using an anti-β-actin monoclonal antibody (Sigma).

RNase protection assay (RPA)

An RNase protection assay (RPA) with Ambion's HybSpeed RPA kit was then performed according to the manufacturer's protocol. Positive-stranded HCV RNA in each fraction was determined using a negative-sense 3'UTR RNA probe. The negative-sense 3'UTR RNA probe for HCV genotype 1b was transcribed by T7 RNA polymerase from pUC19/T7(-)3'UTR DNA linearized with HindIII and labelled with [α-32-P]UTP (16). The negative-sense 3'UTR RNA probe for HCV genotype 2a was transcribed by T7 RNA polymerase from pSGR-JFH1-FL/AR DNA linearized with XbaI and labelled with [α-32-P]UTP (15). The T7 RNA transcripts were purified by using an RNaseasy RNA purification kit (Qiagen, Hilden, Germany). HCV RNA extracted from HCV genotype 1b replicon and 2a producing cell lines was hybridized with 10^5 c.p.m. [α-32-P] UTP-labelled negative-sense 3'UTR RNA probe. After digestion with RNase A/T1, RNA products were analysed on a 6% polyacrylamide/7.7 M urea gel and visualized by autoradiography (17).
RESULTS

Statin inhibited HCV genotype 1b RNA replication and HCV protein expression

To confirm whether statin inhibits HCV genotype 1b RNA replication and HCV protein expression, we treated 50 μM of lovastatin to HCV genotype 1b replicon cells and harvested the cells every 24 hrs during 6 days. HCV genotype 1b replication (HCV proteins expression) was slightly suppressed in the cells treated with lovastatin at 48 hrs, and significantly inhibited after 72 hrs. HCV proteins expression by treatment of lovastatin was gradually suppressed in time-dependant manner (Fig. 1). In order to know whether HCV RNA replication and HCV protein expression are suppressed by concentration of lovastatin, we treated 0, 0.4, 2, 10, and 50 μM of lovastatin to HCV genotype 1b replicon cell during 72 hrs. Suppression of HCV protein expression was weakly shown from 2 μM of lovastatin and was significantly shown by 50 μM of lovastatin (Fig. 2A). Inhibition of HCV RNA replication was shown as same patterns of suppression of HCV protein expression (Fig. 2B).

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dose-dependent manner. To investigate whether inhibition of HCV genotype 1b replication by lovastatin was recovered by mevalonate and geranylgeranyl, we added them into HCV genotype 1b replicon cells treated with 50 μM of lovastatin (Fig. 3), harvested cells, and investigated changes of HCV protein expression. Inhibition of HCV protein expression by statin was recovered by adding with mevalonate and geranylgeranyl (Fig. 3A, 3B). Recovery of HCV

Figure 4. Effects of various statins to HCV replication in HCV genotype 1b replicon S1179I #NS3-3 at 72 hrs by Western blot assay. Total protein from a clarified lysate (25 μg) was separated by SDS-PAGE and subjected to immunoblot analysis using antibodies directed against NS5A and β-actin. *cytotoxic effects.

Figure 5. Effects of various statins in HCV genotype 1b replicon S1179I #NS3-3 at 72 hrs by RNase protection assay. HCV genotype 1b replicon cell is treated with various doses (0, 2, 4, 6, 8 and 10 μM) of lovastatin and incubated for 3 days (Lane 2~6, respectively). RNA was extracted using Trizol solution and chloroform. Positive-stranded HCV RNA in each fraction was determined using a negative-sense 3'UTR RNA probe. RNA products were analyzed on a 6% polyacrylamide/7.7 M urea gel and visualized by autoradiography. (D) The intensities of images on gel were quantified using Phosphor imager (Molecular Device). Each experiment was repeated at least three times, and a representative result is shown here. R, RNA marker; P, probe; C, positive control.
replication was depending on concentration of mevalonate which is turned from acetyl CoA by HMG CoA reductase.

Different suppression of HCV genotype 1b RNA replication and HCV protein expression by treatment of various statins

To know whether different statins could affect the HCV replication, we treated 6 kinds of statins to HCV genotype 1b replicon cell by different doses (0, 0.08, 0.4, 2, 10 and 50 μM), and investigated HCV protein expression in treated cells. Suppression of HCV protein expression was affected by atorvastatin, simvastatin, fluvastatin, mevastatin, and lovastatin in order, but was not affected by pravastatin (Fig. 4A). HCV replication was affected from 2 μM of statins and was not affected under 0.4 μM of statins. HCV replication was strongly suppressed by 50 μM of atorvastatin, simvastatin and fluvastatin which caused cytotoxic effects in the cells. To elucidate more exact concentration to inhibit HCV protein expression, we tested HCV genotype 1b RNA replication and HCV protein expression in HCV genotype 1b replicon cell treated by 0, 2, 4, 6, 8, and 10 μM of various statins. HCV genotype 1b protein expression was inhibited in dose-dependent manner. Inhibition of HCV RNA replication was shown as same patterns of suppression

![Figure 6](image-url)

**Figure 6.** Effects of various statins to HCV RNA replication in HCV genotype 2a producing cells at 72 hrs by different dose. (A) Total protein from a clarified lysate (25 μg) was separated by SDS-PAGE and subjected to immunoblot analysis using antibodies directed against NS3 and β-actin, (B)-(D) HCV genotype 1b replicon cell is treated with different doses (0, 2, 4, 6, 8 and 10 μM) of six statins and incubated for 3 days. RNA was extracted using Trizol solution and chloroform. Positive-stranded HCV RNA in each fraction was determined using a negative-sense 3'UTR RNA probe. RNA products were analyzed on a 6% polyacrylamide/7.7 M urea gel and visualized by autoradiography. R, RNA marker; P, probe; NT, not treated (negative control).
Effect of Statin to HCV Genotypes 105

of HCV protein expression. Atorvastatin, simvastatin, fluvastatin, mevastatin, and lovastatin inhibited half of HCV protein expression and RNA replication by around 2, 3, 4, 5, and 10 \( \mu \text{M} \), respectively (Fig. 4B, Fig. 5). Our data suggest that suppression of HCV replication has been differently affected by structure of statin.

**Replication of HCV genotype 2a was not affected by statins**

In order to know whether statins affect HCV protein expression and HCV RNA replication of another HCV genotype, we treated 0, 2, 4, 6, 8, and 10 \( \mu \text{M} \) of six statins to HCV 2a producing cell line. Suppression of HCV protein expression and HCV RNA replication was not observed from HCV genotype 2a producing cells treated with six statins (Fig. 6). However, there are cytotoxic effects or apoptosis in cell treated with high concentration (50 \( \mu \text{M} \)) (data not shown). Statins did not affect on suppression of HCV RNA replication and HCV protein expression in HCV genotype 2a producing cells.

**Infection of HCV genotype 2a was not affected by statins**

In order to confirm whether high dose of statins affect HCV replication and infection of HCV genotype 2a, we treated 50 \( \mu \text{M} \) of six statins to HCV genotype 2a producing cell line. Any suppression of HCV protein expression was not shown in HCV genotype 2a producing cells treated with high dose of statins (Fig. 7A). To know whether statins affect production of HCV genotype 2a, we treated 0, 2, 4, 6, 8 and 10 \( \mu \text{M} \) of fluvastatin and simvastatin to HCV genotype 2a producing cells. HCV replication was not changed in HCV genotype 2a producing cells (Fig. 7B). In addition, production or infection of HCV genotype 2a was slightly inhibited but not significantly inhibited in the infected Huh7.5 cell when the supernatant from HCV producing cell line was infected to Huh7.5 cell (Fig. 7B). That is, all of statins could not affect on HCV genotype 2a RNA replication, HCV protein expression, and HCV production or infection. As a result, HCV genotype 1b is sensitive by treatment of statins and 2a is resistant by treatment of statin. These data suggest that statins might be used more effective in patients infected with HCV genotype 1b.
Suppression of HCV RNA replication and HCV protein expression only in HCV genotype 1b but not in HCV genotype 2a by cholesterol biosynthesis inhibitor

To compare effects on HCV protein expression by cholesterol that is a material separated from downstream of mevalonate pathway, we suppressed cholesterol by MβCD (cholesterol biosynthesis inhibitor), and observed the changes of HCV protein expression of HCV genotype 1b and 2a. MβCD suppressed HCV protein expression of HCV genotype 1b in dose dependent manner (Fig. 8A). HCV genotype 1b protein expression was suppressed from 200 μM of MβCD. These data shown that control of upstream of mevalonate pathway is easier than control of downstream of that. However, MβCD did not suppress HCV protein expression of HCV genotype 2a (Fig. 8B). Both MβCD and statin did not affect HCV genotype 2a replication. There was no inhibition of HCV protein expression in HCV genotype 2a producing cell treated with MβCD (Fig. 9A). However, HCV production from HCV genotype 2a producing cells was decreased from 0.2 mM of MβCD in dose dependent manner (Fig. 9B). Our data indicate that replication of HCV genotype 2a is resistant though that of HCV genotype 1b is sensitive to the suppression of cholesterol.

DISCUSSION

Combination therapy using interferon (IFN) and ribavirin is useful to treat HCV infection (2). However, HCV genotypes 1 and 4 are resistant to IFN combination therapy, though HCV genotypes 2 and 3 are sensitive to the therapy. Many researchers have studied that HMG CoA reductase inhibitor and cholesterol inhibitor regulate HCV RNA replication and HCV protein expression in HCV genotype 1b cells (4, 5). So, we tested whether statins suppress HCV RNA replication and HCV protein expression of both HCV genotypes 1b and 2a. Interestingly, statins suppressed the replication of HCV genotype 1b but not that of HCV genotype 2a. Statin against replication and infection of HCV genotype 1b showing IFN-resistance can be used in clinic. However, there are still not known the reason why HCV genotype 1b is sensitive to HMG CoA reductase and cholesterol biosynthesis inhibitors, but HCV genotype 2a is.

Figure 8. Effects of HCV replication in HCV genotype 1b replicon S1179I #NS3-3 (A) and HCV genotype 2a replicon (B) treated with different concentration of MβCD at 72 hrs. Total protein from a clarified lysate (25 µg) was separated by SDS-PAGE and subjected to immunoblot analysis using antibodies directed against NS5A, NS3 and β-actin.

Figure 9. Effect of MβCD to HCV RNA replication in HCV genotype 2a producing cell lines at 72 hrs by different concentration. Total protein from a clarified lysate (25 µg) was separated by SDS-PAGE and subjected to immunoblot analysis using antibodies directed against NS3 and β-actin. (A) Effects to HCV replication in HCV producing cell treated with MβCD. (B). Effects to HCV infection in Huh7.5 infected with the sup. A1, treatment after infection; BI, treatment before infection; NI, not infected; HS, supernatant from Huh7.5 cell. *cytotoxic effects.
Effect of Statin to HCV Genotypes

not. Recently, synergic effects by combination treatment with statin and IFN were observed in HCV genotype 1b replicon cells (6). However, HMG-CoA reductase inhibitor like atorvastatin or combination therapy of statin and interferon does not inhibit HCV RNA replication in vitro at conventional dose, though they inhibit HCV RNA replication in vivo (13). Our data elucidated that HMG CoA reductase and cholesterol biosynthesis inhibitors can regulate replication of HCV in HCV genotype-dependent manner.

HCV protein expression was slightly increased but not significantly in HCV genotype 1b replicon cells by adding with mevalonate. Mevalonate and geranylgeranylated recovered inhibition of HCV replication by statin in dose-dependent manner. HCV genotype 1b replicates in mevalonate pathway-dependent manner. However, HCV genotype 2a replicates in mevalonate pathway-independent manner. We have still not understood how HCV genotype 2a has not been affected by statins.

Though there are no any proofs in this paper, there is a hypothesis that HCV genotypes such as HCV genotype 2a not 1b might be recover suppression of mevalonate pathway by HMG CoA reductase inhibitor. The control of cholesterol level in blood is important to adult disease such as diabetes, fats, hypertension, heart diseases and cancer as well as virus infection and replication. Lipid metabolism is very important for viral transfer, packaging, energy and entry.

Interestingly, replication of HCV genotype 2a by treatment of HMG CoA reductase and cholesterol biosynthesis inhibitors was not inhibited but HCV production and infection was slightly suppressed. HMG CoA reductase and cholesterol biosynthesis inhibitors might slightly affect production of human apolipoprotein E (apoE) which is required for HCV infection (18).

Our results suggest that statin can be narrowly selected in HCV genotype 1b infected patients who were not affected by IFN therapy. Though statin has some side effects, statin should be used in some patients. We observed morphological change such as cytopathic effects of HCV replicon cells treated with high concentration of statins except for pravastatin (19). The shape of cell was changed to dried tree shape. In addition, protein concentration and cell growth in affected cells by statins were low and slow compared to those of non-affected cells, respectively. High-dose some statins which strongly decrease cholesterol in blood, should cause apoptosis of cells (19). We have to be careful to use high-dose statins to patients who have been diseased with cholesterolemia and HCV infection.

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