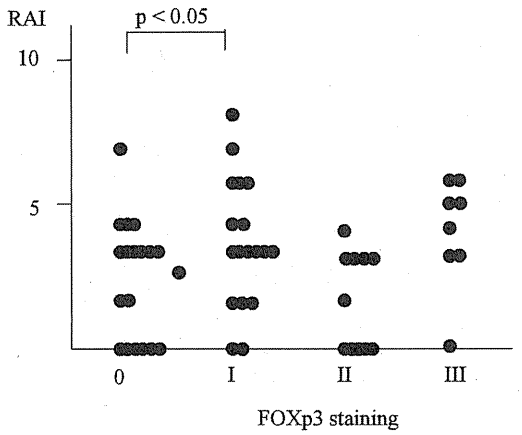

Fig. 2. The relationship between the rejection activity index (RAI) and FOXp3 staining. A significant difference was only seen between 0 and I in terms of FOXp3 staining with regard to the RAI.

Results

Table 1 showed characteristics of the patients and a summary of the histologic findings. The median days from the time of liver biopsy was 270 days (range: 14–2000 days) after LT. The median grade, based on the HAI, was 4 (0–10). The median degree of rejection, based on the RAI, was 3 (0–8). The difference in HAI was significant between 0 vs. I, as well as II vs. III, based on the number of FOXp3-positive cells (Fig. 1). On the other hand, a significant difference in the RAI was only seen for 0 vs. I (Fig. 2).

Figure 3 shows a representative liver biopsy specimen of a patient with recurrent HCV infection after LT. The patient was a 58-year-old woman who had undergone LT 4 years earlier. Her aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels were elevated at 107/80. H&E staining revealed an HAI grade of 10 and RAI of 5.

Figure 4 shows another representative liver biopsy specimen. This patient was a 62-year-old woman who underwent LT 4 months before the biopsy. Her AST/ALT levels were elevated at 68/65. H&E staining revealed an HAI grade of 9 and RAI of 6.

On the other hand, Figure 5 shows a representative liver biopsy specimen from a patient with ACR. The patient was 58-year-old woman who had undergone LT 4 years before the biopsy samples were taken. Her AST/ALT levels were elevated to 120/108. H&E staining revealed HAI grade of 3 and RAI of 6.

Discussion

This study examined the distribution and frequency of the appearance of Tregs in the liver after LT. After LT, in patients with hepatitis C, both ACR and recurrent HCV can lead to elevation of transaminases, in

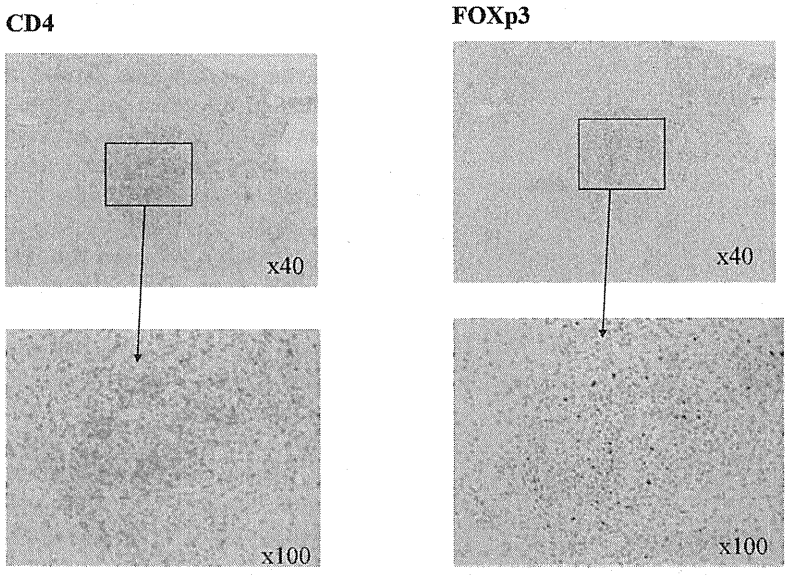

Fig. 3. Case 13. Representative findings in a liver with recurrent hepatitis C. Many liver infiltrating lymphocytes were positive for CD4 and FOXp3. HAI, hepatitis activity index; RAI, rejection activity index.

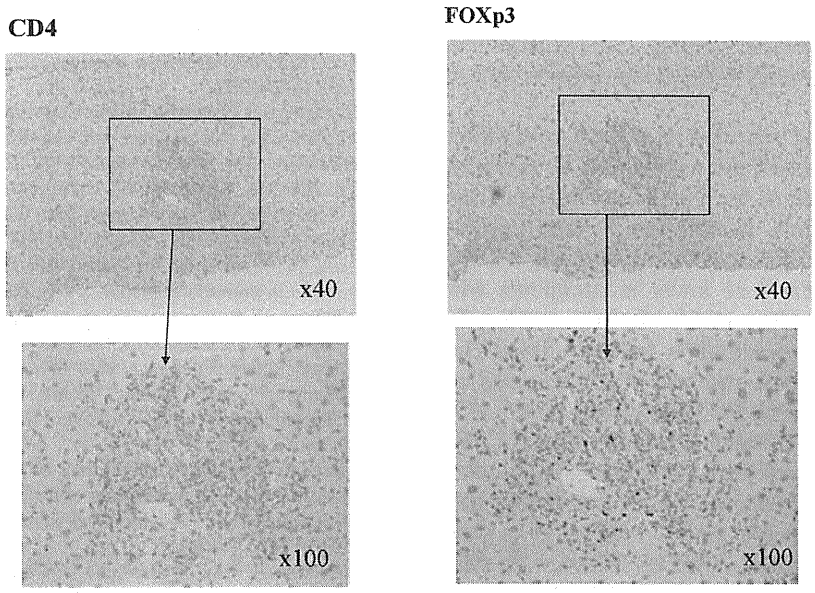

Fig. 4. Case 18. Representative findings in a liver with recurrent hepatitis C. Many liver infiltrating lymphocytes were positive for CD4 and FOXp3. HAI, hepatitis activity index; RAI, rejection activity index.

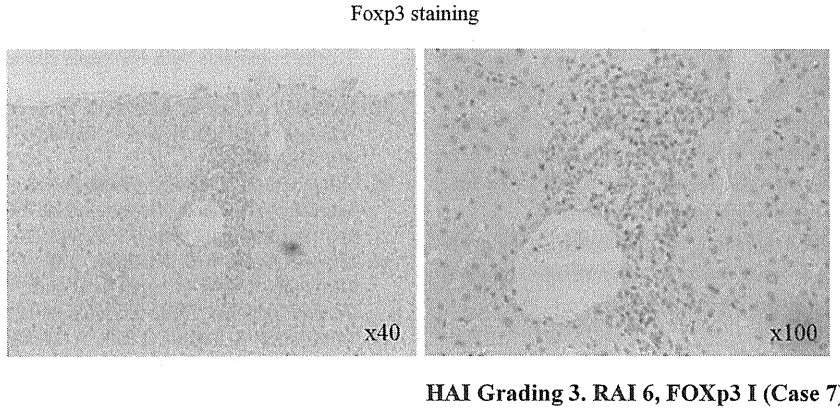

Fig. 5. Case 7. Representative findings in a liver with acute cellular rejection. Few liver infiltrating lymphocytes were positive for both CD4 and FOXp3. HAI, hepatitis activity index; RAI, rejection activity index.

addition to possible vascular abnormalities. Previously, Jain et al. (21) reported the significance of C4d expression in infiltrating lymphocytes, as CD4, CD8, and CD56 were similar in both ACR and recurrent HCV infection. However, accurately differentiating ACR from hepatitis C can sometimes be very difficult.

In previous reports regarding LT, the significance of Tregs in the grafted liver has been controversial. One report showed a relationship between ACR and an increase in Tregs. Intrahepatic detection of FOXp3 gene expression after LT can be accomplished using minimally invasive aspiration biopsy (15). With regard to recurrent hepatitis C, FOXp3 mRNA expression was used to differentiate between the two conditions. Based on needle biopsy, they reported that intrahepatic FOXp3 levels are associated with HCV re-infection and a history of acute rejection, and that the level increased within the first year after LT (15).

Generally speaking, Tregs are associated with graft tolerance in organ transplantation. It seems likely that FOXp3 mRNA expression is associated with graft acceptance (22). It was reported that CD4+ FOXp3 cells are present within grafts in a subset of tolerant patients after human LT (23). However, in the present study, no clear relationship was observed between

ACR and Tregs, except to find a statistical difference between 0 and I in FOXp3 staining. This relationship needs further investigation without the interference of HCV infection.

Sakamoto et al. (24) reported increased expression of FOXp3 mRNA immediately after LDLT, probably because of the activation of T cells, including Tregs and other T-cell subsets. In addition, it was reported that expression of FOXp3 mRNA on days 14, 21, and 28 after transplantation were lower in recipients with ACR within 60 days after LDLT. In our study, the median time since transplantation was 270 days. This is different from previous reports, which focused on short-term diagnosis using FOXp3 staining in the liver and peripheral blood. Usually, 6 months after LT, the level of immunosuppression is stabilized. HCV infection could occur during this period, and antiviral therapy is often initiated. In our study, most patients were undergoing or had already received antiviral therapy with IFN and ribavirin. Although we showed a relationship with FOXp3 expression, we were unable to clarify the function of Tregs in recurrent HCV infection after LT. Further investigation will be needed.

After effective IFN therapy, the number of infiltrating lymphocytes seemed to decrease, which made scoring FOXp3 staining difficult. It was unclear whether the character of the infiltrating lymphocytes changed over the course of treatment. In settings other than transplantation, the FOXp3 staining system may be used to differentiate hepatitis C from autoimmune-like disease or other causes of hepatitis.

In conclusion, FOXp3 staining in infiltrating lymphocytes in the liver may represent a surrogate marker for recurrent HCV infection after LDLT.

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Authors' contributions: S.E., T.K., and K.N carried out study conception and design. M.H. and A.S. provided acquisition of data. M.T., T.I., and H.M. performed analysis and interpretation of data. M.T. and S.E. were responsible for drafting of the manuscript. T.K., S.E., and K.N. performed critical revision.

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Anti-hepatitis C virus activity of geranylgeranylacetone treatment in hepatitis C-infected patients

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Background. Geranylgeranylacetone (GGA), which is an isoprenoid compound, has been used orally as an antiulcer drug in Japan. GGA induces antiviral gene expression by stimulating the formation of interferon-stimulated gene factor 3 in human hepatoma cells. This study verified the anti-hepatitis C virus (HCV) activity of GGA in chronic hepatitis C-infected patients.
Methods. The present prospective study included 20 consecutive anti-HCV antibody-positive, HCV-genotype 1b, and chronic gastritis patients who visited Nagasaki University Hospital between January 1999 and December 1999. GGA (150 mg per day, which is the dose generally used for chronic gastritis) was taken orally for four weeks. We evaluated HCV-RNA titers and other clinical parameters at pretreatment, posttreatment, and at the endpoint of the study. Pretreatment was the beginning point of GGA treatment. Posttreatment was the termination point of GGA treatment. The endpoint was the point four weeks after the posttreatment point.
Results. All patients completed four weeks of GGA treatment and four weeks of observation. HCV-RNA titers at postpoint were not significantly diminished compared to those at pretreatment. However, HCV-RNA titers were significantly diminished at endtreatment compared to pretreatment. Unfortunately, we did not observe a case with no titer of HCV-RNA. Alanine aminotransferase values and other parameters were not affected by GGA treatment.
Conclusion. GGA has anti-HCV activities in chronic hepatitis C-infected patients. In the future, it will be necessary to examine the clinical effectiveness of the combination of treatment with both GGA and interferon in HCV patients.
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Keywords: Hepatitis C virus, geranylgeranylacetone, chronic hepatitis C

Introduction

Currently, chronic hepatitis C virus (HCV) infections are the major cause of hepatocellular carcinoma (HCC) worldwide (1). Therefore, an anti-HCV strategy is important for the prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases but is less than 50% effective in genotype 1 cases. New anti-HCV agents designed to inhibit the life cycle of HCV have been developed and are used in combination with IFN-α to ameliorate the salvage rate of HCV infection (2). However, this combination therapy cannot completely eliminate chronic HCV infections. Therefore, long-term management and safety drugs for chronic hepatitis C (CHC) patients are required.

Geranylgeranylacetone (GGA) is an isoprenoid compound, which includes retinoids. GGA was developed in Japan and has been used orally as an antiulcer drug (3). GGA protects the gastric mucosa from various types of stress without affecting gastric acid secretion (4,5). Moreover, GGA suppresses cell growth and induces differentiation or apoptosis

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in several human leukemia cells (6,7). 3,7,11,15-Tetramethyl-2,4,6,-10,14-hexadecapentaenoic acid is another isoprenoid compound that was designated as an acyclic retinoid because it has the ability to interact with nuclear retinoid receptors (8) that cause apoptosis in certain human hepatoma cells (9). GGA acts as a potent inducer of antiviral gene expression, and it induces the expression by stimulating the formation of IFN-stimulated gene factor 3 (ISGF3) in human hepatoma cells (10). GGA induces the expression of antiviral proteins such as 2′5′-oligoadenylate synthetase (2′5′-OAS) and double-stranded RNA-dependent protein kinase (PKR) in hepatoma cell lines. GGA stimulates 2′5′-OAS and PKR gene expression at the transcriptional level through the formation of ISGF-3, which regulates the transcription of both genes. GGA induces the expression of signal transducers and activators of transcription 1, 2 (STAT-1, STAT-2) and p48 proteins, components of ISGF3, together with the phosphorylation of STAT1 (10). However, the anti-HCV activity of GGA has not been observed in vivo and in vitro.

At present, new treatments for CHC patients are necessary, and GGA has an IFN-like action in hepatoma cells (10). Therefore, we attempted to verify the anti-HCV activity of GGA in CHC patients.

Methods

Patients

The present prospective study included 20 consecutive anti-HCV antibody-positive, HCV-genotype 1b, and chronic gastritis patients who visited the Nagasaki University Hospital between January 1999 and December 1999. Patients were enrolled in the study after informed consent was obtained. The patients had not been previously treated with IFN therapy and were diagnosed with CHC on the basis of clinical data. The patients were evaluated with a HCV-RNA polymerase chain reaction (PCR) method (Amplicor method). The HCV-RNA high group (100,000 IU/mL or more in the serum) was identified by quantitative PCR. The criteria for HCC were assessed by abdominal imaging methods and by HCC history. The patients who were not previously diagnosed with diabetes mellitus (DM) were evaluated by the 75-g oral glucose tolerance test (OGTT). All subjects underwent OGTT with 75 g of glucose according to the recommendations of the National Diabetes Data Group of the National Institute of Health. Blood samples were taken at 0, 30, 60, 90, 120, and 180 min after administration in order to measure the plasma glucose (PG) and insulin concentrations. In this study, the DM group consisted of patients with clinically diagnosed DM or ≥110 mg/dL fasting PG and/or 140 mg/dL or high PG at 120 min.

White blood cell counts, red blood cell counts, platelet counts, hemoglobin A1c levels, alanine aminotransferase (ALT) levels, aspartate aminotransferase (AST) levels, and γ-glutamyl transpeptidase (GTP) levels were determined by hematometry and standard laboratory techniques. Clinical characteristics are shown in the Table.g

Table. Clinical characteristics at pre-GGA treatment
Characteristic mean (SD) or number

Age (years)	56 (16)
Sex (F/M)	10/10
BMI	21.0 (3.02)
Genotype 1b	20
HCV high titer	14
HCV-RNA titer	489 (378)
HCC +/-	0/20
WBC count	6004 (1585)
RBC count	447 (60)
Plt count	18.9 (7.9)
Alb level	4.46 (3.0)
AST level	49.5 (21.2)
ALT level	71 (28)
γ-GTP level	50.5 (32)
DM +/-	0/20
HbA1c level	5.05 (0.8)
FPG level	96 (13)

Data are shown as means (standard deviation) and numbers.
BMI, body mass index; HCV, Hepatitis C virus; HCC, hepatocellular carcinoma; WBC, white blood cells; RBC, red blood cells; Plt, platelets; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; DM, diabetes mellitus; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose.
Normal values in laboratory tests: ALT (IU/L), 5-40; AST (IU/L), 10-40; γ-GTP (IU/L), <70 in men, <30 in women; Alb (g/dL), 4.0-5.0; WBC (cells/μL), 3500-9000; RBC ($\times 10^4$ cells/μL), 450-580 in men, 380-480 in women; Plt ($\times 10^4$ platelets/μL), 14-33; ferritin (ng/mL), 39.4-340 in men, 3.6-114 in women; FPG (mg/dL), 70-110; HbA1c (%), 4.3-5.8; BMI, body weight (kg)/height^2 (m).

Methods

The dose of 150 mg of GGA per day, which is generally used to treat chronic gastritis in Japan, was taken orally for four weeks, and it was assumed that patients took one dose a day. Pretreatment was the beginning point of GGA treatment. We evaluated HCV-RNA titers and other clinical parameters at pretreatment, posttreatment, and study endpoint. Posttreatment was the termination point of GGA treatment. Endpoint was the point four weeks after the

posttreatment of GGA. During this study, all patients were not treated with Stronger Neo-Minophagen C (Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) because of its anti-hepatitis effects or with IFN because of its anti-HCV effects.

Statistical analysis

Data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Inc., Cary, NC). The differences in the values of each laboratory parameter were analyzed with a t-test. P values less than 0.05 were considered statistically significant.

Results

GGA decreased the HCV-RNA titers in patients but did not affect the values of ALT

All patients completed four weeks of GGA treatment and four weeks of observation. Adverse effects were not observed in any patient. The titers of HCV-RNA (Fig. 1A) changed after the patients completed GGA treatment. Compared with HCV-RNA titers at pretreatment, titers at endpoint did not diminish significantly. However, compared to HCV-RNA titers at pretreatment, the titers were significantly diminished at posttreatment. Unfortunately, we did not observe a case with no titer of HCV-RNA. Values of ALT (Fig. 1B) and other parameters were not changed by GGA

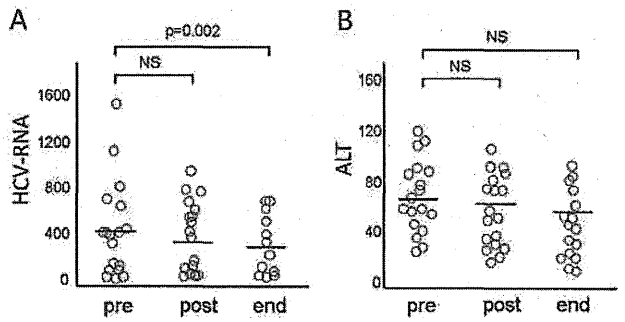

Figure 1. Titers of hepatitis C virus (HCV)-RNA at the endpoint were decreased compared to the levels at pretreatment (A), but alanine aminotransferase (ALT) levels were not changed (B).

Panel A shows serum HCV-RNA titers, and panel B shows serum ALT levels at each of the indicated points. The bar indicates the mean value. Statistical significance was accepted with p-values less than 0.05. "Pre" indicates the point of pre-geranylgeranylacetone (GGA) treatment. "Post" indicates the termination point of GGA treatment. "End" indicates the point four weeks after posttreatment of GGA. Compared to HCV-RNA titers at pretreatment, GGA treatment decreased the titer at pretreatment but not at posttreatment. The diminished HCV-RNA titers at the posttreatment point were increased at the endpoint, which was four weeks after the posttreatment point.

In Fig. 2, we present the case of a patient who had the most diminished HCV-RNA titers among the 20 GGA-treated patients (Fig. 2). This case had mild fluctuations of ALT levels before GGA treatment. The HCV-RNA titer was 420 K copies/mL and 380 K copies/mL at 12 weeks before treatment and at the pretreatment point, respectively. After GGA treatment, HCV-RNA titers were decreased to 2 K copies/mL and 4 K copies/mL at the endpoint and at the posttreatment point, respectively. In this case, the ALT values were also diminished in a similar manner as HCV-RNA. After the observation period, +12 weeks, HCV-RNA titers and ALT values were increased compared to those at the pretreatment point.

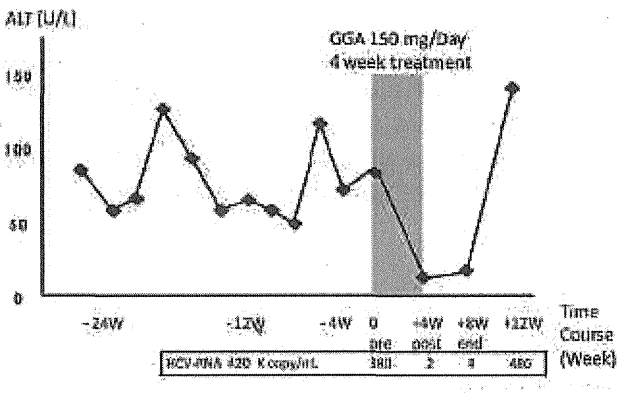

Figure 2. The clinical course of a geranylgeranylacetone (GGA)-treated chronic hepatitis C (CHC) patient.

Here, we present a case of a 53-year-old man who was an outpatient of our hospital for 5 years. He was diagnosed with chronic hepatitis on the basis of clinical data. The patient had not been previously treated with interferon (IFN). The y-axis indicates alanine aminotransferase (ALT) levels, and the x-axis indicates the time course. The duration of the GGA treatment periods is shown in the gray field. The zero point on the x-axis is the GGA treatment-starting day. HCV-RNA titers are 420, 380, 2, and 4 K copies/mL at -12 weeks, 0 weeks (pretreatment), +4 weeks (posttreatment), and +8 weeks (end of follow-up period), respectively.

Discussion

GGA demonstrated anti-HCV activity in this study. The anti-HCV effect that was due to GGA did not result in a disappearance of HCV-RNA titers in CHC patients. An adverse effect was not observed with GGA treatment.

GGA is a non-toxic heat shock protein (HSP) 70 inducer (11). Various GGA activities outside of the stomach are also related to HSP induction (12,13,14). GGA induces

thioredoxin, as well as HSP-70, in hepatocytes and other cells (15). The antiviral activity of thioredoxin is induced by AP-1 and NF-κB but not by HSP-70 (16). GGA, which has potent antiviral activities through the enhancement of antiviral factors, can clinically provide protection from influenza viral infections (17). Previously, we reported that GGA induction of antiviral proteins was dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 with which HCV was not infected (10). However, HCV products inhibit the Jak-STAT pathway in HCV-infected hepatocytes (18). The mechanism of inhibition of the Jak-STAT pathway is multifactorial and includes the expression of suppressor of cytokine signaling 3 (SOCS-3) (19), protein phosphatase 2A induction (20), STAT-3 expression (21), and IL-8 expression (22). A clarification of GGA-induced anti-HCV activity is necessary for further examination of the *in vitro* and *in vivo* effects.

The peak venous blood concentration after taking 150 mg of GGA orally is 5-7 μmol/L (23), but 50 μmol/L is the best dose for induction of PKR and 2′5′-OAS in hepatoma cell lines (10). In this study, we employed the usual dosage of GGA used to treat chronic gastritis in Japan, which is 150 mg per day. In a previous study, it was reported that portal blood concentration after taking 150 mg of GGA orally was several-fold that of the venous blood concentration (23). The usual dosage of GGA also may have a possible antiviral gene expression effect in the liver.

In conclusion, GGA, a drug that can be safely administered orally, has anti-HCV activity. Unfortunately, we did not observe a case that exhibited disappearance of HCV-RNA titers. GGA treatment is insufficient for clearance of HCV, and, therefore, it will be necessary to examine the clinical effectiveness of the combination treatment with GGA and IFN in HCV patients in the future.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells

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Abstract
Background Geranylgeranylacetone (GGA), an isoprenoid compound which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating ISGF3 formation in human hepatoma cells. This drug has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.
Methods OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. After treatment, the cells were harvested with Renilla lysis reagent and then subjected to a luciferase assay according to the manufacturer's protocol.
Result The results showed that GGA had anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1, and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.
Conclusions GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

Keywords mTOR · STAT-1 · Interferon · HCV · GGA

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
GGA	Geranylgeranylacetone
siRNA	Small interfering RNA

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Introduction

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are

used in combination with IFN-α to ameliorate the salvage rate of HCV infection [2]. It is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment since IFN-α is the most basic agent for HCV treatment. Any agents that can support IFN activity will improve the therapeutic effect for HCV infected patients.

Geranylgeranylacetone (GGA), an isoprenoid compound, which includes retinoids, has been used orally as an anti-ulcer drug developed in Japan [3]. GGA protects the gastric mucosa from various types of stress without affecting gastric acid secretion [4, 5]. Moreover, GGA suppresses cell growth and induces differentiation or apoptosis in several human leukemia cells [6, 7]. Another isoprenoid compound, 3,7,11,15-tetramethyl-2,4,6,-10,14-hexadecapentaenoic acid, which is designated as an acyclic retinoid because it has the ability to interact with nuclear retinoid receptors [8], causes apoptosis in certain human hepatoma cells [9]. GGA acts as a potent inducer of antiviral gene expression by stimulating the ISGF3 formation in human hepatoma cells [10]. GGA induces the expression of antiviral proteins such as 2′5′-oligoadenylate synthetase (2′5′-OAS) and double-stranded RNA-dependent protein kinase (PKR) in hepatoma cell lines. GGA stimulates 2′5′-OAS and PKR gene expression at the transcriptional level through the formation of interferon-stimulated gene factor 3 (ISGF-3), which regulates the transcription of both genes. GGA induces the expression of signal transducers and activators of transcription 1, 2 (STAT-1, STAT-2) and p48 proteins, components of ISGF3, together with the phosphorylation of STAT1 [10]. However, no anti-HCV activity was observed.

A cell culture HCV replicon system has been developed as a useful tool for the study of HCV replication and mass screening for anti-HCV reagents. OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE [11], were used to examine the influence of the anti-HCV effect of IFN. The luciferase activity in cell lysate of OR6 was correlated with the HCV-RNA concentration, and the IC50 of IFN-α was less than 10 IU/mL [11]. The OR6 system is a useful and sensitive cell culture replicon system.

This study verified the anti-HCV activity of GGA in the OR6 system. In addition, the mechanisms of anti-HCV activity were examined in OR6 cells.

Materials and methods

Reagents

GGA was a generous gift from Eisai Co. (Tokyo, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). Wortmannin, LY294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fatal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication.

Reporter gene assay

The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and GGA. After treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI, USA) and luciferase activity in the cells was determined using a luciferase reporter assay system and a TD-20/20 luminometer. The data were expressed as the relative luciferase activity.

Western blotting and antibodies

Western blotting with anti-STAT-1, anti-PKR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-serine-2448 phosphorylated mTOR, anti-mTOR, anti-threonine-389 phosphorylated p70S6K, anti-p70S6K (Cell Signaling, Beverly, MA, USA) and anti-HSP70 (Stressmarq Biosciences Inc, Victoria, Canada) was performed as described previously [10]. Briefly, OR6 cells were lysed by the addition of a lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium *o*-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

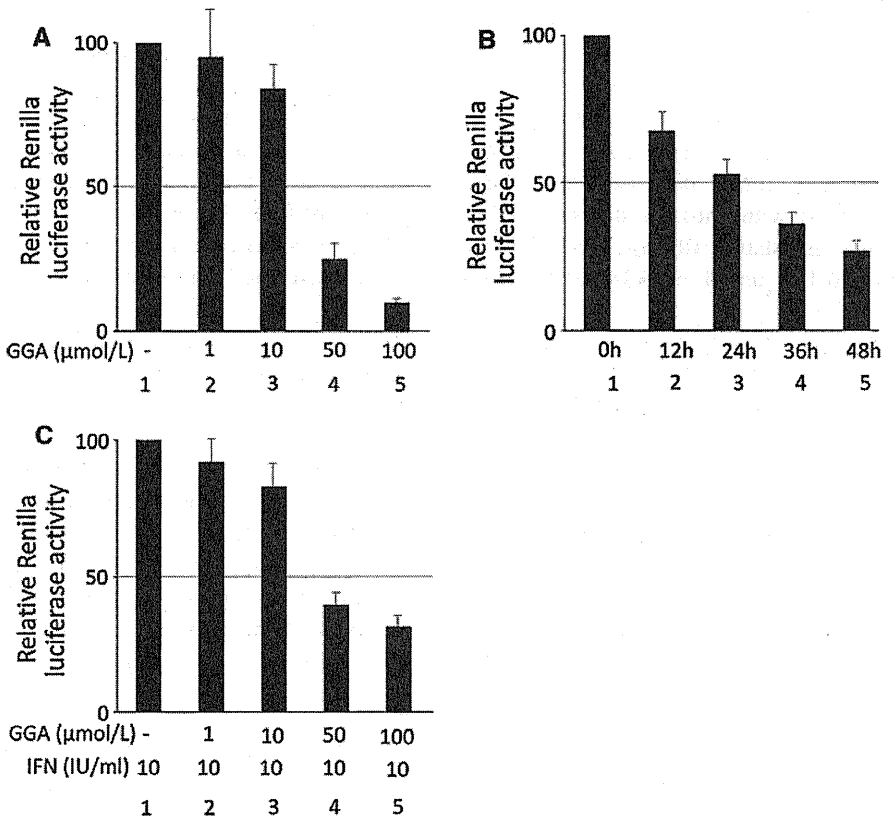

Fig. 1 The effect of GGA on the genome-length HCV RNA replication system. **a** Dose dependent effect of GGA. **b** Time course of GGA suppressed HCV replication. **c** The additive effect of GGA with IFN-α suppressed HCV replication. **a** The OR6 cells were treated with 1–100 μmol/L of GGA (*lanes 2–5*) and *lane 1* was not treated. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments. The differences between *lane 3* versus *4*, *lane 3* versus *5* and *lane 3* versus *5* were statistically significant. **b** The OR6 cells were treated 50 μmol/L of GGA and at the indicated time, HCV replicon assay was done ($n = 4$). The differences between *lane 1* versus *3–5* and *lane 2* versus *4*, *5* were statistically significant. **c** The OR6 cells were treated with 10 IU/mL of IFN-α in the absence (*lane 1*) or presence of treatment with 1–100 μmol/L of GGA (*lanes 2–5*). Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The differences between *lane 1* versus *4*, *5* were statistically significant. Statistical significance was accepted as a P value of <0.05. The data are expressed as the mean ± SD and are representative of four similar experiments

siRNA transfection assay

mTOR gene knockdown was performed using siRNA (Cell Signaling, Beverly, MA, USA). OR6 cells were transfected with100 nmol/L mTOR specific and non-targeted siRNA as a control in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 50 μmol/L GGA.

mTOR kinase activity assay

The cells were washed two times with TBS and lysed by addition of lysis buffer [50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol (w/v), 1% Tween-20 detergent (w/v), 1 mM EDTA, 20 nM microcystin-LR, 25 mM NaF, and a cocktail of protease inhibitors]. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity using a commercially available kit (Calbiochem, San Diego, USA) according to the manufacturer's instructions.

Results

GGA with or without IFN had anti-HCV activity

OR6 cells, the full-length HCV replication system, were used to examine the effect of GGA. The cells were treated with 1–100 μmol/L of GGA for 24 h and the amount of HCV replicon was measured by the *Renilla* luciferase assay (Fig. 1a). The relative *Renilla* luciferase activity decreased in a dose-dependent manner. Furthermore, GGA

induced anti-HCV replicon activity was time dependent (Fig. 1b). GGA was combined with IFN-α to examine the additive effect (Fig. 1c). One or 10 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity slightly (Fig. 1c). However, 50 or 100 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity with statistical difference. GGA treatment did not have any statistically significant effect on cell viability from 1 to 100 μmol/L of GGA for 24 h (data not shown).

GGA did not activate the tyrosine-701 and serine-727 on STAT-1, and did not induce PKR and HSP-70 in OR6 cells

GGA mediated phosphorylation of STAT-1 at the tyrosine-701 and serine-727 residues was investigated using antibodies to phospho-specific STAT-1 on OR6 cells. No phosphorylation of tyrosine-701 and serine-727 on STAT-1 was detected in OR6 cells (Fig. 2a). IFN induce anti-viral protein, PKR, and STAT-1 has an interferon stimulating responsive element (ISRE) in the promoter region [12]. The expression levels of both proteins did not change throughout this study, as indicated by a Western blotting analysis (Fig. 2b, c). Next, the role of HSP in the mechanism of GGA activity was examined because GGA is an inducer of HSP. The HSP-70 expression was increased by pre-exposure to heat shock (Fig. 2d, lanes 2, 4), but it did not increase due to the effects of GGA (Fig. 2d, lanes 3, 4).

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor, were able to cancel the GGA induced anti-HCV activity

The role of the PI3-K-Akt-mTOR pathway the anti-HCV activity of GGA was examined in OR6 cells. The cells were treated with GGA after 3 h in the presence or absence of rapamycin as an mTOR inhibitor, Akt inhibitor, or wortmannin as a PI3-K inhibitor (Fig. 3). Pretreatment with rapamycin attenuated the anti-HCV replication effect

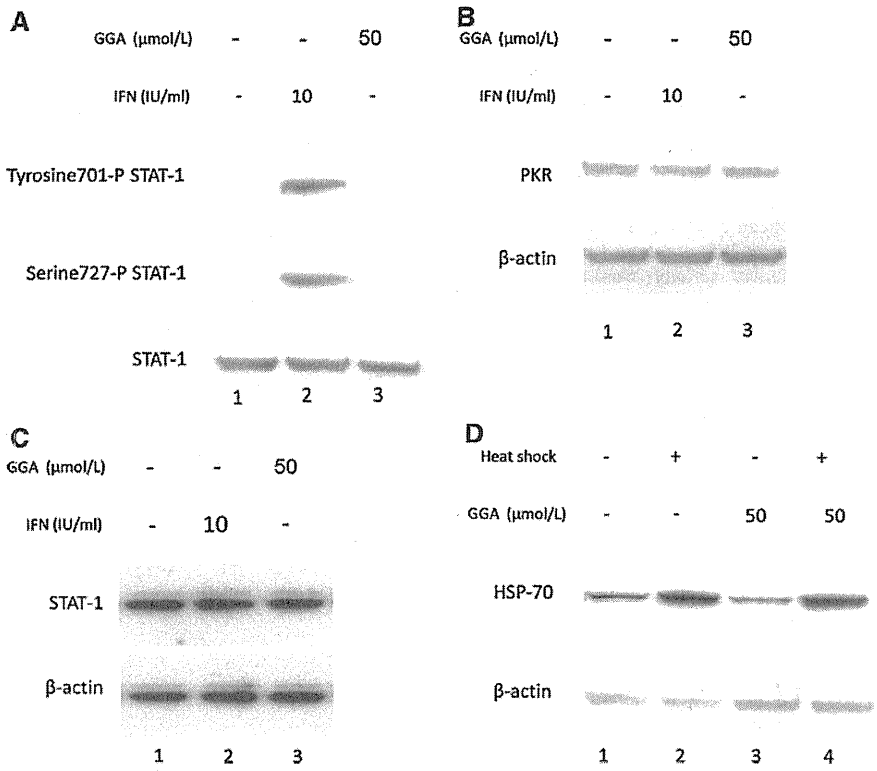

Fig. 2 Effect of GGA on STAT-1 (**a**), PKR (**b**) and HSP-70 (**c**). **a** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*) and then were phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and at serine-727 residue (*middle panel*), the expression STAT-1 (*lower panel*) was analyzed by Western blotting. **b** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*), and then the expression of PKR (*upper panel*) was analyzed by a Western blotting analysis. The β-actin (*lower panel*) protein expression was used as an internal control. **c** The OR6 cells were either untreated (*lane 1*) or given heat shock (at 42°C 15 min, overnight recovery at 37°C) (*lanes 2, 4*) or treated with 50 μmol/L of GGA (*lanes 3, 4*) and then the expression HSP-70 (*upper panel*) was analyzed by Western blotting. β-Actin (*lower panel*) protein is the internal control

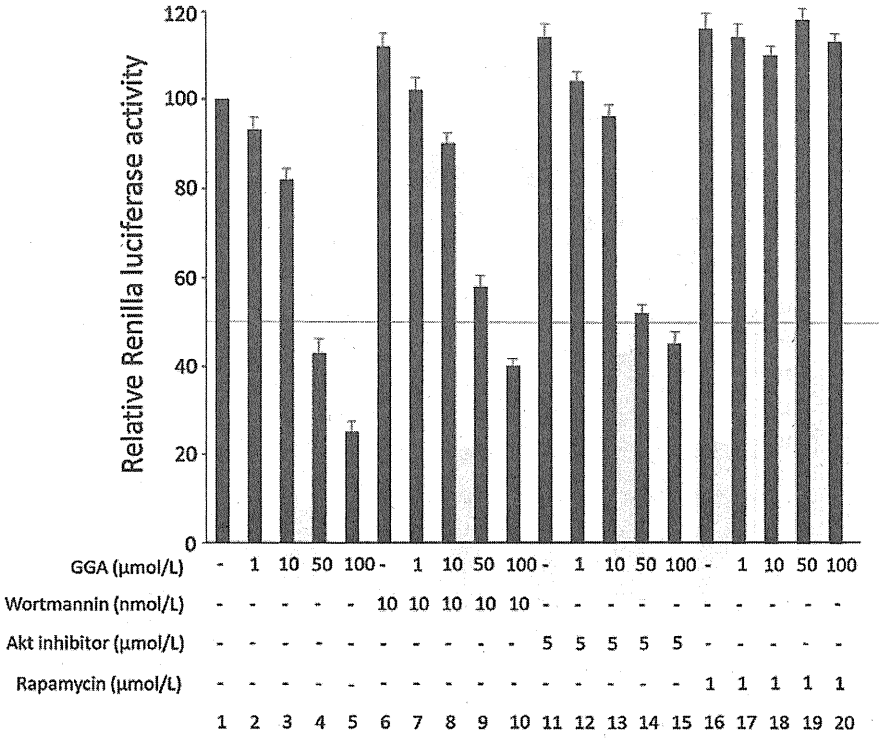

Fig. 3 Changes in GGA suppressed HCV replication by rapamycin, but not PI3-K inhibitor and Akt inhibitor. OR6 cells were treated with 1–100 μmol/L of GGA in the absence (*lanes 2–5*) or presence of pretreatment (*lanes 7–10, 12–15, 17–20*) for 3 h. *Lanes 1, 6, 11* and *16* were not treated with GGA. *Lanes 6, 11* and *16* were treated with wortmannin, an Akt inhibitor, and rapamycin, respectively. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments

in comparison to GGA alone (Fig. 3, lanes 17–20), whereas pretreatment with wortmannin and Akt inhibitor did not increase the *Renilla* luciferase activity (Fig. 3, lanes 7–10, 12–15). siRNA transfection was used for mTOR knockdown to explore role of mTOR in the anti-HCV activity (Fig. 4). The transfection efficiency of the siRNA was confirmed by a Western blotting analysis. In this experiment, the detectable band intensities were quantified by the National Institutes of Health image software program. Although the transfection efficiency of siRNA was barely 46% (Fig. 4a), GGA-induced anti-HCV activity was clearly inhibited in mTOR-siRNA transfected cells (Fig. 4b, lane 4, 6) in comparison to the control cells (Fig. 4b, lanes 3, 5).

GGA induced mTOR activity, mTOR phosphorylation and p70S6K phosphorylation in OR6 cells

The phosphorylation of the serine-2448 residues of mTOR by 50 μmol/L of GGA was detected 30 min after GGA treatment. The band intensity of serine-2448 phosphorylated mTOR decreased by pretreatment with rapamycin but was almost same as with GGA alone following pretreatment with LY294002 (Fig. 5a). Furthermore, an mTOR activity assay was conducted to confirm the activity mechanism of GGA (Fig. 5b). The mTOR activity was increased by treatment with GGA alone (Fig. 5b, lane 4) and was inhibited by pretreatment with rapamycin (Fig. 5b, lane 6), whereas pretreatment with LY94002 did not suppress the mTOR activity (Fig. 5b, lane 5). Furthermore, to evaluate the mTOR activity, we investigated the level of phospholylated-p70S6K by a Western blotting analysis (Fig. 5c). The phosphorylation of the threonine-389 residue of p70S6K by 50 μmol/L of GGA was detected. Similar to mTOR, the band intensity of phospho-threonine-389 of p70S6K decreased after pretreatment with rapamycin, but the intensity was almost the same as that seen following treatment with GGA alone after pretreatment with LY294002 (Fig. 5c).

Discussion

GGA demonstrated the anti-HCV activity in this study. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity. An additive effect was observed with the combination of IFN and GGA.

GGA is a non-toxic heat shock protein (HSP) 70 inducer [13]. Various GGA activities outside of the stomach are also related to HSP induction [14–16]. GGA induced HSP-70 exerts an anti-ischemic stress activity in the heart and liver [16, 17], an anti-inflammatory activity in various cell types [18] and promotes liver regeneration [19]. GGA induces thioredoxin as well as HSP-70 in hepatocytes and other cells [20]. Thioredoxin anti-virus activity, is induced by AP-1 and NF-κB but not HSP-70 [21]. GGA has potent

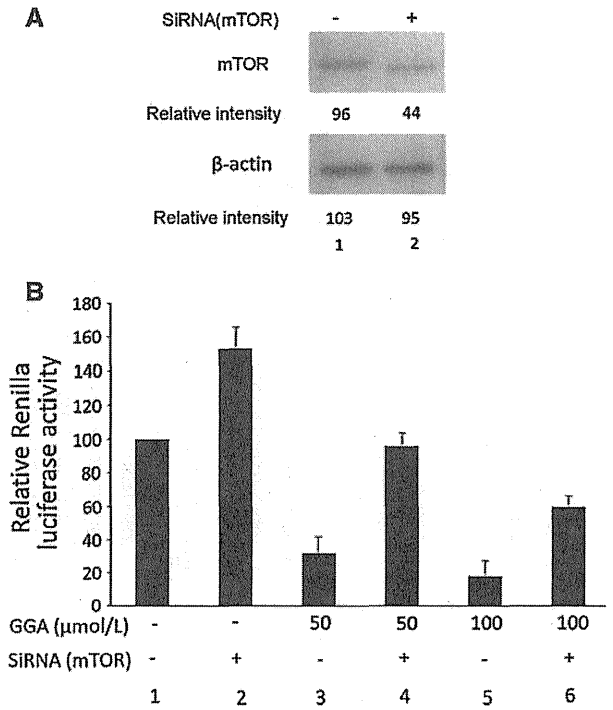

Fig. 4 Changes in GGA suppressed HCV replication by mTOR-siRNA. **a** OR6 cells were transfected with mTOR-siRNA (*lane 1*) or the non-targeted siRNA (*lane 2*). The expression of mTOR was evaluated by a Western blotting analysis. **b** The OR6 cells were transfected with mTOR-siRNA (*lanes 2, 4* and *6*) and the non-targeted siRNA (*lanes 1, 3* and *5*). One day later, the cells were treated with GGA (*lanes 3–6*). The HCV replicon assay is the same as Fig. 3. Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The *Renilla* luciferase activity increased in the OR6 cells transfected with mTOR-siRNA (*lane 2*) in comparison to the non-targeted siRNA (*lane 1*). However, in OR6 cells treated with GGA, there was a greater elevation of *Renilla* luciferase activity in OR6 cells transfected with mTOR-siRNA (*lanes 4* and *6*) as compared to that with the non-targeted siRNA (*lanes 3* and *5*). The data are expressed as the mean ± SD and are representative example of four similar experiments

antiviral activity via the enhancement of antiviral factors and can clinically provide protection from influenza virus infection [22]. GGA significantly inhibits the synthesis of influenza virus-associated proteins and prominently enhances the expression of human myxovirus resistance 1 (MxA) followed by increased HSP-70 transcription [22]. Moreover, GGA augments the expression of an interferon-inducible double-strand RNA-activated protein kinase (PKR) gene and promotes PKR autophosphorylation and concomitantly alpha subunit of eukaryotic initiation factor 2 phosphorylation during influenza virus infection [22]. These anti-virus activities are related to GGA induced HSP-70. But, HSP-70 protein and PKR were not induced by GGA in OR6 cells in the current study. There is apparently no relationship between the GGA induced anti-HCV activity and HSP, PKR in OR6 cells. Therefore, we thought that HSP and PKR-independent anti-HCV activity induced by GGA was present in this hepatoma-derived cell line.

GGA induction of anti-viral protein is dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 [10]. However, GGA did not induce STAT-1 tyrosine phosphorylation and anti-virus protein, PKR, in OR6 cells in this study. Moreover, the GGA induced anti-HCV activity depended on mTOR activity, not STAT-1. OR6 cells are full length HCV replicon transfected HuH-7 cells [11]. HCV virus products inhibit the Jak-STAT pathway [23–25]. The mechanism of inhibition of the Jak-STAT pathway is multi-factorial including the suppressor of cytokine signaling 3 (SOCS-3) expression [26], protein phosphatase 2A (PP2A) induction [27], STAT-3 expression [28] and IL-8 expression [29]. GGA induced STAT-1 tyrosine phosphorylation and inducible PKR protein levels are also minor. Generally, the replicon transfection induces the intrinsic IFN [30], but STAT-1 tyrosine phosphorylation was not detected in combined OR6 cells. HCV replicon produced viral product might be inhibiting GGA-induced STAT-1 tyrosine phosphorylation.

mTOR is associated with the IFN induced anti-HCV signal [31]. The IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalomyocarditis virus effect [32]. IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. A relationship has been observed between the replication of the hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV [33]. The IFN induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity and Jak independent TOR activity involves STAT-1 phosphorylation and nuclear localization, and then PKR is expressed in hepatocytes [31]. No relationship between GGA and mTOR has been reported. However, GGA induced anti-HCV activity depended on mTOR activity independent of PI3-K-Akt, as observed with IFN induced mTOR activity.

When 150 mg of GGA was administered orally, the serum concentration of GGA was approximately 7 μmol/L [34]. The concentration of GGA in the portal blood would be several-fold higher than the serum concentration of GGA; therefore, we speculated that the pharmacological action that would be obtained in clinical practice would be the same as that observed in this study.

GGA, a drug that can be safely administered orally, has mTOR dependent anti-HCV activity. The combination of IFN and GGA has an additive effect on anti-HCV activity. The current results suggest that combination therapy with

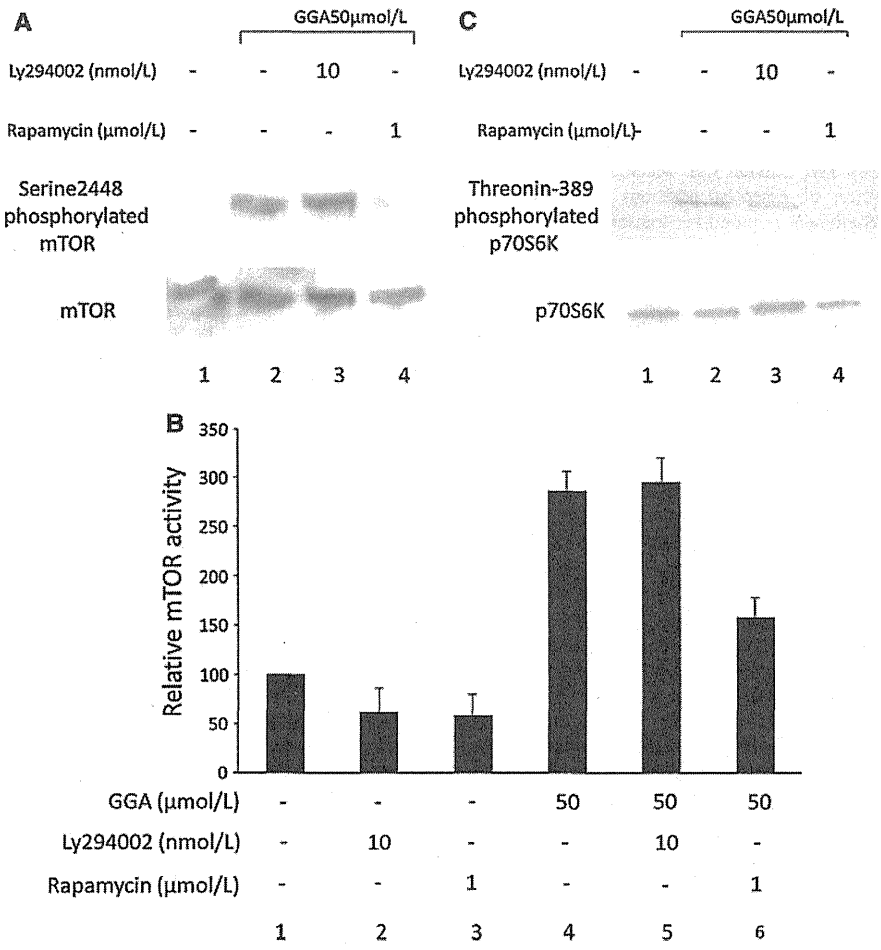

Fig. 5 Effect of GGA on mTOR and effect of LY294002 and rapamycin on GGA-induced serine phosphorylated mTOR and threonine phosphorylated p70S6K. **a** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min and then were phosphorylated mTOR at serine-2448 residue (*upper panel*), the expression of mTOR (*lower panel*) was analyzed by Western blotting. **b** After pretreatment with 10 nmol/L LY294002 (*lanes 2* and *5*) and 1 μmol/L rapamycin (*lanes 3* and *6*) for 3 h, the OR6 cells were either untreated (*lanes 1–3*) or treated with 50 μmol/L GGA (*lanes 4–6*) for 30 min. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit ($n = 4$). The differences between *lanes 1* and *4*, *lanes 4* and *6*, and *lanes 5* and *6* were statistically significant. The data are expressed as the mean ± SD and are representative of four similar experiments. **c** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L, and with rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min, and then were examined for phosphorylated p70S6K at the threonine-389 residue (*upper panel*), or the expression of p70S6K (*lower panel*) by a Western blotting analysis

GGA and IFN is, therefore, expected to improve the anti-HCV activity. It will, therefore, be necessary to examine the clinical effectiveness of the combination with GGA and IFN for HCV patients in the future.

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Insulin-induced mTOR activity exhibits anti-hepatitis C virus activity

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Abstract. The mammalian target of rapamycin (mTOR) is one of the influential molecules for the anti-hepatitis C virus (HCV) action of interferon (IFN). IFN-induced mTOR activity, independent of phosphatidylinositol-3-kinase (PI3K) and Akt, is a critical factor for anti-HCV activity. mTOR activity is involved in signal transducers and activators of transcription (STAT)-1 phosphorylation and nuclear localization, and then double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes. Insulin (INS) is a major cytokine for metabolism and regulates the PI3K-Akt-mTOR signaling pathway in hepatocytes. Changes in mTOR activity have been reported in chronic HCV-infected patients with excess nutrition and INS resistance. Therefore, this experiment investigated whether INS increases anti-HCV activity via mTOR activity. This study used a genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), derived from HuH7 cells. OR6 cells were pre-treated with rapamycin or LY294002 or siRNA, and the cells were treated with INS (0-300 nmol/l) or IFN (0-50 IU/ml) for 30 min to 48 h. The cells were lysed and analyses were carried out using the *Renilla* luciferase assay, western blotting or ELISA. INS induced the anti-HCV effects via mTOR activity, independently of STAT-1 tyrosine phosphorylation, in a dose- and time-dependent manner. INS-induced mTOR activation was found to be PI3K-Akt-dependent in OR6 cells. The combination of IFN and INS had an additive anti-HCV effect. The INS-induced mTOR activity was identified to be an anti-HCV signal independent of the STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should, therefore, attempt to identify new agents that activate mTOR to promote anti-HCV activity.

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Abbreviations: IFN, interferon; INS, insulin; HCV, hepatitis C virus; CHC, chronic hepatitis C; Jak, Janus kinase; STAT, signal transducers and activators of transcription; ISRE, IFN-stimulated regulatory element; PKR, double-stranded RNA-dependent protein kinase; Rapa, rapamycin; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA

Key words: mammalian target of rapamycin, signal transducers and activators of transcription-1, interferon, hepatitis C virus, insulin

Introduction

At present, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide (1). Although HCV is a hepatotropic virus, chronic HCV infection causes many metabolic disorders, including diabetes (2), insulin resistance (3) and hepatic steatosis (4). These metabolic disorders are related to an interferon (IFN) refractory condition (5-7). Treatment of HCV using a combination of pegylated IFN and ribavirin is effective in less than 50% of chronic hepatitis C (CHC) patients with genotype 1 virus (8). New anti-HCV agents, such as protease (9), polymerase (10) and cyclophilin inhibitors (11), have been developed to inhibit the HCV life cycle, but single application of these newly developed drugs is not effective enough for HCV eradication. Since IFN-α is the most basic agent for HCV treatment, it is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The most important intra-hepatocellular signal transduction of the IFN pathways is the Janus kinase (Jak)-signal transducers and activators of transcription (STAT) signal (12). Mammalian target of rapamycin (mTOR) is one of the influential molecules associated with IFN-induced anti-HCV action (13). The IFN-activated mTOR pathway plays an important regulatory role in the promotion of the IFN effect, including the anti-encephalomyocarditis virus effect (14). IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE)-dependent promoter gene activity. Human cytomegalovirus is inhibited by AMP-activated protein kinase-mediated inhibition of mTOR kinase (15). By contrast, vesicular stomatitis virus is mTOR-dependent (16). A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70

S6 kinase pathway and regulates the replication of HCV (17). IFN-induced mTOR activity, independent of PI3K and Akt, is a critical factor for the anti-HCV activity, and Jak-independent mTOR activity involves STAT-1 phosphorylation and nuclear localization; subsequently, double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes (13).

mTOR activity has pleiotrophic functions, such as cell growth (18), nutrition control (19) and immunoregulation (20). However, there has been little examination of the influence that mTOR activity has on HCV proliferation. mTOR activity does not influence HCV-ISRE activity as the viral promoter has cap-independent translation (21). Although mTOR is a mRNA translational regulator that acts through phosphorylation of a downstream target such as 4E-BP and S6K (22), IFN-induced mTOR activity influences the phosphorylation of STAT-1 (13). Changes in mTOR activity in CHC patients with extra nutrition or with insulin resistance have been reported (23). However, the anti-HCV effect associated with mTOR activity remains to be sufficiently elucidated.

Insulin (INS) is a major cytokine for metabolism and acts via the PI3K-Akt-mTOR signaling pathway in hepatocytes (24). INS is indirectly related to HCV-associated INS resistance. Therefore, this study investigated whether INS increases the anti-HCV activity via mTOR activity.

Materials and methods

Reagents. INS was purchased from Eli Lilly Japan (Kobe, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). LY294002 and rapamycin (Rapa) were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system. OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE (25), were used to examine the influence of the anti-HCV effect of IFN and INS. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and maintained in the presence of G418 (300 mg/l; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI, USA) and then subjected to a luciferase assay according to the manufacturer's protocol. The data were expressed as the relative luciferase activity.

Reporter gene assay. The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers, IFN and INS, and the luciferase activities in the cells were determined using a luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative luciferase activity.

Cell viability assay. The cells were assessed using a colorimetric cell viability assay method. Cell viability was determined by a colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). Cell viability after 2 days of 100 IU/ml of IFN-α and 1000 nmol/l of Rapa treatment was expressed as a percentage of the viability in standard media without IFN-α and Rapa. Statistical significance was assessed using the Student's t-test, and a difference was considered to be statistically significant at $P<0.05$.

Western blotting and antibodies. Western blotting with anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-mTOR and anti-serine-2448 phosphorylated mTOR (Cell Signaling, Beverly, MA, USA) was performed as described later. OR6 cells were lysed by the addition of a lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mmol/l sodium o-vanadate and 1 mmol/l NaF). The samples were separated by electrophoresis on 8-12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling), and the immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

mTOR kinase assay. The K-LISA mTOR Activity kit (Calbiochem) was used in this study. OR6 cells were incubated in the absence or presence of INS for 30 min with or without pre-treatment with Rapa for 12 h. The cells were washed two times with TBS and lysed by addition of a lysis buffer that was recommended by the manufacturer. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity according to the manufacturer's protocol. The absorbance was measured with a Multiskan JX microplate reader.

siRNA transfection assay. Knockdown of the mTOR gene was performed using siRNA (Cell Signaling), and 100 nmol/l mTOR-specific and non-targeted siRNA as a control was used to transfect OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/ml IFN or 300 nmol/l INS.

Results

INS exhibits anti-HCV activity with or without IFN. OR6 cells, a full-length HCV replication system, were used to examine the anti-viral effect of INS and IFN. The cells were incubated in medium that contained 0-100 nM of INS and 0-50 IU/ml of IFN for 48 h, and were harvested for a *Renilla* luciferase assay (Fig. 1). The relative *Renilla* luciferase activity decreased in an INS dose-dependent manner when the IFN concentration was 0 or 1 U/l. Cell viability in the presence of INS showed no statistically significant difference with or without IFN treatment for 24 or 48 h (data not shown). Rapa and LY294002 also showed no significant difference in comparison to the control (data not shown).

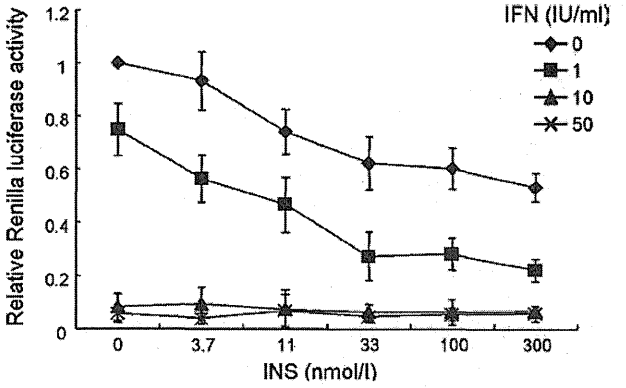

Figure 1. Effect of INS on HCV replication with or without IFN-α. OR6 cells were treated with 0-100 nmol/l INS and 0-50 IU/ml IFN. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). The data are expressed as the means ± SD and are a representative example of four similar experiments.

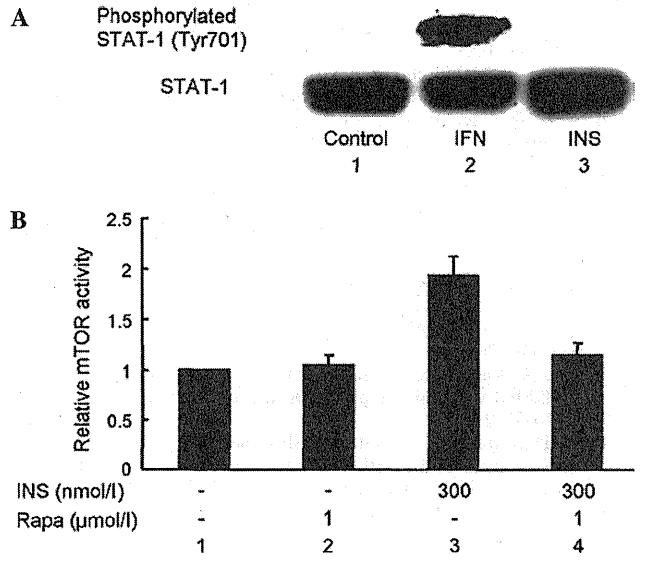

Figure 2. INS does not induce tyrosine phosphorylation of STAT-1 but induces mTOR kinase activity. (A) OR6 cells were incubated in medium only (control), and with IFN or INS for 30 min. Phosphorylation of STAT-1 at tyrosine-701 residue was analyzed by western blotting. (B) OR6 cells were not pre-treated (bars 1 and 3) or pre-treated with 1 μM Rapa (bars 2 and 4) for 12 h, and the cells were treated with 300 nM of INS (bars 3 and 4) for 30 min. Bars 1 and 2, cells not INS-treated. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 3 vs. bars 1, 2 and 4 by the Student's t-test (P<0.05).

INS does not activate tyrosine on STAT-1, but induces mTOR kinase activity. OR6 cells were incubated in medium only or with 50 IU/ml of IFN or 300 nmol/l of INS for 30 min, and were lysed for western blotting (Fig. 2A). INS did not induce phosphorylation of Tyr 701 of STAT-1 (Fig. 2A, lane 3). IFN-induced anti-viral protein PKR was also not detected by western blotting under the same condition (data not shown). Treatment with 300 nmol/l of INS induced mTOR activity in OR6 cells. INS-induced mTOR kinase activity was suppressed in OR6 cells pre-treated with Rapa for 12 h before INS stimulation (Fig. 2B, bar 4).

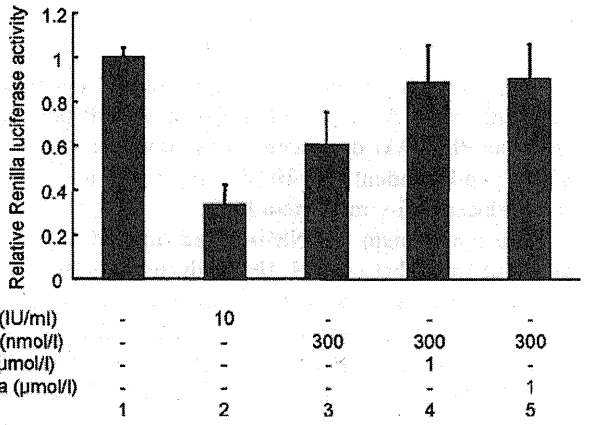

Figure 3. Changes in INS suppress HCV replication by PI3K and mTOR. OR6 cells were treated with 10 IU/ml IFN (bar 2) or with 300 nmol/l INS (bars 3-5) in the absence (bar 3) or presence of pre-treatment (bars 4 and 5) with LY294002 (LY) or rapamycin (Rapa) for 12 h. Bar 1, not treated. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). Data are expressed as the means ± SD and are a representative example of four similar experiments. A statistically significant difference was observed between bar 3 vs. bars 1, 4 and 5 by the Student's t-test (P<0.05).

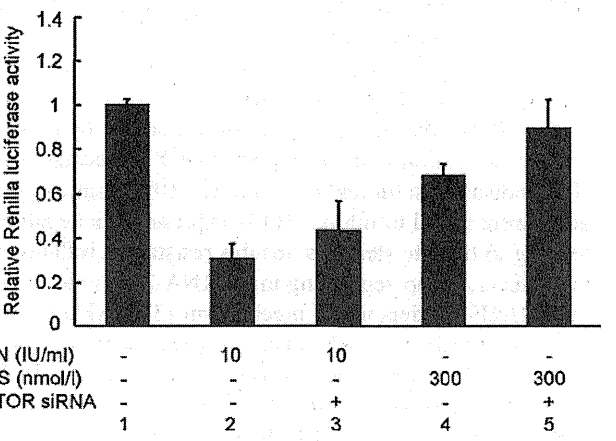

Figure 4. Changes in INS suppress HCV replication by siRNA against mTOR. OR6 cells were transfected by siRNA against mTOR (bars 3 and 5) or vehicle siRNA (bars 1, 2 and 4). One day later, the cells were treated with IFN (bars 2 and 3) or INS (bars 4 and 5). Thereafter, 1 day later, the *Renilla* luciferase activity was determined (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 4 vs. bars 1 and 5 by the Student's t-test (P<0.05).

INS-induced anti-HCV activity is inhibited by blunted mTOR activity. The role of the PI3K-Akt-mTOR pathway in INS-induced anti-HCV activity was examined in OR6 cells. The cells were treated with INS after 12 h in the presence or absence of Rapa as an mTOR inhibitor, or LY294002 as a PI3K inhibitor (Fig. 3). Pre-treatment with LY294002 or Rapa attenuated the anti-HCV effect in comparison to INS alone. siRNA transfection for mTOR knockdown was used to explore the role of mTOR activity in INS-induced anti-HCV activity (Fig. 4). IFN-induced anti-HCV activity dependent on mTOR was inhibited by mTOR siRNA. Although the transfection efficiency of siRNA was barely 10%, INS-induced anti-HCV activity was clearly inhibited in mTOR siRNA-transfected cells (Fig. 4, bar 5).

Discussion

INS-induced mTOR activity exhibits anti-HCV action independently of STAT-1 phosphorylation. mTOR activated by INS was PI3K-Akt-dependent in hepatocytes. Notably, The STAT-1-independent anti-HCV activity did not induce the IFN-induced anti-viral protein PKR.

The mechanism of INS-induced anti-HCV activity is different from that of IFN. IFN-induced anti-HCV activity depends on IFN-induced anti-viral protein via Jak-STAT signaling (12). A genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), which is an effective screening tool (25) has been used to identify a more effective therapy, especially for CHC patients with genotype 1. The OR6 system demonstrated the IFN-independent anti-HCV activity of statins (26). Another replicon system was used to demonstrate the IFN-independent anti-HCV activity of cyclosporine A (27). Both drugs have been associated with the life cycle of HCV and have an inhibitory effect on HCV replication (28,29). INS-induced anti-HCV activity may be associated with the life cycle of HCV, but not IFN-induced anti-virus protein. The susceptible point in the HCV life cycle is uncertain, but mTOR activity is an important factor which contributes to the inhibition of HCV proliferation.

Various relationships have been reported between mTOR activity and insulin resistance. mTOR was found to play a key role in IL-6-induced hepatic insulin resistance by regulating STAT3 activation and subsequent SOCS3 expression in an IL-6-induced insulin resistance model (30). Unsaturated fatty acids were found to inhibit PTEN expression in hepatocytes, leading to hepatic steatosis, insulin resistance, inflammation and cancer, by up-regulating microRNA-21 synthesis via an mTOR/NF-κB-dependent mechanism (31). mTOR activity was found to induce IRS serine phosphorylation leading to IRS degradation resulting in insulin resistance (23). HCV infection of hepatocytes stimulates insulin resistance through multifactors, including IRS degradation (32) and SOCS-3 expression (33). The present study of the association of mTOR activity and HCV proliferation suggests that insulin resistance is a condition that suppresses excessive proliferation of HCV and may be associated with chronic HCV infection.

INS-induced anti-HCV activity may be dependent on the PI3K-Akt-mTOR pathway, and one aspect of IFN-induced anti-HCV activity depends on mTOR activity (13). In this study, the combination of IFN and INS had an additive anti-HCV effect. Amino acids and INS have been demonstrated to have an additive effect on mTOR activity, since these effectors use different pathways to induce mTOR activity (34). Efficient anti-HCV activity via mTOR activity requires a combination of effectors that operate via different mTOR activation pathways. mTOR can be activated via three different pathways. The IRS-PI3K-Akt-mTOR pathway is stimulated by extracellular effectors, including INS and INS-like growth hormone (35). Activated mTOR inhibits the IRS function and generates negative feedback for IRS signaling (36). There is also nutrition-associated Akt-independent and wartmannin-dependent, or Akt-independent and adenosine monophosphate kinase-dependent mTOR activation. Amino acids and glucose inhibit Akt-dependent glycolysis through mTOR activation (37). Future studies should investigate agents that enhance IFN-induced Akt-independent mTOR activity via other mTOR-activated pathways.

INS-induced mTOR activity was found to be an anti-HCV signal-independent STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should therefore identify new anti-HCV agents that activate mTOR activity.

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