Table 5
Factors associated with the yearly rate of fibrosis progression

	n	Mean	SD	*p* Value
Gender				
Male	50	−0.01	0.19	0.12
Female	47	0.06	0.23	
Age at biopsy				
<60 years	73	−0.0002	0.21	0.06
⩾60 years	24	0.10	0.23	
HCV genotype				
1b	83	0.02	0.20	0.37
non-1b	14	0.08	0.32	
BMI				
<25 kg/m^2	53	0.004	0.24	0.32
⩾25 kg/m^2	44	0.05	0.19	
Steatosis on first biopsy				
0–1	58	−0.03	0.20	0.004
2–3	39	0.10	0.21	
Activity on first biopsy				
0–1	51	−0.001	0.21	0.24
2–3	46	0.05	0.22	
Fibrosis on first biopsy				
1–2	71	0.03	0.20	0.43
3	26	−0.01	0.25	
Average ALT between paired biopsies				
<100 IU/l	80	−0.01	0.17	0.0005
⩾100 IU/l	17	0.18	0.31	

4. Discussion

In the present study, we found that a higher grade of hepatic steatosis at baseline and a higher average value of ALT are independent risk factors for the progression of fibrosis over time in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy. These two factors may be involved in promoting the progression of fibrosis. The association between steatosis and progression of fibrosis in untreated patients had been suggested by previous studies but this study is the first to demonstrate a similar association for treated patients. These findings are particularly important to establish a rationale for identifying therapeutic targets to halt potentially progressive disease independent of antiviral therapy.

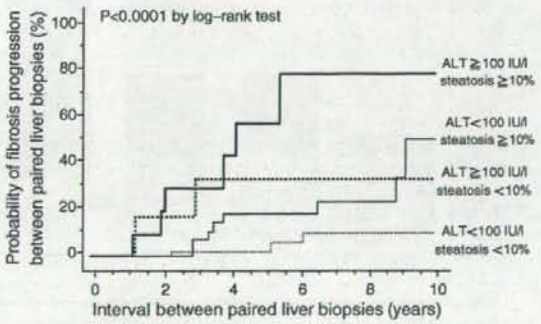

Fig. 3. Probability of fibrosis progression according to the presence of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors and the time to progression of fibrosis was analyzed.

There have been many studies that analyzed the association between steatosis and progression of liver fibrosis in HCV-infected patients, and the majority have shown a positive association [10–13], including a large scale meta-analysis [14]. However, some studies did not report this association [15–18]. There are two possible reasons for these conflicting results. First, longitudinal studies, rather than cross-sectional studies, are particularly important in the analysis of the role of steatosis in time-dependent progression of hepatic fibrosis, because cross-sectional studies involve patients with an unknown duration of steatosis. Three of four longitudinal studies that analyzed the progression of fibrosis through paired biopsies in untreated patients showed that the presence or worsening of steatosis was associated with the progression of fibrosis [12,13,20], and the probability of progression of fibrosis was significantly related to the grade of steatosis [13]. In one study, however, progression of fibrosis was correlated with older age, periportal necroinflammation and ALT elevations but not with steatosis [17]. Interestingly, steatosis was associated with older age, higher body mass index and ALT elevations in that study, indicating an indirect association of steatosis and fibrosis progression. The authors assumed that steatosis was the result rather than the cause of inflammation. This observation highlights the second reason for the controversies over a correlation between the presence of steatosis and progression of fibrosis, that is, there are so many confounding factors associated with both steatosis and fibrosis progression such as older age, advanced stage of fibrosis, higher degree of inflammation, elevated ALT, increased body mass index and insulin resistance. Because it is very difficult to prove a causal relationship between these confounding factors through clinical observations, steatosis may be a hallmark of the progression of fibrosis but it is unclear whether the effect of steatosis on progression of fibrosis is direct or mediated by other confounding factors.

Hepatic steatosis is a common pathological finding in patients with chronic hepatitis C [9]. Because the proportion of patients with steatosis is higher than would be expected from a chance association, a direct role of HCV in the pathogenesis of steatosis is suggested, at least in some patients with genotype 3 infection [21]. Furthermore, other observations suggest that steatosis may be metabolic; it is correlated with a high body mass index, visceral adiposity and insulin resistance, especially in non-3a genotypes and metabolic steatosis also is correlated with progression of fibrosis [11,22]. The

most reliable evidence that metabolic steatosis is associated with progression of fibrosis is shown by a study indicating that weight reduction in patients with chronic hepatitis C leads to a reduction in steatosis and an improvement in fibrosis, despite the persistence of HCV infection. A reduction in steatosis was significantly associated with a decrease in stellate cell activation and regression of hepatic fibrosis in 56% of patients. Thus, weight reduction may provide an important new adjunct treatment strategy for patients with chronic hepatitis C [23]. A recent study showed that the administration of pioglitazone led to metabolic and histological improvement in subjects with non-alcoholic steatohepatitis [24]. Whether amelioration of insulin resistance could improve steatosis and fibrosis in chronic hepatitis C awaits future investigation.

The mechanism by which steatosis could aggravate hepatic fibrosis in chronic hepatitis C patients remains largely hypothetical. Steatosis related insulin resistance may contribute to hyperinsulinemia and increased hepatic expression of connective tissue growth factor leading to progression of fibrosis [25]. Alternatively, a steatohepatitis-like pathway may be involved where steatosis requires a second hit for progression to fibrosis [26]. The most likely candidate is an oxidative stress with subsequent lipid peroxidation which is reported to correlate with the stage of fibrosis [27]. Another important candidate is an antiviral inflammatory response. It is reported that steatotic liver has increased susceptibility to inflammatory response [28] and that a higher grade of steatosis is correlated with a higher degree of inflammation or elevated ALT [14,15,17]. Higher degree of inflammation or elevated ALTs are associated with the progression of fibrosis [29,30], but hepatic steatosis may be responsible for the amplification of hepatic inflammation and vice versa, and the coexistence of these two factors may lead to further progression of fibrosis, as in patients with non-alcoholic steatohepatitis. In our study, average value of ALT between two biopsies was associated with fibrosis progression, whereas histological inflammation at first liver biopsy was not. The reason for this discordance may be explained by the dynamic process of hepatic necroinflammation. Severity of histological inflammation at the time of biopsy may not reflect subsequent inflammation process, whereas average value of regularly determined ALT may reflect entire fluctuation of hepatic inflammation. If so, our finding may support the hypothesis that co-operation of steatosis as the first hit and dynamic process of hepatic inflammation as the second hit promotes fibrosis progression. On the other hand, elevation of ALT may not be a mere reflection of hepatic inflammation so much as hepatocellular death such as apoptosis. Since it is reported that apoptotic caspase activation is elevated in HCV-associated steatosis [31] and that steatotic liver has increased susceptibility to apoptosis [28], elevation of ALT may also reflect an apoptosis amplified by steatosis which may lead to fibrosis progression.

Regardless of the precise mechanism, the results of the present study suggest that lowering of ALT levels may be beneficial in preventing progression of fibrosis in patients who failed to achieve a SVR. In our population, all patients received 24 weeks of IFN therapy and none received long-term maintenance therapy aiming to ameliorate hepatic inflammation. However, we speculate that amelioration of hepatic inflammation and lowering ALT levels by long-term IFN may prevent fibrosis progression in patients who remain viremic since it has been reported that IFN slowed the natural progression of fibrosis in patients who failed IFN therapy when the rate of progression of fibrosis after IFN therapy was compared to the estimated rate of progression before therapy [2,32], and that treatment duration was associated with the reduction of fibrosis independent of virological response [2]. Another possible approach to lower ALT levels may be the use of ursodeoxycholic acid, which has been reported to induce an almost 30% decrease in serum ALT levels [33,34]. The long-term efficacy of therapies targeted to the reduction of hepatic fibrosis needs future verification.

Some factors related to fibrosis progression in previous studies such as obesity [35] and worsening of steatosis [20] were not significant in our study. In our study where the majority of the population had normal body weight and very few had obesity (BMI $\geqslant 30\ kg/m^2$), impact of increased BMI on fibrosis progression may not be evaluated. Also, a smaller number of patients with worsening of steatosis (11.3% in present study and 34% in previous study [20]) may be the reason for the discrepancy. This may be due to difference in patient selection since no patients in that study had antiviral treatment between two biopsies.

In conclusion, the presence of hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who failed to achieve a SVR to IFN therapy. These two factors may be a therapeutic target to halt the potentially progressive disease independent of antiviral therapy.

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External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a noninvasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] × AST [U/L])/ ((PLT[10^9/L]) × (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

		METAVIR Fibrosis Score			Diagnostic Accuracy
	FIB-4	F0-2	F3-4	Total	
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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別紙３

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

データマイニング手法を用いた効果的なC型肝炎治療法に関する研究：C型慢性肝炎のインターフェロン治療における宿主側とウイルス側因子の相互関係

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研究要旨：C型肝炎ウイルスの病態や治療感受性はHCVの遺伝子構造と宿主側因子に密接に関与していると考えられることからHCVコア領域のアミノ酸変異およびISDR変異を含めたウイルスおよび宿主因子解析による治療効果予測について検討し、以下の結果を得た。(1)ISDR変異数が2以上の症例は治療早期陰性化症例が多く有意なSVR上昇を認めた。(2)コア変異に関しては70/91のdouble mutantの女性で有意なSVR低下およびNR症例の増加をみた。(3)治療効果に関与する因子の解析では、 ISDR, Hb値, F因子で有意差を認めた。 ISDR変異数2以上の症例では良好な治療効果が期待されるが、ISDR変異数1以下、コア領域のdM変異の女性では難治が予想される。これらウイルスおよび宿主因子(Hb値、肝線維化)により症例ごとの治療前効果予測を行うことが治療の質の向上につながるものと考えられる。

A．研究目的

C型肝炎ウイルスの病態や治療感受性はHCVの遺伝子構造と宿主側因子に密接に関与していると考えられる。そこで、HCVコア領域のアミノ酸変異およびISDR変異を含めたウイルスおよび宿主因子解析による治療効果予測について検討した。

B．研究方法

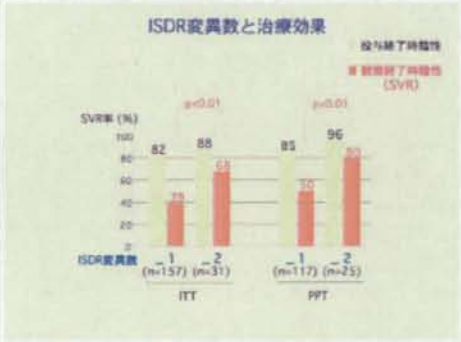
お茶の水リバーカンファレンス共同研究関連施設でインターフェロン治療を導入した607症例の中で最終効果判定可能であったPeg-riba48W療法255例、Peg単独48週療法34例、Peg-riba24週療法84例の解析を行った。当研究は厚生科学審議会の「遺伝子解析研究に付随する倫理問題等に対応するための指針」などに準じて、当該施設倫理審査委員会で、研究の適否などを議論・審査し承認を得た上で、人権及び利益の確保を行うよう配慮した。

C．研究結果

・Peg-riba48W療法255例のITT解析ではSVR 39%、PP解析ではSVR 49%であった。Othersに対するPeg単独48週療法、Peg-riba24週療法ではITT、PP解析ともに70-80%とほぼ同等に良好なSVRが得られた。

・難治例に対しさらに詳細な検討を行った。ISDRとコア70/91変異の解析では、ISDR変異2以上の症例ではコア変異の有無に関わらず良好なSVRが得られた。

・ ISDR変異数１以下と2以上の症例にわけて治療効果を解析したところ、投与終了時



は両群ともに80-90%で陰性化が得られたがISDR 0,1群では再燃症例が多く、SVRはISDR変異2以上の症例で有意に高く、others同様70-80%でSVRが得られることがわかった。この差が生じる原因の一つとしてウイルス陰性化時期の違いがあり、ISDR変異が２以上の症例の多くが治療開始早期にウイルス陰性化が得られていた。

・ 当施設のC型肝炎症例787例の解析では難治例といわれるGenotype1の中でもOthers 同様に良好な治療効果が期待できるISDR変異数2以上の症例は平均で18%、どの年代でも20ー30%の比率で存在していたが、インターフェロン使用歴の有無に関わらず女性は男性の半分、それ以下の比率であり、ISDR変異症例の比率が男女で異なることが分かった。女性のSVR低下の原因として、SVRの得られやすいISDR変異症例の比率が女性では低いことが関与していることが示唆された。

・ コア変異に関しては全体の解析では変異の有無と治療効果に有意な相関は認めないが、女性ではコア抗原のDouble mutant症例で有意なSVR低下、NR増加を認め、コアdM変異は女性においてSVR低下に関与している可能性が示唆された。

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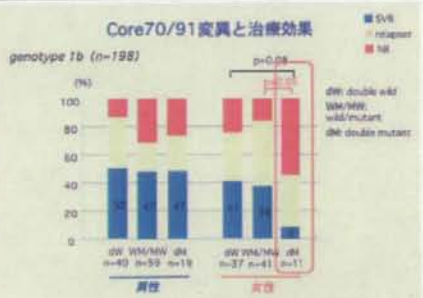
分担研究報告書



・治療前のパラメータではISDR，Hb，F因子 で有意差を認め、これらが治療効果予測因子として重要であることがわかった。

D．考察

・ISDR変異数が2以上の症例は治療早期陰性化症例が多く、有意にSVR上昇を認めた($p<0.01$)。ISDR変異数が2以上の症例は全体の約20%程度存在したが、女性では年代に関係なく比率が低いことが示され、ISDR変異例が少ないことが女性のSVR低下に関与している可能性が示唆された。

・コア変異に関しては70 or 91単独の変異と治療効果の関係は認めなかったが、70/91のdouble mutantの女性で有意なSVR低下およびNR症例の増加をみた($p<0.05$)。

・治療効果に関与する因子の解析では、 ISDR，Hb値，F因子で有意差を認め、これらが治療前の効果予測因子として重要であると考えられた。

E．結論

ISDR変異数2以上の症例では良好な治療効果が期待されるが、ISDR変異数1以下、コア領域のdM変異の女性では難治が予想される。これらウイルスおよび宿主因子（Hb値、肝線維化）により症例ごとの治療前効果予測を行うことが治療の質の向上につながるものと考えられる。

G．研究発表

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H．知的財産権の出願・登録状況

（予定を含む。）

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Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae radix*. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneodelS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

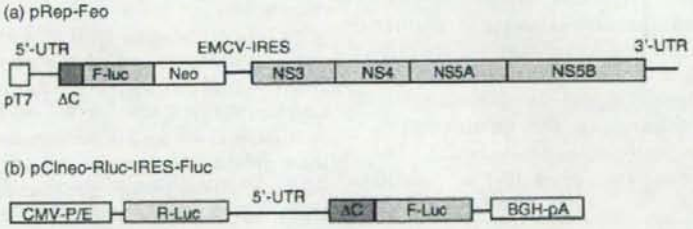

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3′ UTR, 3′ untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency. The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

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Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[9] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

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RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

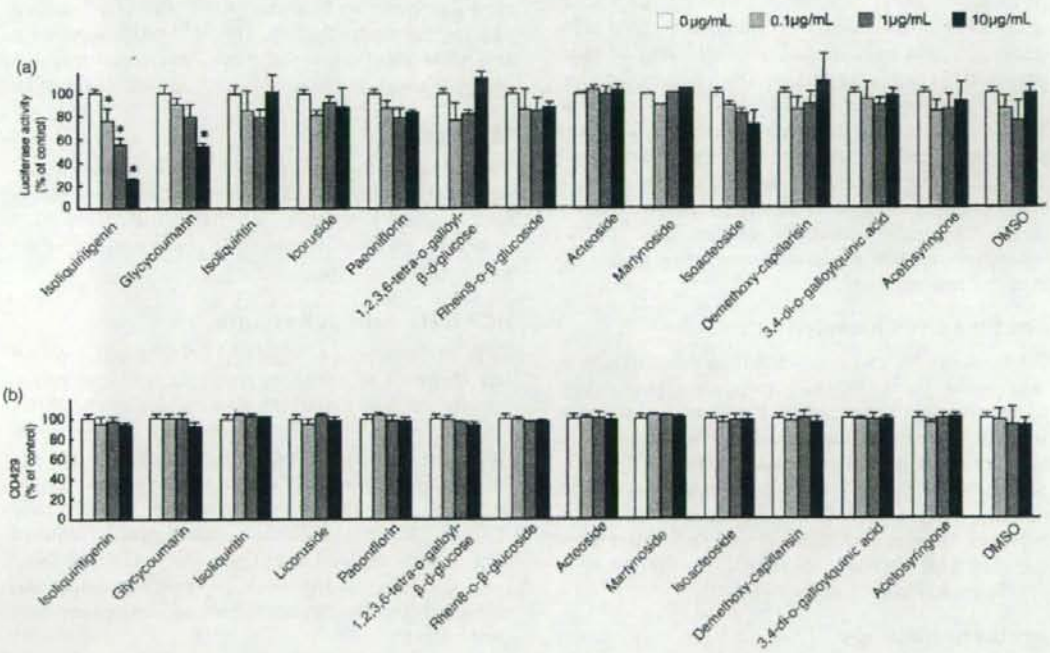

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

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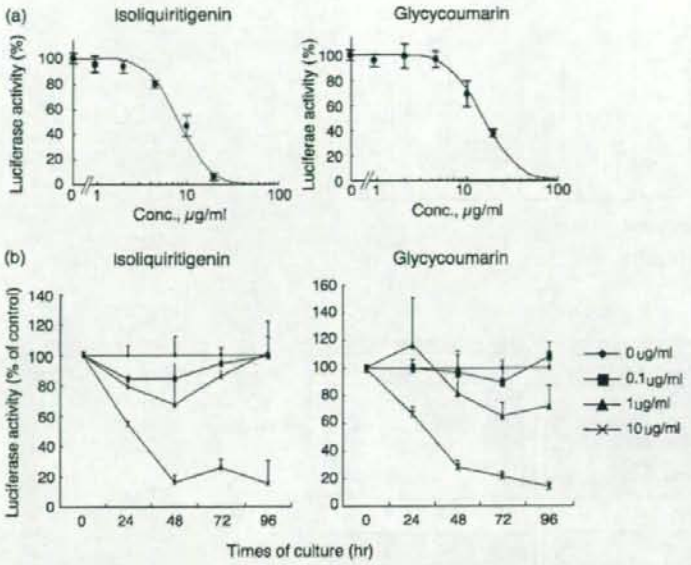

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

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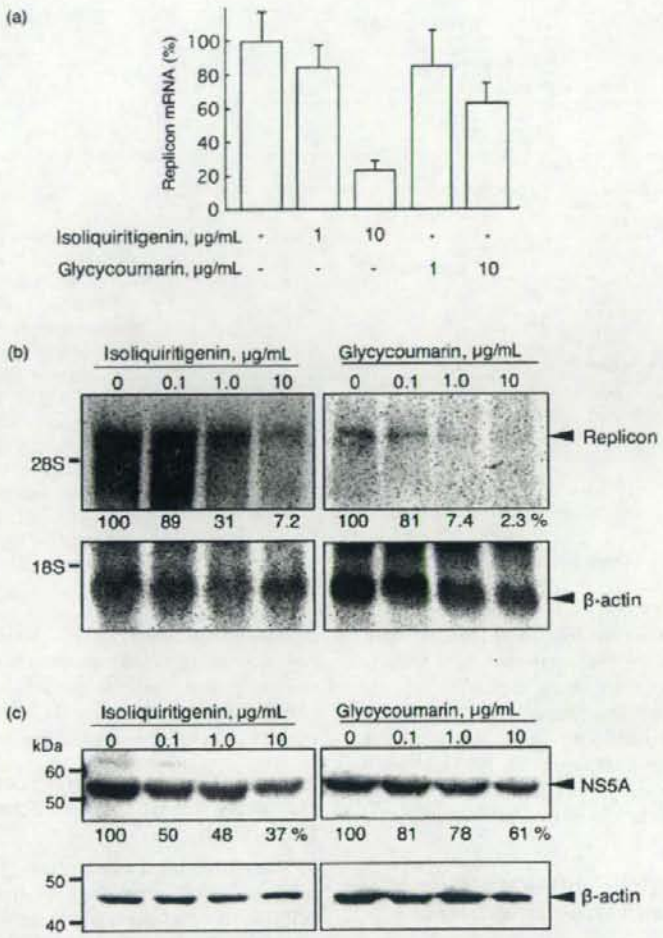

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycycoumarin than the untreated culture. The effect of glycycoumarin was partly reversed on day six probably due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycycoumarin (Fig. 7b).

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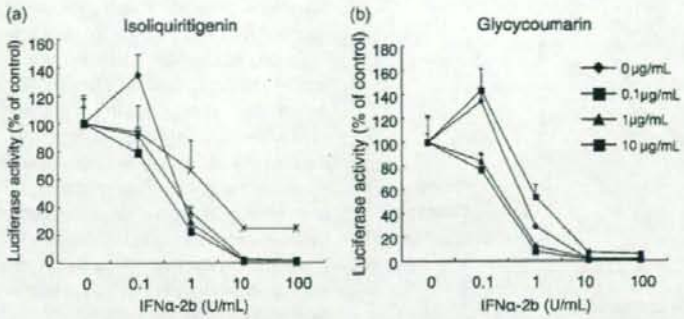

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

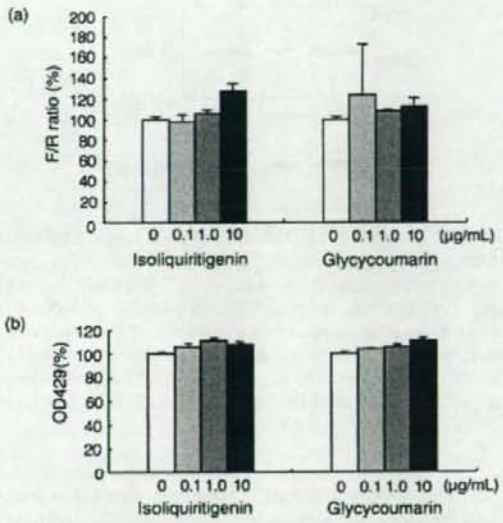

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.

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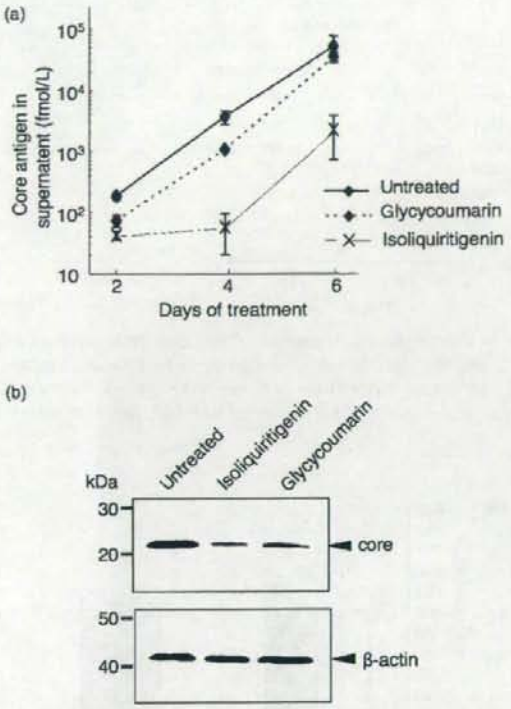

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes, Haemophilus infuenzae* and *Moraxella catarrhalis*,[36] and methicillin-resistant *Staphylococcus aureus*,[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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HEPATOLOGY

Inhibition of hepatitis C virus infection and expression *in vitro* and *in vivo* by recombinant adenovirus expressing short hairpin RNA

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Key words
adenovirus vector, hepatitis C virus, RNA interference.

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Abstract

Background and Aim: We have reported previously that synthetic small interfering RNA (siRNA) and DNA-based siRNA expression vectors efficiently and specifically suppress hepatitis C virus (HCV) replication *in vitro*. In this study, we investigated the effects of the siRNA targeting HCV-RNA *in vivo*.

Methods: We constructed recombinant retrovirus and adenovirus expressing short hairpin RNA (shRNA), and transfected into replicon-expressing cells *in vitro* and transgenic mice *in vivo*.

Results: Retroviral transduction of Huh7 cells to express shRNA and subsequent transfection of an HCV replicon into the cells showed that the cells had acquired resistance to HCV replication. Infection of cells expressing the HCV replicon with an adenovirus expressing shRNA resulted in efficient vector delivery and expression of shRNA, leading to suppression of the replicon in the cells by $\sim 10^{-3}$. Intravenous delivery of the adenovirus expressing shRNA into transgenic mice that can be induced to express HCV structural proteins by the Cre/*lox*P switching system resulted in specific suppression of virus protein synthesis in the liver.

Conclusion: Taken together, our results support the feasibility of utilizing gene targeting therapy based on siRNA and/or shRNA expression to counteract HCV replication, which might prove valuable in the treatment of hepatitis C.

Introduction

Hepatitis C virus (HCV), which affects 170 million people worldwide, is one of the most important pathogens causing liver-related morbidity and mortality.[1] The difficulty in eradicating HCV is attributable to limited treatment options against the virus and their unsatisfactory efficacies. Even with the most effective regimen with pegylated interferon (IFN) and ribavirin in combination, the efficacies are limited to less than half of the patients treated.[2] Given this situation, the development of safe and effective anti-HCV therapies is one of our high-priority goals.

RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA.[3,4] Because of its potency and specificity, RNAi rapidly has become a powerful tool for basic research to analyze gene functions and for potential therapeutic applications. Recently, successful suppression of various human pathogens by RNAi have been reported, including human immunodeficiency viruses,[5,6] poliovirus,[7] influenza virus,[8] severe acute respiratory syndrome (SARS) virus[9] and hepatitis B virus (HBV).[10–13]

We and other researchers have reported that appropriately designed small interfering RNA (siRNA) targeting HCV genomic RNA can efficiently and specifically suppress HCV replication *in vitro*.[14–19] We have tested siRNA designed to target the well-conserved 5′-untranslated region (5′-UTR) of HCV-RNA, and identified the most effective target, just upstream of the translation initiation codon. Furthermore, transfection of DNA-based vectors expressing siRNA was as effective as that of synthetic siRNA in suppressing HCV replication.[14]

In this study, we explored the further possibility that efficient delivery and expression of siRNA may be effective in suppression and elimination of HCV replication and that delivery of such

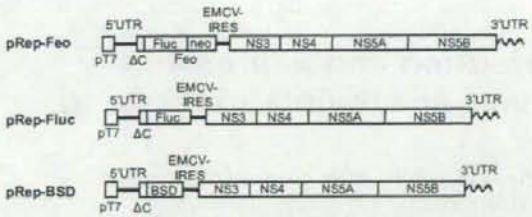

Figure 1 Structures of HCV replicon plasmids. The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase (GenBank accession No. AB119282).[14,20] The pRep-Fluc expressed the Fluc protein. The pRep-BSD expressed the blasticidin S (BSD) resistance gene. pT7, T7 promoter; 5'UTR, HCV 5'-untranslated region; ΔC, truncated HCV core region (nt. 342–377); neo, neomycin phosphotransferase gene; EMCV, encephalomyocarditis virus; NS3, NS4, NS5A and NS5B, genes that encode HCV nonstructural proteins; 3'UTR, HCV 3'-untranslated region.

HCV-directed siRNA *in vivo* may be effective in silencing viral protein expression in the liver. Here, we report that HCV replication was suppressed *in vitro* by recombinant retrovirus and adenovirus vectors expressing short hairpin RNA (shRNA) and that the delivery of the adenovirus vector to mice *in vivo* specifically inhibited viral protein synthesis in the liver.

Methods

Cells and cell culture

Huh7 and Retro Pack PT67 cells (Clontech, Palo Alto, CA, USA) were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon, G418 (Wako, Osaka, Japan) was added to the culture medium to a final concentration of 500 μg/mL.

HCV replicon constructs and transfection

HCV replicon plasmids, pRep-Feo, pRep-Fluc and pRep-BSD were constructed from were constructed from a virus, HCV-N strain, genotype 1b.[21] The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase.[14,20] The pRep-Fluc and the pRep-BSD expressed the Fluc and blasticidin S (BSD) resistance genes, respectively (Fig. 1). The replicon RNA synthesis and the transfection protocol have been described previously.[22]

Synthetic siRNA and siRNA-expression plasmid

The design and construction of HCV-directed siRNA vectors have been described.[14] Briefly, five siRNA targeting the 5'-UTR of HCV RNA were tested for their efficiency to inhibit HCV replication, and the most effective sequence, which targeted nucleotide position of 331 though 351, was used in the present study. To construct shRNA-expressing DNA cassettes, oligonucleotide inserts were synthesized that contained the loop sequence (5'-TTC AAG AGA-3') flanked by sense and antisense siRNA sequences (Fig. 2a). These were inserted immediately downstream of the human U6 promoter. To avoid a problem in transcribing shRNA because of instability of the DNA strands arising from the tight palindrome structure, several C-to-T point mutations, which retained completely the silencing activity of the shRNA, were introduced into the sense strand of the shRNA sequences (referred to as 'm').[23] A control plasmid, pUC19-shRNA-Control, expressed shRNA directed towards the Machado–Joseph disease gene, which is a mutant of ataxin-3 gene and is not normally expressed. We have previously described the sequence specific activity of the shRNA-Control.[24]

Prior to construction of the virus vectors, we tested silencing efficiency of five shRNA constructs of different lengths that covered the target sequence (Fig. 2a). The shRNA-HCV-19, shRNA-HCV-21 and shRNA-HCV-27 had target sequences of 19, 21 and 27 nucleotides, respectively. Transfection of these shRNA constructs into Huh7/pRep-Feo showed that shRNA with longer target sequences had better suppressive effects (Fig. 2b). Therefore, we used shRNA-HCV-27m (abbreviated as shRNA-HCV) in the following study.

Recombinant retrovirus vectors

The U6-shRNA expression cassettes were inserted into the *StuI*/*Hin*dIII site of a retrovirus vector, pLNCX2 (Clontech) to construct pLNCshRNA-HCV and pLNCshRNA-Control (Fig. 2c). The plasmids were transfected into the packaging cells, Retro Pack PT67. The culture supernatant was filtered and added onto Huh7 cells with 4 μg/mL of polybrene. Huh7 cell lines stably expressing shRNA were established by culture in the presence of 500 μg/mL of G418.

Recombinant adenovirus

Recombinant adenoviruses expressing shRNA were constructed using an Adenovirus Expression Vector Kit (Takara, Otsu, Japan). The U6-shRNA expression DNA cassette was inserted into the *SwaI* site of pAxcw to construct pAxshRNA-HCV and pAxshRNA-Control. The adenoviruses were propagated according to the manufacturer's protocol (AxshRNA-HCV and AxshRNA-Control; Fig. 2c). A 'multiplicity of infection' (MOI) was used to standardize infecting doses of adenovirus. The MOI stands for the ratio of infectious virus particles to the number of cells being infected. An MOI = 1 represents equivalent dose to introduce one infectious virus particle to every host cell that is present in the culture.

Plasmids for assays of interferon responses

pISRE-TA-Luc (Invitrogen, Carlsbad, CA, USA) contained five copies of the consensus interferon stimulated response element (ISRE) motifs upstream of the Fluc gene. pTA-Luc (Invitrogen), which lacks the enhancer element, was used for background determination. The pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega, Madison, WI, USA), which expresses the *Renilla* luciferase protein, was used for normalization of transfection efficiency.[25] A plasmid, peGFPneo (Invitrogen), was used to monitor percentages of transduced cells.

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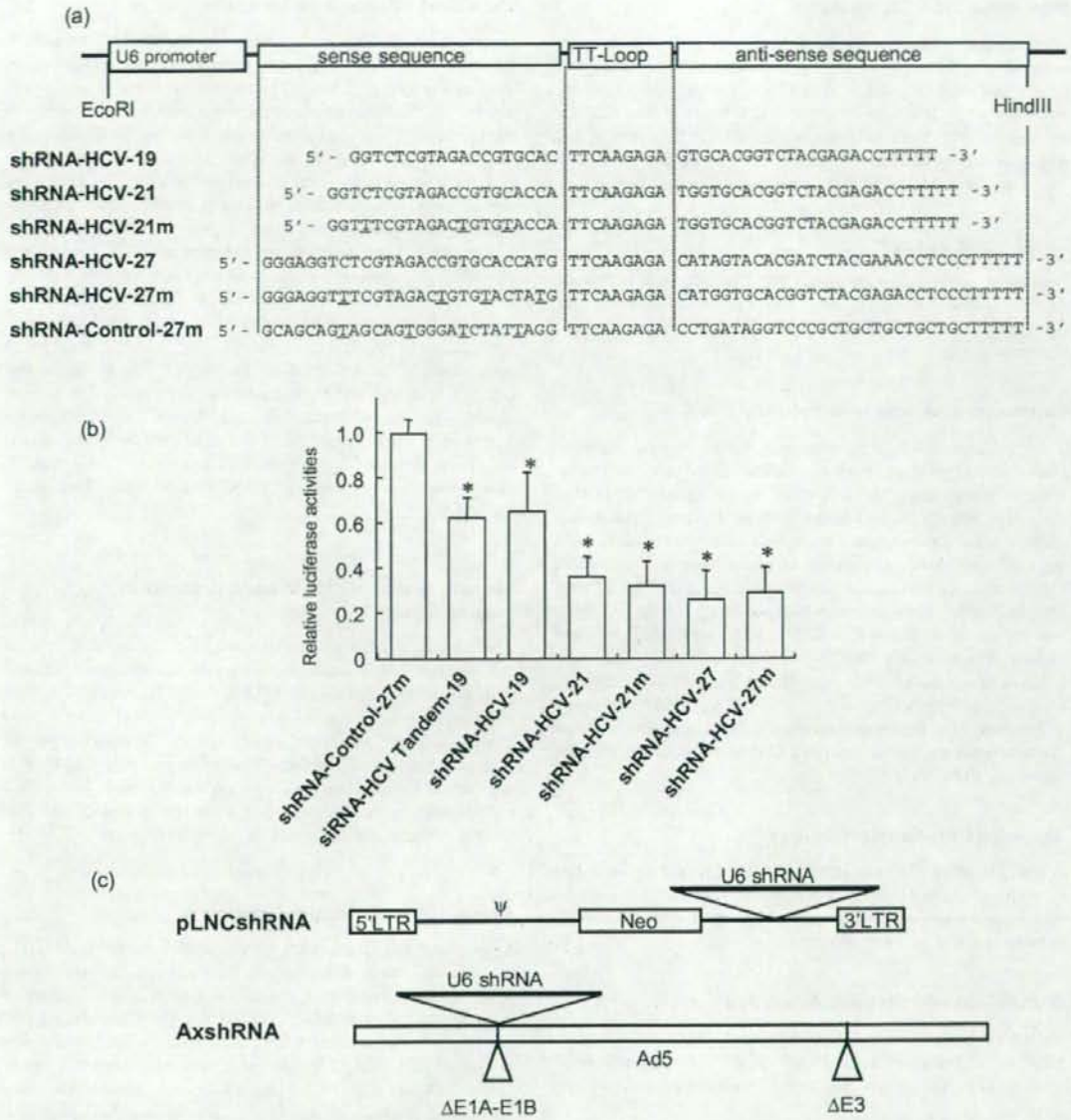

Figure 2 Structure of shRNA-expression constructs and shRNA sequences. (a) Structure of shRNA-expression cassette and shRNA sequences. TT-Loop, the loop sequence. The shRNA-Control was directed toward an unrelated target, Machado–Joseph disease gene. Underlined letters indicate C-to-T point mutations in the sense strand. (b) The shRNA-expression plasmids were transfected into Huh7/pRep-Feo cells, and internal luciferase activities were measured at 48 h of transfection. Each assay was done in triplicate, and the values are displayed as mean + SD. $^*P < 0.05$. (c) pLNCshRNA, structure of a recombinant retrovirus expressing shRNA. Ψ, the retroviral packaging signal sequence. AxshRNA, structure of a recombinant adenovirus expressing shRNA.