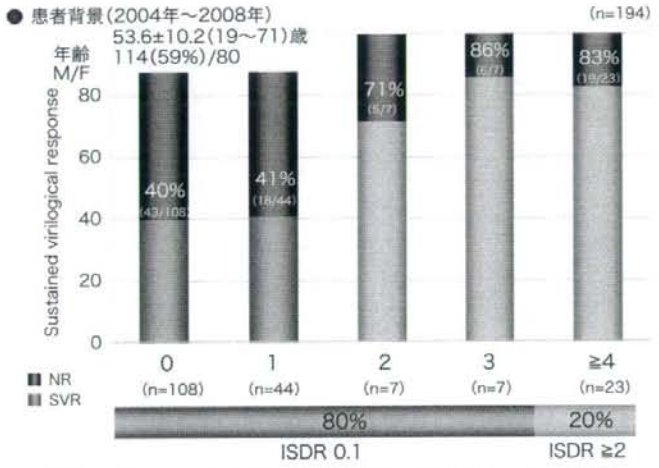

図5　PEG + Riba治療(12カ月)のISDR別SVR率(高ウイルス症例)

表1　PEG + Riba治療(1b)のSVRに寄与する因子(12カ月治療)(n=194)

		odds比	95% CI	p
年齢	＜60/≧60	0.255	0.057-0.886	0.0329
F因子	0-1/2-4	0.139	0.034-0.563	0.0061
白血球数	＜4600/≧4600	3.427	0.582-13.787	0.0828
ISDR変異数	0-1/2-	164.571	8.458-＞999.9	0.0008
コアAA70	Q/R	13.840	2.464-77.739	0.0028

多変量ロジステック回帰分析

別に検討すると，Wild type, Intermediate type, Mutant typeのSVR率はそれぞれ5%，10%，64%であり，ISDRにおけるアミノ酸変異数の増加に伴い，著効率は上昇するもののISDR変異数4個以上が重要であることが明らかにされている(図3)．インターフェロン単独療法では，治療効果はウイルス量と相関し，HCVRNA量が高いほど治療効果が劣るが，ISDR変異とHCVRNA量は相関することから，ISDR変異は単にウイルス量を反映したに過ぎないとの指摘もある．しかし，多変量解析では，ISDRはウイルス量とは関係なく，SVRに寄与する独立した因子として認められるし，ウイルス量が同等であれば，ISDR変異数が多いほど治療効果は高い(図4)．一方，genotype 2aないしは2bにおいては，総じて治療効果が高いため，ISDRは1b型ほど有意な治療効果規定因子とはなりえないが，ISDRのアミノ酸変異数が多いほど治療効果が高い傾向がある．また，最近では，インターフェロン単独療法はペグインターフェロン(PEG-IFN α 2a: ペガシス)を用いることが多いが，この現象はPEG-IFN α 2a (12カ月治療)でも認められる．

表2　PEG/Riba治療(1b-ISDR 0-1)のSVRに寄与する因子(n=157)

		odds比	95% CI	p
年齢	＜60/≧60	0.122	0.023-0.641	0.0129
F因子	0-1/2-4	0.066	0.011-0.407	0.0035
T.Chol	＜160/≧160	10.757	1.630-70.990	0.0136
コアAA70	Q/R	49.457	4.499-543.583	0.0014

多変量ロジステック回帰分析

4　ペグインターフェロン＋リバビリン併用療法とISDR変異

現在，1b型のHCVに対する標準治療は，ペグインターフェロン＋リバビリン併用療法であり，難治である1b型かつ高ウイルス量症例の治療成績を大きく向上させた．この治療法においても，ISDR変異はSVRを規定する独立した因子であり，宿主因子である肝の線維化とならび，最も重要な因子であった[4]．しかし，インターフェロン単独療法でSVRが得られるためにはISDRのアミノ酸変異は4個以上必要であったが，ペグインターフェロン＋リバビリン併用療法においては，2個以上の変異があれば80%以上の高い確率でSVRを期待できることが明らかになった(表1)．これは，インターフェロン単独療法に比較して，PEG-IFN＋リバビリン療法の抗ウイルス効果が格段に高いことに由来していると考えられるが，臨床的な血中リバビリン濃度では，HCVに対する単独の抗ウイルス効果はわずかに過ぎない．しかし，レプリコンシステムを用いた*in vitro*の検討では，インターフェロンにリバビリンを少量添加すると，濃度依存的にインターフェロンのHCV増殖抑制効果を高めることが確認され，リバビリンはインターフェロンの効果を相乗的に高めることが報告されている．さらに，リバビリンは，ウイルスのmutagenとして作用して，NS5A領域の遺伝子変異を増加させる現象がみいだされており，リバビリンの作用機序のひとつとして注目されている[5]．しかし，ISDRのアミノ酸変異数が0ないし1個であっても，SVRとなる症例は存在する．そこで，ISDRのアミノ酸変異数が0ないし1個の症例に限り，治療開始後4週間でウイルス量が法が2 log以上低下したsteep responderと1 log未満しか低下しなかったflat responderにつき，HCV全ゲノムの相違を検討した．その結果，両者に相違がみられたのは，コア領域とNS2領域のアミノ酸であり，統計学に有意な相違がみられたのはコア領域の70番目のアミノ酸のみであった．さらにこの領域に注目してretrospectiveに，IFN治療効果とこのアミノ酸変異との関連を検討すると，ISDRが0ないしは1の難治が予測される症例であっても，このアミノ酸が，HCVのプロトタイプのHCV-Jにみられるアルギニン(R)であれば高率にSVRが期待できるものの，グルタミン(Q)に変異していると，IFN治療反応性が極めて悪く，主治医の判断で治療中断した症例や，治療完遂してもSVRにならない症例が多数を占め，ISDR変異数0ないし1個の症例ではコア70番のアミノ酸変異が，SVRを予測する最も重要な因子であった(表2)．1b型症例全体でもコア70番のアミノ酸変異は治療効果予測因子のひとつであるが，ISDR変異により，症例を細分化した

表3　C型慢性肝炎に対するテーラーメイド治療

1）遺伝子型
　2型：　インターフェロン単独
　　高ウイルス量ではペグインターフェロン＋リバビリン24週
　1型：　ISDRとコア領域変異測定による方針決定
2）1型におけるISDRとコアアミノ酸70番変異
　ISDR変異数4個以上：　インターフェロン単独
　ISDR変異数2個以上：　ペグインターフェロン＋リバビリン48週
　ISDR変異数0・1個の場合：　下記のペグインターフェロン＋リバビリンの成績をふまえ，検討する

ISDR変位数 / コア70番アミノ酸	0	1	2個以上
R	72週投与投与により 70%	86%	81%
NonR（Q）	72週投与（8週以内の陰性化例）により 40%		

うえで，難治が予測されるISDR変異数0ないし1の症例においては，コアアミノ酸変異を検討することが重要であると考えられる．

5　ISDRからみたC型慢性肝炎に対する治療方針

HCVのゲノム解析により，インターフェロン治療の効果予測は可能となりつつある．すなわち遺伝子型が2aないしは2b型であれば，遺伝子変異に関わらず，インターフェロン単独療法でもある程度効果期待できるが，ウイルス量が多い場合は，ペグインターフェロン＋リバビリン併用療法が望ましい．さらに，1b型でもISDR変異数が4個以上あれば，単独療法でも治癒可能であるし，2個以上あればペグインターフェロン＋リバビリン療法48週間の治療でSVRが期待できる．またISDR変異数が0ないし1であってもコア70番のアミノ酸がRであればSVRが期待できるが，Qであれば治療には慎重である必要がある．しかし，ISDR変異数0・1でコア70番がQであっても，治療早期（8週以内）にウイルス陰性化が得られれば，72週間投与によりSVRとなる可能性が残されており，さらに，宿主因子である性別，年齢，肝線維化・脂肪化，初回・再治療，前治療の効果を考慮することによって，さらに詳細な治療効果予測が可能になると思われ，近い将来の，いわゆる個別化医療にHCVのゲノム解析の臨床応用が重要な意味を持っているものと考えられる（表3）．

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Hepatology Research 2008; **38**: 909–918 doi: 10.1111/j.1872-034X.2008.00352.x

Original Article

Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication *in vitro*

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Aim: Hepatitis C virus (HCV), which infects an estimated 170 million people worldwide, is a major cause of chronic liver disease. The current standard therapy for chronic hepatitis C is based on pegylated interferon (IFN)α in combination with ribavirin. However, the success rate remains at approximately 50%. Therefore, alternative agents are needed for the treatment of HCV infection.

Methods: Using an HCV-1b subgenomic replicon cell culture system (Huh7/Rep-Feo), we found that griseofulvin, an oral antifungal agent, suppressed HCV-RNA replication and protein expression in a dose-dependent manner. We also found that griseofulvin suppressed the replication of infectious HCV JFH-1. A combination of IFNα and griseofulvin exhibited a synergistic inhibitory effect in Huh7/Rep-Feo cells.

Results: We found that griseofulvin blocked the cell cycle at the G_2/M phase in the HCV subgenomic replicon cells, but did not inhibit HCV internal ribosome entry site-dependent translation.

Conclusion: Our results suggest that griseofulvin may represent a new approach to the development of a novel therapy for HCV infection.

Key words: cell cycle, griseofulvin, hepatitis C virus internal ribosome entry site, hepatitis C virus replicon, JFH-1

INTRODUCTION

HEPATITIS C VIRUS (HCV) is an etiologic agent of chronic liver disease,[1,2] and it is estimated that approximately 170 million people worldwide are infected with the virus. Chronic hepatitis C can lead to severe liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma.[3] With advancements in HCV therapy, including the most recent combination of pegylated interferon (IFN)α and ribavirin, up to one-half of patients achieve a sustained virological response. However, the remainder cannot clear the virus, demonstrating a great need for more powerful therapeutic modalities.[4]

Investigations have been hampered by the lack of an efficient HCV cell culture system. In 1999, the establishment of an HCV subgenomic replicon cell culture system improved the situation. The subgenomic replicon RNA is composed of the HCV 5′ untranslated region (UTR) containing the internal ribosomal entry site (IRES), a neomycin phosphotransferase (neo) gene and the HCV non-structural (NS) proteins through 3–5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′ UTR.[5] A HCV replicon carrying, in addition to the selectable marker, a gene encoding luciferase, can be used to screen a large number of compounds for antiviral activity.[6–8] The recent development of an *in vitro* HCV infection system provides an opportunity to evaluate inhibitors of all stages of the HCV life cycle.[9–11]

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Received 12 September 2007; revision 31 January 2008; accepted 11 February 2008.

Currently, proof of concept has been obtained in clinical trials of three different HCV NS3 protease inhibitors, BILN 2061,[12,13] telaprevir (VX-950),[14] and SCH 503034.[15] However, because of many factors, including possible side-effects and the emergence of drug-resistant mutants, there is still great need for improved therapies. We focused therefore on screening a set of licensed drugs which have not been recommended previously for antiviral use. Here, we found that the oral antifungal agent, griseofulvin, had a suppressive effect on HCV replication, assessed using the HCV-1b subgenomic replicon system and the particle-producing cell culture HCV-2a model of JFH-1. The mechanism of the anti-HCV activity of griseofulvin also was studied.

METHODS

Cell cultures and HCV replicon

THE HUMAN HEPATOMA cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For subgenomic replicon Huh7/Rep-Feo (HCV 1b replicon that expresses a chimeric protein consisting of neomycin phosphotransferase and firefly luciferase) cells,[7,8] the culture medium was supplemented with 250 g/mL G418. Huh 7.5.1/JFH-1 cells (Huh 7.5.1 chronically infected HCV JFH-1) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.[16]

Reagents

Griseofulvin and fluconazole were purchased from Wako Pure Chemical (Tokyo, Japan). Itraconazole was purchased from LKT Laboratories (St Paul, MN, USA). Recombinant human IFNα-2b was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assays

For griseofulvin and fluconazole, viable cell growth was determined by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) reduction assay using the Cell Titer 96 aqueous one solution cell proliferation assay (Promega, Madison, WI, USA), according to the manufacturer's protocol.

For itraconazole, viable cell growth was determined using the CellTiter-Glo luminescent cell viability assay (Promega, USA), according to the manufacturer's protocol.

Luciferase activity assays

Typically, Huh7/Rep-Feo cells were seeded in a 48-well plate at a density of 2×10^4 cells per well. Compounds were added to the culture medium at various concentrations. After 72 h of culture, the expression levels of the HCV replicon were measured by luciferase assay using the luciferase assay system (Promega, USA) and the Luminescencer-JNR AB-2100 (Atto, Tokyo, Japan).

The Huh7 cells stably transfected with the pEF Fluc IN vector were mock treated (control) or treated with 20 μM or 40 μM griseofulvin. After 72 h of culture, luciferase assays were performed using the luciferase assay system and the Luminescencer-JNR AB-2100. Luciferase activity was normalized by the protein concentration, measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

The Huh7 cells stably transfected with the pEF Rluc-HCV IRES Feo vector were mock treated (control) or treated with 20 μM griseofulvin. Dual luciferase activities were carried out at 8, 16, 24, and 32 h after exposure to griseofulvin using the dual luciferase reporter assay system and the Luminescencer-JNR AB-2100.

All assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

RNA analysis

Total cellular RNA was extracted from the Huh7/Rep-Feo cells using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA). RNA was reverse transcribed with a Thermoscript reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (PCR) was carried out using ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The forward and reverse primers for the 5′ UTR of HCV-RNA were 5′-TGCGGAACCGGTGAGTACA-3′ and 5′-CTTAAGG TTTAGGATTCGTGCTCAT-3′, respectively. The fluorogenic probe used for the quantification of HCV-RNA was 5′-(FAM)-CACCCTATCAGGCAGTA-CCACAAGG CC-(TAMRA)-3′. Human 18S ribosomal RNA levels in the samples were analyzed by quantitative real-time PCR to normalize the RNA content. The forward and reverse primers for human 18S ribosomal RNA were 5′-ACTCTAGATAACCTCGGGCCGA-3′ and 5′-GATGT GGTAGCCGTTTCTCAGG-3′, respectively. The fluorogenic probe used for quantification of human 18S ribo-

somal RNA was 5′-(FAM)-CCATTCGAACGTCTGCCC TATCAACTTT-(TAMRA)-3′. The method has been described elsewhere.[17]

The primers used for reverse transcription (RT)–PCR were as follows: human 2′,5′-oligoadenylate synthetase (2′,5′-OAS): forward primer, 5′-CAATCAGCGAGGCC AGTAATC-3′ and reverse primer, 5′-TGGTGAGAAGTGC TGGGGTC-3′; human myxovirus resistance protein A (MxA): forward primer, 5′-GTCAGGAGT-TGCCCTT CCCA-3′ and reverse primer, 5′-GGCCCCTTCGTT ACCCTTA-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′-GAAG GTGAAGGTCGGAGTC-3′ and reverse primer, 5′-GTT TAGGGTAGTGGTAGAAG-3′, respectively. Each reaction mixture contained cDNA (3 μL), 1.5 mM $MgCl_2$, 200 μM dNTP, 1 μM each primer, and 1.25 U AmpliTaq Gold (Applied Biosystems, USA) with 1× supplied reaction buffer. After activation of AmpliTaq Gold activity at 95°C for 10 min, the temperature cycling conditions for MxA were 29 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min. For 2′,5′-OAS, the conditions were 32 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min. For GAPDH, the conditions were 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min. PCR products were subjected to electrophoresis in a 3% agarose gel.

Western blotting

Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously.[18] The antibodies used in this study were the anti-NS3 antibody (Santa Cruz Biotechnology, USA) anti-NS5A antibody (Virogen, Watertown, MA, USA) and anti-β-actin antibody (Cell Signaling, Danvers, MA, USA). Alkaline phosphatase-conjugated secondary antibodies and CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA, USA) were used for detection.

Cell cycle analysis

Harvested cells were washed once with phosphate-buffered saline (PBS) and fixed with 70% ethanol at 4°C for 1 h. After an additional wash, the cells were treated with 250 μg/mL RNase A at 37°C for 1 h and subsequently stained with 50 μg/mL propidium iodide at 4°C for 1 h. The DNA content was then analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) with ModFit LT software (Verity Software House, Topsham, ME, USA).

Analyses of drug synergy

The effects of the treatment of Huh7/Rep-Feo cells with griseofulvin and IFNα, alone and in combination, were analyzed with CalcuSyn, a computer program based on the method of Chou and Talalaly.[19] After converting the dose-effect curves for each drug or drug combination to median-effect plots, the program calculated a combination index (CI). The CI of <1, 1, and >1 indicate synergy, an additive effect, and antagonism, respectively.

Plasmids and stable transfection

The plasmid pEF-Fluc-IN was constructed as follows. The fragment carrying the firefly luciferase was amplified from the pGL3 control vector (Promega, USA) by PCR using a pair of primers (5′-GAATTCATGGAAGAC GCCAAAAACATAAA-3′ [*Eco*RI site] and 5′-GCGGC CGCTTACACGGCGATCTTTCCGCCC-3′ [*Not*I site]). The PCR product was cloned into the pGEM-T Easy vector (Promega, USA). The EMCV IRES Neo fragment was excised from the pMXs-IN vector by *Not*I and *Sal*I digestion.[20] The *Eco*RI–*Sal*I fragment of the pCHO vector was excised from the pGag-pol-IRES-bsr vector by *Eco*RI and *Sal*I digestion.[21] To construct pEF-Fluc-IB, the *Eco*RI–*Not*I fragment of firefly luciferase, and the *Not*I–*Sal*I fragment of the EMCV IRES Neo were inserted into the *Eco*RI and the *Sal*I site of pCHO by triple ligation.

The plasmid pEF Rluc-HCV IRES Feo was constructed as follows. The fragment carrying the Renilla luciferase was amplified from the phRL-TK vector (Promega, USA) by PCR using a pair of primers (5′-GAATTCATGGCT TCCAAGGTGTACGACCC-3′ [*Eco*RI site] and 5′-GGAT CCTTACTGCTCGTTCTTCAGCACGC-3′ [*Bam*HI site]). The fragment carrying the HCV IRES Feo was amplified from the pRep-Feo vector[7] by PCR using a pair of primers (5′-GGATCCGCCAGCCCCCGATTGGGGGCG AC-3′ [*Bam*HI site] and 5′-GTCGACTCAGAAGAAC TCGTCAAGAAGGC-3′ [*Sal*I site]). Each PCR product was cloned into the pGEM-T Easy vector. To construct pEF Rluc-HCV IRES Feo, the *Eco*RI–*Bam*HI fragment of Renilla luciferase, and the *Bam*HI–*Sal*I fragment of HCV IRES Feo were inserted into the *Eco*RI and *Sal*I site of pCHO by triple ligation.

The pEF-Fluc-IB and pEF Rluc-HCV IRES Feo was transfected into Huh7 cells using Effectene transfection reagent (QIAGEN, Hilden, Germany), according to the manufacturer's recommendation. Two days after trans-

fection, the Huh7 cells were selected in a medium containing 250 μg/mL G418.

Immunofluorescent staining

After treatment with griseofulvin for 72 h, HCV JFH-1-infected cells were fixed with cold methanol and blocked using Blocking One (Nacalai Tesque, Kyoto, Japan). For the detection of the NS3 protein, the cells were incubated with the anti-NS3 antibody (Virogen, USA) for 1 h at room temperature. After washing with PBS, the cells were incubated with an Alexa Fluor 488 goat antimouse immunoglobulin G antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing with PBS, the cells were stained with 7-aminoactinomycin D for nuclear counterstaining, and analyzed using fluorescence microscopy.

RESULTS

Replication of a subgenomic HCV-1b replicon is suppressed by griseofulvin

WE INVESTIGATED THE anti-HCV effect and cell toxicity of griseofulvin in the HCV subgenomic replicon cells, Huh7/Rep-Feo. The luciferase activities of the Huh7/Rep-Feo cells showed that replication of the HCV replicon was suppressed by griseofulvin in a dose-dependent manner (Fig. 1a). Next, we performed a time-course experiment in which the luciferase activities of Huh7/Rep-Feo cells were measured at various time points after treatment with griseofulvin. As shown in Figure 1b, griseofulvin induced a decrease in the luciferase activities of Huh7/Rep-Feo cells over time. The treatment with griseofulvin had little effect on cellular viability at this range of concentration, as revealed by the MTS assay (Fig. 1c). The 50% effective concentration (EC_{50}) of griseofulvin was 6.13 ± 0.17 μM. The 50% cytotoxic concentration of this compound (CC_{50}) was 217.93 ± 3.49 μM. Thus the selectivity index (ratio of CC_{50} to EC_{50}) was 35.5 (Table 1). Furthermore, we examined the effect of other antifungal agents, fluconazole and itraconazole, on HCV-RNA replication. In contrast, fluconazole and itraconazole had little effect on HCV-RNA replication (Table 1).

We analyzed HCV-RNA levels in Huh7/Rep-Feo cells treated or not treated with griseofulvin using real-time RT–PCR. As shown in Figure 2a, treatment with griseofulvin decreased the replicon RNA titer in a dose-dependent manner. Similar results were seen at the protein level by monitoring the HCV non-structural proteins NS3 and NS5A. The Western blot analysis demon-

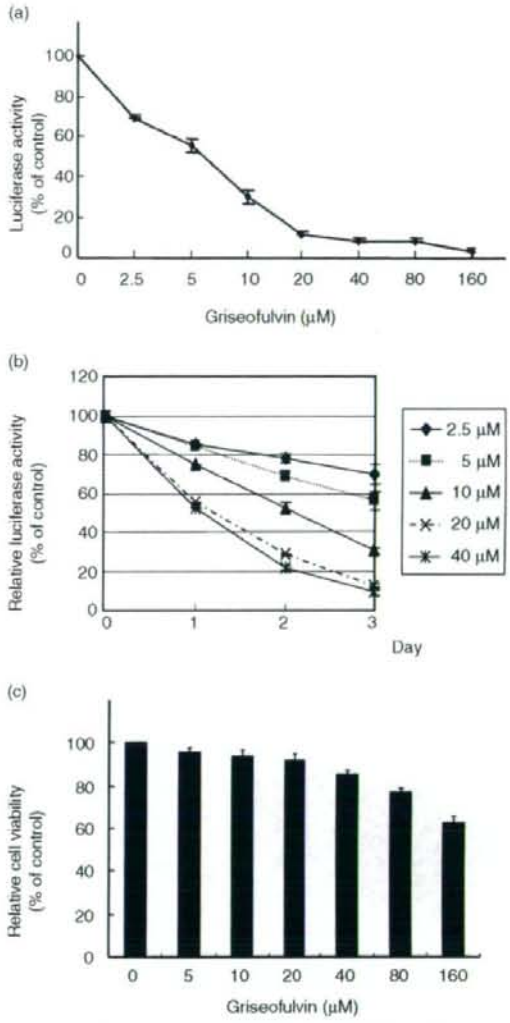

Figure 1 Inhibition of hepatitis C virus replication in Huh7/Rep-Feo cells by griseofulvin. (a) Huh7/Rep-Feo cells were cultured with various concentrations of griseofulvin in the medium and luciferase assays were performed after 72 h of culture. Luciferase assays were performed in triplicate. Error bars indicate mean ± standard deviation. (b) Huh7/Rep-Feo cells were treated with various concentrations of griseofulvin (2.5–40.0 μM). Luciferase activity was measured at the time points indicated after exposure to griseofulvin. (C) 5-(3-Carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt of Huh7/Rep-Feo cells cultured with the concentration of griseofulvin indicated.

Table 1 Antihepatitis C virus activities of oral antifungal agents in Huh7/Rep-Feo cells†

	EC_{50} (μM)	CC_{50} (μM)	SI
Griseofulvin	6.13 ± 0.17	217.93 ± 3.49	35.5
Fluconazole	135.6 ± 1.25	159.06 ± 1.07	1.2
Itraconazole	1.24 ± 0.21	3.35 ± 0.17	2.7

† All data represent means ± standard deviation for three separate experiments. CC_{50}, 50% cytotoxicity concentration based on the reduction of cell viability; EC_{50}, 50% effective concentration based on the inhibition of HCV replication; SI, selectivity index (CC_{50}/EC_{50}).

strated that griseofulvin treatment results in reduced levels of these viral proteins (Fig. 2b).

However, it remains to be clarified whether the griseofulvin inhibits firefly luciferase directly. To investigate this possibility, we examined the effect of griseofulvin on firefly luciferase activity using Huh7 cells expressing firefly luciferase constitutively. The treatment of these cells with griseofulvin resulted in no significant change in the firefly luciferase activity (Fig. 3). This result excludes the possibility that griseofulvin inhibits firefly luciferase activity directly.

Anti-HCV activity of griseofulvin is not mediated by the IFN signaling pathway

It has been reported that the HCV replicon is highly sensitive to IFN.[22,23] To determine whether the action of griseofulvin on the HCV subgenomic replicon involves the activation of IFN-stimulated gene responses, we analyzed the expression of IFN inducible genes in HCV replicon cells. The RT–PCR analysis showed that the messenger RNA for MxA and 2′,5′-OAS, which are both IFN inducible genes, were induced by IFNα-2b, but not by griseofulvin (Fig. 4). These results suggest that the action of griseofulvin on the intracellular replication of HCV replicon is independent of the IFN signaling pathway.

Synergistic inhibitory effect of griseofulvin and IFNα on HCV replicon

Whether a combination of griseofulvin and IFNα exhibits a synergistic, additive, or antagonistic effect was assessed using an isobologram method.[19] An isobologram analysis is an approach used in preclinical studies to quantify the extent of synergistic, additive, or antagonistic effects between drugs used in combination. For instance, a representation of an isobologram to evaluate a drug–drug interaction is shown in Figure 5a. It is understood that synergy, additivity, and antagonism are represented by concave, liner, and convex isoeffective curves (isoboles), respectively. The combined anti-HCV effects of griseofulvin and IFNα were evaluated. Prior to the combination experiments, the optimal concentration ratio of two compounds (combination ratio) had to be determined. After preliminary experiments, three different rations were chosen for each combination

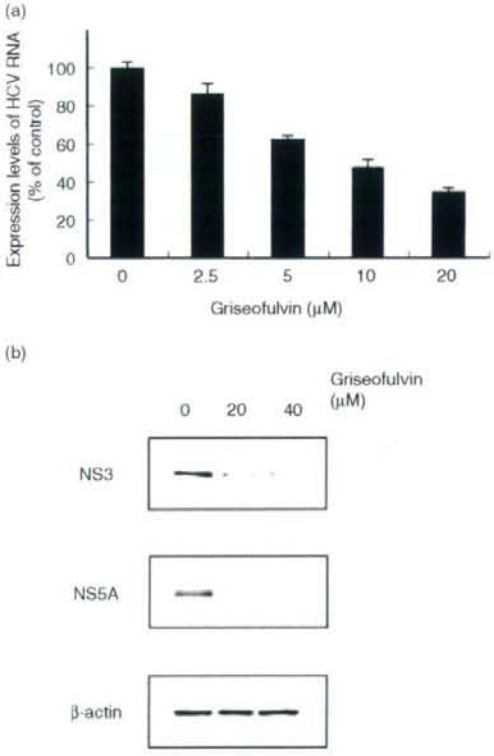

Figure 2 Suppressive effect of griseofulvin for hepatitis C virus (HCV) replicon was confirmed by real-time reverse transcription–polymerase chain reaction (RT–PCR) and Western blot analysis. (a) Incubation of Huh7/Rep-Feo cells with griseofulvin for 72 h resulted in dose-dependent antiviral effects. Real-time RT–PCR was performed on the extracted RNA. HCV-RNA levels are shown as relative percentages of untreated control. Error bars indicate mean ± SD. (b) Western blot analyses of NS3 and NS5A protein expressions were performed on protein extracts from cells that were treated for 72 h with varying dose of griseofulvin. β-Actin was used as a loading control.

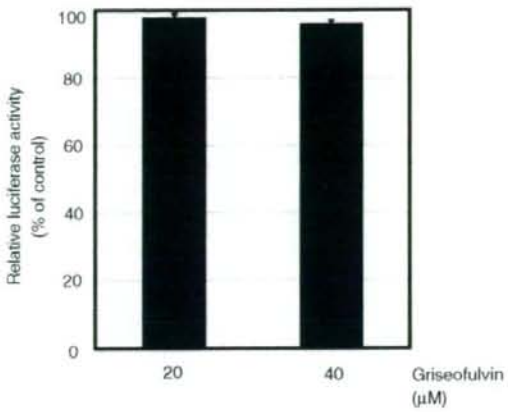

Figure 3 No inhibition of firefly luciferase activity by griseofulvin. pEF Fluc IN vector was stably transfected into Huh7 cells. Cells were cultured without (control) and with 20 μM or 40 μM griseofulvin for 72 h. Firefly luciferase assay was performed. Luciferase activity was normalized by the protein concentration. Error bars indicate mean ± SD.

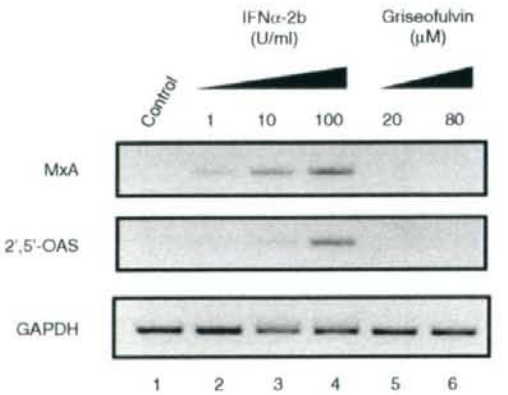

Figure 4 Griseofulvin elicited an interferon (IFN) response. Huh7/Rep-Feo cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 20 (lane 5) or 80 μM griseofulvin (lane 6) for 72 h. Messenger RNA of human myxovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (2′,5′-OAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse transcription–polymerase chain reaction analysis.

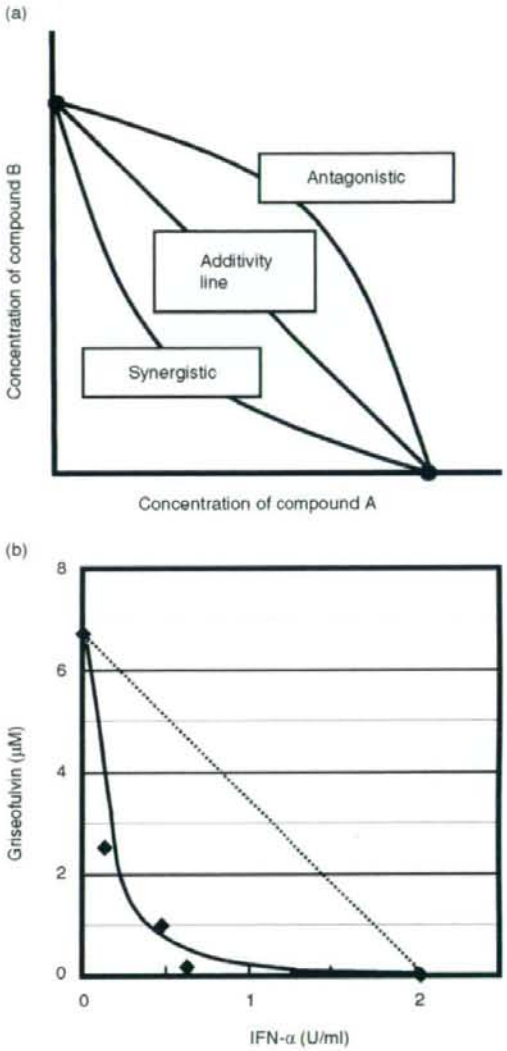

Figure 5 Effect of a combination of griseofulvin and interferon (IFN)α on intracellular hepatitis C virus (HCV)-RNA replication. (a) Representative isobologram for analyzing the interaction between two drugs. (b) Isobole plot of 50% inhibition of HCV-RNA replication. Huh7/Rep-Feo cells were treated with griseofulvin in combination with IFNα, and a luciferase assay was performed after 72 h of culture to obtain each isobole plot. Dotted line indicates an additive effect in the isobologram method used.

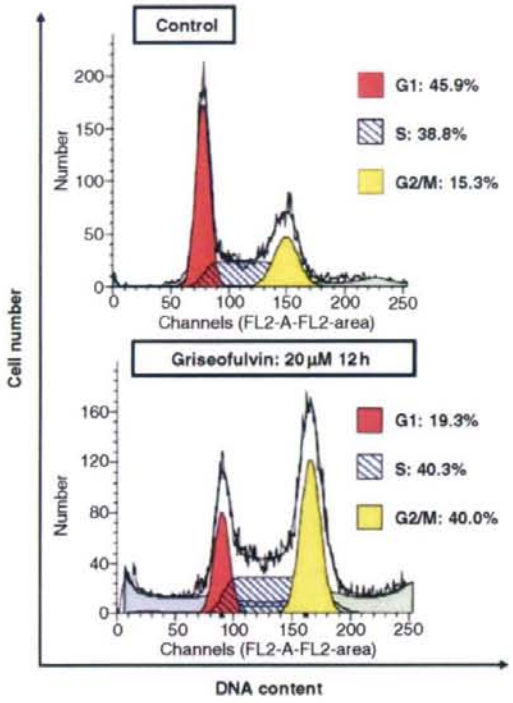

Figure 6 Griseofulvin induced G_2/M phase arrest in Huh7/Rep-Feo cells. Flow cytometry analysis of DNA content of untreated Huh7/Rep-Feo cells (control) and cells treated for 12 h with 20 μM of griseofulvin. [■ G_1: 45.9%, ▨ S: 38.8%, □ G_2/M: 15.3%; ■ G_1: 19.3%, ▨ S: 40.3%, □ G_2/M: 40.0%.]

(data not shown). The ratios of griseofulvin and IFNα were 9:1, 1:1, and 1:9. Each concentration of griseofulvin and IFNα at 50% inhibition was plotted on the X- and Y-axes, respectively, to generate an isobologram (Fig. 5b). As shown in Figure 5b, each plot fell far below the line showing additivity, indicating that the effect of the griseofulvin and IFNα combination on HCV-RNA replication is strongly synergistic.

Griseofulvin induces G_2/M cell cycle arrest in HCV replicon cells

As described previously, griseofulvin blocks cell cycle progression at the G_2/M phase in several human cell lines.[24] Here, we examined the effect of griseofulvin on cell cycle progression in Huh7/Rep-Feo cells. As shown in Figure 6, the population of griseofulvin-treated Huh7/Rep-Feo cells in the G_2/M phase at 12 h was 40%, compared to 15.3% for the control cell populations. These data imply that griseofulvin might have the potential to arrest Huh7/Rep-Feo cells in the G_2/M phase.

As described earlier, the treatment of Huh7/Rep-Feo cells with 20 μM griseofulvin for 12 h results in G_2/M arrest (Fig. 6), while treatment for 72 h had no effect on cell growth (Fig. 1c). To explain this discrepancy, we examined the growth kinetics of griseofulvin-treated Huh7/Rep-Feo cells. The cells were cultured with 20 μM griseofulvin, and cell growth was monitored by MTS assay. The cell viability declined gradually until 48 h after treatment with 20 μM griseofulvin, but increased from 48 h to 72 h (Fig. 7). These data indicate that treatment with 20 μM griseofulvin arrests Huh7/Rep-Feo cells in the G_2/M phase, but does not inhibit cell growth completely.

Griseofulvin does not inhibit HCV IRES-dependent translation

Previous studies have shown that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization and the cell cycle in G_2/M, inhibit

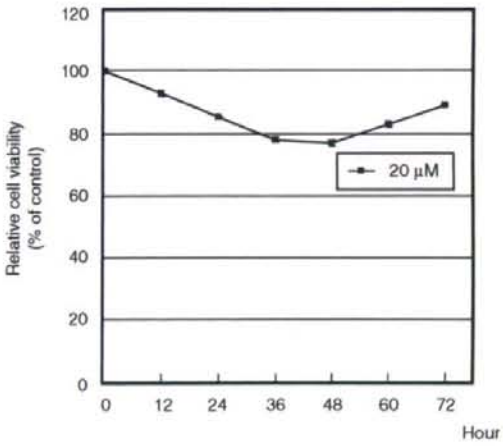

Figure 7 Growth kinetics of griseofulvin treatment of Huh7/Rep-Feo cells. Cells were cultured with [—●—] 20 μM griseofulvin, and cell viability was monitored by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt assay at the times indicated. Error bars indicate mean ± SD.

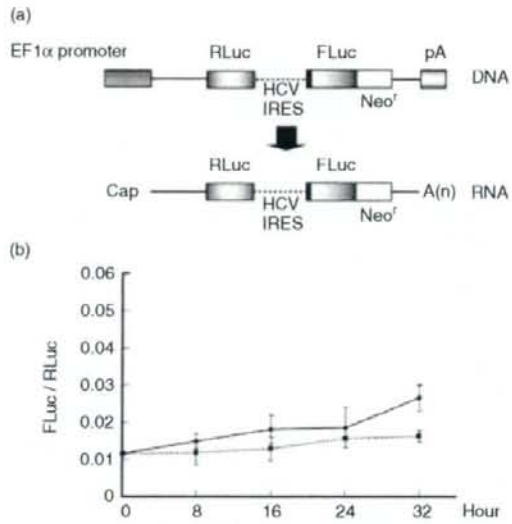

Figure 8 Griseofulvin does not influence hepatitis C virus (HCV) internal ribosomal entry site (IRES)-mediated translation. (a) Structure of the plasmid, pEF-Rluc-HCV IRES Feo. Transcription is initiated under the control of a composite elongation factor 1α (EF1α) promoter. Upstream cistron encodes Renilla luciferase (RLuc) and is translated by a cap-dependent mechanism in transfected cells, while the downstream cistron encodes a fusion (Feo) of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r) genes, translated under the control of the HCV IRES. (b) pEF-Rluc-HCV IRES Feo was stably transfected into Huh7 cells. Cells were treated without (control –◆–) and with 20 μM griseofulvin –■–. Dual luciferase activities were measured at the indicated time points after exposure to griseofulvin. Values are displayed as ratios of Fluc to Rluc. Error bars indicate mean ± SD.

HCV replication, but not HCV IRES-dependent translation.[25] Therefore, we determined whether G_2/M cell cycle arrest by griseofulvin affects HCV IRES-dependent translation using Huh7 cells transfected with pEF Rluc-HCV IRES Feo (Fig. 8a). The treatment of these cells with 20 μM griseofulvin resulted in no significant change of the internal luciferase activities, a concentration that suppressed the expression of the HCV replicon and arrested the HCV replicon cells in the G_2/M phase (Fig. 8b). These results suggested that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation, as shown previously for vinblastine sulfate and nocodazole.

Griseofulvin suppressed JFH-1 HCV replication

The studies described thus far were carried out using the subgenomic HCV-1b replicon system. Recently, Wakita *et al.* established a cell culture model for HCV. This system, known as the JFH-1 system and based on genotype 2a HCV, allows the production of a virus that can be efficiently propagated in cell culture (HCVcc).[10] Therefore, we examined the effect of griseofulvin using the JFH-1 system. The Huh7.5.1/JFH-1 cells (cells persistently infected with HCV JFH-1) were cultured with 10 μM or 20 μM griseofulvin for 72 h. We detected the HCV NS3 protein in Huh7.5.1/JFH-1 HCV cells by immunostaining. As shown in Figure 9, in the absence of griseofulvin treatment, the NS3 protein was localized predominantly in the perinuclear region. After treatment of griseofulvin, the NS3 protein expression level was reduced substantially (Fig. 9). This result indicates that griseofulvin also suppresses HCV replication in the JFH-1 HCVcc system.

DISCUSSION

WE HAVE SHOWN here that griseofulvin inhibits the replication of HCV in the HCV subgenomic replicon cells, Huh7/Rep-Feo. In this reporter-based subgenomic replicon system, the EC_{50} of griseofulvin for the inhibition of HCV replication, determined by measurement of the luciferase activity, was approximately 6.13 μM. The real-time RT–PCR and Western blot analyses revealed that both RNA synthesis and its translation were inhibited by griseofulvin in a dose-dependent manner. The treatment of Huh7/Rep-Feo cells with griseofulvin did not activate the IFN inducible gene responses, suggesting that the inhibitory mechanism of griseofulvin in HCV replication is independent of the IFN signaling pathway. Moreover, we demonstrated that the combination treatment of griseofulvin and IFNα had a synergistic inhibitory effect in Huh7/Rep-Feo cells. We also demonstrated that griseofulvin suppressed replication of JFH-1 HCV.

A previous study demonstrated that griseofulvin induces G_2/M arrest in several human cell lines.[24] Here, we show that griseofulvin arrested the Huh7/Rep-Feo cells in the G_2/M phase. Recently, several studies have shown a correlation between HCV IRES-mediated translation and the cell cycle. Honda *et al.* reported that the HCV IRES activity was highest in the G_2/M phase.[26] In contrast, Venkatesan *et al.* reported that the HCV IRES activity was lowest in the G_2/M,[27] while other studies

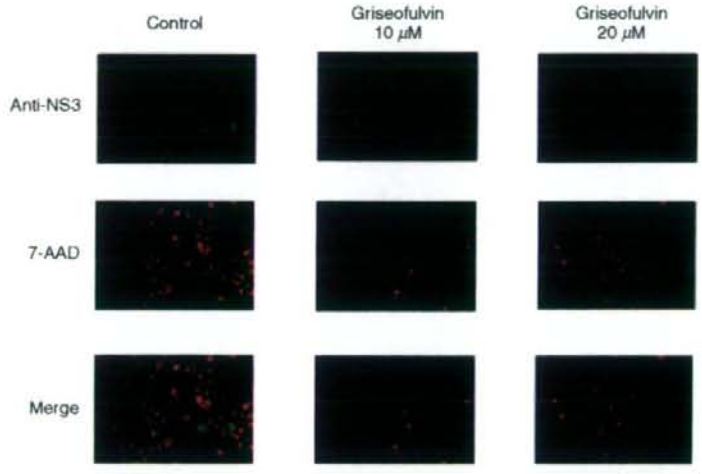

Figure 9 Griseofulvin suppresses JFH-1 replication. Immunofluorescent staining of Huh7.5.1/JFH-1 cells treated with various concentrations of griseofulvin. Hepatitis C virus NS3 protein is stained green and nuclei are stained with 7-aminoactinomycin D (7-AAD; red).

reported that the HCV IRES activity was independent of the stage of the cell cycle.[28,29] In addition, Bost *et al.* reported that several cell cycle inhibitors (vinblastine sulfate, colchicine, nocodazole, and cytochalasin D) did not affect HCV IRES-dependent translation.[25] We also have shown that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation. Accordingly, our results support the hypothesis that the HCV IRES activity is independent of the cell cycle.

Previous studies have demonstrated that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization, are able to inhibit HCV-RNA replication in HCV subgenomic replicon cells.[25] These findings indicate that microtubule polymerization is required for the formation of the HCV replication complex. Griseofulvin has been shown to arrest human cells in the G_2/M phase by acting on microtubule polymerization.[30] Thus it is speculated that the inhibition of microtubule polymerization by griseofulvin may influence the formation of the HCV-RNA replication complex. Further, defining the mechanism of action of griseofulvin against HCV replication may be important for defining a novel target for anti-HCV therapy.

Griseofulvin has been used for many years for the treatment of ringworm and other dermatophyte infections. Moreover, griseofulvin does not have significant toxicity for humans. Consequently, the development of derivatives of this compound may be a useful strategy for future therapeutic intervention in chronic hepatitis C.

ACKNOWLEDGMENTS

WE THANK DR Toshio Kitamura (University of Tokyo, Tokyo, Japan) for kindly providing the pGag-pol-IRES-bsr and pMXs-IN plasmids; Dr Francis V Chisari (The Scripps Research Institute, La Jolla, CA, USA) for kindly providing the Huh7.5.1 cell line; Dr Iyoko Katoh for helpful comments and discussion; and Mr Masashi Osano and Ms Ikuko Kayama (University of Yamanashi, Yamanashi, Japan) for technical assistance. This study was supported by a grant of Health Science from the Ministry of Health, Labor, and Welfare of Japan, and by a grant from the Human Sciences Foundation.

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Erratum

An author would like to draw the reader's attention to some errors in the following article:

Jin H, Yamashita A, Maekawa S, Yang P, He L, Takayanagi S, Wakita T, Sakamoto N, Enomoto N and Ito M. Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication *in vitro*. *Hepatology Research* 2008; **38**: 909–918.

On page 909, the *Methods* and *Results* under the Abstract section should read as follows:

Methods: Using an HCV-1b subgenomic replicon cell culture system, we focused on screening a set of marketed drugs which have not been previously recommended for antiviral use.

Results: We found that griseofulvin, an oral antifungal agent, suppressed HCV RNA replication and protein expression in a dose dependent manner. We also found that griseofulvin suppressed the replication of infectious 2a HCV JFH-1. A combination of IFN-a and griseofulvin exhibited a synergistic inhibitory effect in HCV-1b subgenomic replicon cells. Furthermore, we found that griseofulvin blocked the cell cycle at G2/M in the HCV subgenomic replicon cells, but did not inhibit HCV internal ribosome entry site (IRES)-dependent translation.

Potential Relevance of Cytoplasmic Viral Sensors and Related Regulators Involving Innate Immunity in Antiviral Response

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Background & Aims: **Clinical significance of molecules involving innate immunity in treatment response remains unclear. The aim is to elucidate the mechanisms underlying resistance to antiviral therapy and predictive usefulness of gene quantification in chronic hepatitis C (CH-C).** ***Methods:*** **We conducted a human study in 74 CH-C patients treated with pegylated interferon α-2b and ribavirin and 5 nonviral control patients. Expression of viral sensors, adaptor molecule, related ubiquitin E3-ligase, and modulators were quantified.** ***Results:*** **Hepatic RIG-I, MDA5, LGP2, ISG15, and USP18 in CH-C patients were up-regulated at 2- to 8-fold compared with non-hepatitis C virus patients with a relatively constitutive Cardif. Hepatic RIG-I, MDA5, and LGP2 were significantly up-regulated in nonvirologic responders (NVR) compared with transient (TR) or sustained virologic responders (SVR). Cardif and RNF125 were negatively correlated with RIG-I and significantly suppressed in NVR. Differences among clinical responses in RIG-I/Cardif and RIG-I/RNF125 ratios were conspicuous (NVR/TR/SVR = 1.3:0.6:0.4 and 2.3:1.3:0.8, respectively). Like viral sensors, ISG15 and USP18 were significantly up-regulated in NVR (4-fold and 2.3-fold, respectively). Multivariate and receiver operator characteristic analyses revealed higher RIG-I/Cardif ratio, ISG15, and USP18 predicted NVR. Lower Cardif in NVR was confirmed by its protein level in Western blot. Also, transcriptional responses in peripheral blood mononuclear cells to the therapy were rapid and strong except for Cardif in not only a positive (RIG-I, ISG15, and USP18) but also in a negative regulatory manner (RNF125).** ***Conclusions:*** **NVR may have adopted a different equilibrium in their innate immune response. High RIG-I/Cardif and RIG-I/RNF125 ratios and ISG15 and USP18 are useful in identifying NVR.**

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to cirrhosis and hepatocellular carcinoma in many patients.[1] Although combination therapy with pegylated interferon (PEG-IFN) α and ribavirin is now established as the standard treatment for chronic HCV infection genotype 1b, the sustained virologic response rate in these patients is still around 50%.[2–4] Moreover, physicians have also found that 20% of patients are nonvirologic responders (NVR; those whose HCV-RNA does not become negative during 48 weeks of combination therapy).[5] Prediction of NVR status is of clinical importance because these patients have no chance of achieving a sustained virologic response even after prolonged combination therapy.[6] However, mechanisms involving resistance to PEG-IFN-α and ribavirin have not been fully elucidated, and it is difficult to predict treatment responses before initiation of PEG-IFN-α and ribavirin combination therapy.

In vitro studies have suggested that an innate immune response in viral infection is an essential part of the host antiviral defense system.[7] HCV evades the host immune response through a complex combination of processes that include signaling interference, effector modulation, and continual viral genetic variation.[8] We hypothesized that liver tissue would show a consistent difference between responders and nonresponders in expression levels of the gene involved in innate immunity and IFN signal transduction. These differences could be used to predict treatment outcomes.

The retinoic acid–inducible gene I (RIG-I), a cytoplasmic RNA helicase, and the related melanoma differentia-

Abbreviations used in this paper: CARD, Caspase-recruiting domain; Cardif, caspase-recruiting domain adaptor inducing IFN-β; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; IPS-1, IFN-β promoter stimulator I; ISG15, IFN-stimulated gene 15; PEG-IFN, pegylated interferon; MDA5, melanoma differentiation associated gene 5; MAVS, mitochondrial antiviral signaling protein; NVR, nonvirologic responders; PBMC, peripheral blood mononuclear cell; RIG-I, retinoic acid-inducible gene I; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TR, transient responder; UBP43, ubiquitin-specific protease 43; USP18, ubiquitin-specific protease 18; VISA, virus-induced signaling adaptor.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.02.019

Table 1. Patient Characteristics at Baseline According to Final Virologic Response

	SVR n = 30	TR n = 24	NVR n = 20	*P* value
Age (*y*)	52 ± 13	60 ± 8.7	60 ± 10	.04[a]
Female % (M/F)	47% (16/14)	63% (9/15)	60% (8/12)	.5[b]
Naïve & Relapser[c]/Non-responder[c]	26/4	20/4	14/6	.3[b]
BMI	24.6 ± 3.0	24.9 ± 4.4	24.0 ± 2.1	.6[a]
ALT (*IU/L*)	75 ± 57	65 ± 35	68 ± 41	1.0[a]
Hemoglobin (*g/dL*)	14.3 ± 1.6	14.1 ± 1.1	14.5 ± 1.7	.6[a]
Platelet count ($\times 10^3/\mu L$)	182 ± 62	169 ± 48	140 ± 39	.04[a]
Liver histology				
A1/A2/A3	19/8/3	14/8/1	10/10/0	.3[b]
F1/F2/F3	14/9/7	11/7/5	7/5/8	.7[b]
Viral load ($\times 10^6$ *IU/mL*)	1.6 ± 1.2	1.8 ± 1.1	1.6 ± 1.1	.8[a]
Viral decline rate (log_{10}/day)				
First phase	2.1 ± 0.9	1.5 ± 0.6	0.7 ± 0.5	<.0001[a]
Second phase	0.05 ± 0.05	0.04 ± 0.02	0.006 ± 0.008	<.0001[a]

ALT, alanine aminotransferase; BMI, body mass index.
[a]*P* values were determined by Kruskal–Wallis test.
[b]*P* values were determined by chi-square test.
[c]Response to previous IFN treatment.

tion–associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral dsRNA.[9,10] Caspase-recruiting domain (CARD) adaptor inducing IFN-β (Cardif), also called IFN-β promoter stimulator 1 (IPS-1), mitochondrial antiviral signaling protein (MAVS), and virus-induced signaling adaptor (VISA), is an adaptor molecule. Cardif connects RIG-I sensing to downstream signaling, resulting in IFN-β gene activation.[11–14] On the other hand, RIG-I sensing has been shown to be negatively regulated in a dominant-negative manner by LGP2,[10,15] a helicase related to RIG-I and MDA5 lacking CARD. Interestingly, the ubiquitin ligase ring-finger protein 125 (RNF125) has been recently shown to conjugate ubiquitin to RIG-I, MDA5 as well as Cardif, which results in suppressing the functions of these proteins.[16] Furthermore, these molecules are conjugated (ISGylated) by IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[17] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18), also called ubiquitin-specific protease 43 (UBP43).[18,19] Moreover, the NS3/4A protease of HCV specifically cleaves Cardif as part of its immune evasion strategy.[11,20] Therefore, the RIG-I/Cardif system and its regulatory systems have essential key functions in the innate antiviral response (see Supplementary Figure 1 online at www.gastrojournal.org). However, the clinical significance of these innate immune systems, especially in relevance to the treatment response, is unclear because findings in this field have been mainly obtained by in vitro experiments using cell lines.

The aims of this study were to elucidate the mechanisms underlying resistance to antiviral therapy in the clinical setting and to determine whether quantification of transcripts of positive and negative cytoplasmic viral sensors and related regulatory molecules involving innate immune system is useful in predicting responses to PEG-IFN-α and ribavirin combination therapy.

Patients and Methods

Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital, 74 patients of HCV genotype 1b with a high viral load (>100,000 IU/mL by Amplicor-HCV Monitor Assay; Roche Molecular Diagnostics Co, Tokyo, Japan) were included in the present study (Table 1). Patients with cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient was positive for hepatitis B virus–associated antigen/antibody or anti–human immunodeficiency virus antibody. No patient received immunomodulatory therapy prior to the enrollment. Written informed consent was obtained from all the patients, and this study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration. Five patients with nonviral liver disease (2 had autoimmune hepatitis and 3 had primary biliary cirrhosis) were included in the present study as controls.

Treatment Protocol

The patients were treated for 48 weeks with subcutaneous injections of PEG-IFN-α-2b (PegIntron; Schering-Plough Corporation, Kenilworth, NJ) at a dose of 1.5 $\mu g \cdot kg^{-1} \cdot week^{-1}$. Ribavirin (Rebetol; Schering-Plough Corporation) was administered concomitantly over the 48-week period, given orally twice daily at a total daily dose of 600 mg for the patients who weighed less than 60 kg and 800 mg for the patients who weighed between 60 and 80 kg. The dose of PEG-IFN-α-2b was reduced to 0.75 $\mu g \cdot kg^{-1} \cdot week^{-1}$ when either the neutrophil count was $<750/mm^3$ or the platelet count was $<80 \times 10^3/mm^3$. The dose of ribavirin was reduced to 600 mg/day when the hemoglobin concentration decreased to <10 g/dL.

Measurement of Gene Expression in the Liver

Liver biopsy was performed immediately before starting the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of positive and negative cytoplasmic viral sensors (RIG-I, MDA5, and LGP2), the adaptor molecule (Cardif), related ubiquitin E3-ligase (RNF125), and the modulators of these molecules (ISG15 and USP18) was quantified by real-time quantitative polymerase chain reaction (PCR) using primers specific for target genes. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen (Nippon Gene Co Ltd, Toyama, Japan) from the liver biopsy specimen, which was 0.2–0.4 cm in length and 13 gauge in diameter. Complementary DNA (cDNA) was transcribed from 2 μg total RNA template in a 140-μL reaction mixture using a SYBR RT-PCR Kit (Takara Bio Co Ltd, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio Co Ltd) with the SYBR RT-PCR Kit (Takara Bio Co Ltd) according to the manufacturer's instructions, and intercalating SYBR Green I (Molecular Probes Inc, Eugene, Oregon) was detected. Assays were performed in duplicate, and the expression levels of target genes were normalized to expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene and hydroxymethylbilane synthase, which is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of 2 housekeeping genes was used in the present study. Sequences of primer sets were as follows: RIG-I: 5′-AAAGCATGCATGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTCACTGATAA-3′; MDA5: 5′-ACATAACAGCAACATGGGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGGAG-3′; LGP2: 5′-ACAGCCTTGCAAACAGTACAACCTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; Cardif: 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGCACGCCAGGCTTACTCA-3′; RNF125: 5′-AGGGCACATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAACGGCAAAGTGG-3′; ISG15: 5′-AGCGAACTCATCTTTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACATGGA-3′; USP18: 5′-TGGTTCTGCTTCAATGACTCCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACTC-3′; GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′, 5′-TGGTGGTGAAGACGCCAGT-3′. hydroxymethylbilane synthase: 5′-AAGCGGAGCCATGTCTGGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Sequential Measurement of Gene Expression in Peripheral Blood Mononuclear Cells Before and During Therapy

To understand transcriptional response of the genes to PEG-IFN-α-2b and ribavirin therapy, serial expression of RIG-I, RNF125, Cardif, ISG15, and USP18 were determined before and during treatment in peripheral blood mononuclear cells (PBMC) in 14 patients (7 were sustained viral responders [SVR] and 7 were NVR). PBMC was obtained from whole blood samples collected before and at 4, 8, 24, 48, and 168 hours after the initiation of PEG-IFN-α-2b and ribavirin combination therapy. After extraction of total RNA from the PBMC, the expression of mRNA was quantified at each specified time point using real-time quantitative PCR as described above. Gene expression levels at each time point during treatment were calculated relative to baseline expression levels measured prior to IFN treatment.

Western Blotting

Western blotting was carried out in 9 patients (5 were SVR and 4 were NVR) and 3 non-HCV control subjects as described previously.[21] Liver biopsy specimen of ~10 mg was homogenized in 100 μL Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Twenty micrograms of the homogenates were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride Western blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, United Kingdom). The anti-VISA mouse monoclonal antibody (BioDesign, Saco, ME) and anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO) were used.

HCV Dynamics in Serum

To analyze the viral dynamics, HCV RNA was quantified just before and at 4, 8, and 24 hours and 2, 7, 14, 28, 56, and 84 days after the initiation of PEG-IFN-α-2b and ribavirin combination therapy, using real-time detection PCR, as reported previously.[22] For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase with a straight-line fit of the data.

Definitions of Response to Therapy

A patient negative for serum HCV RNA during the first 6 months after the completion of PEG-IFN-α-2b and ribavirin combination therapy was defined as an SVR, and a patient for whom HCV RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient responder (TR). A patient who was positive for HCV RNA even during the course of therapy was defined as an NVR. HCV RNA was determined with the Amplicor qualitative assay (Roche Molecular Diagnostics Co, Tokyo, Japan). The detection sensitivity of this assay is approximately 50 IU/mL.

Statistical Analysis

Categorical data were compared by the χ^2 test and Fisher exact test. Distributions of continuous variables were analyzed by Mann–Whitney U test for 2 groups. Kruskal–Wallis test was used for multiple group comparisons. All tests of significance were 2-tailed, and P values $< .05$ were considered statistically significant.

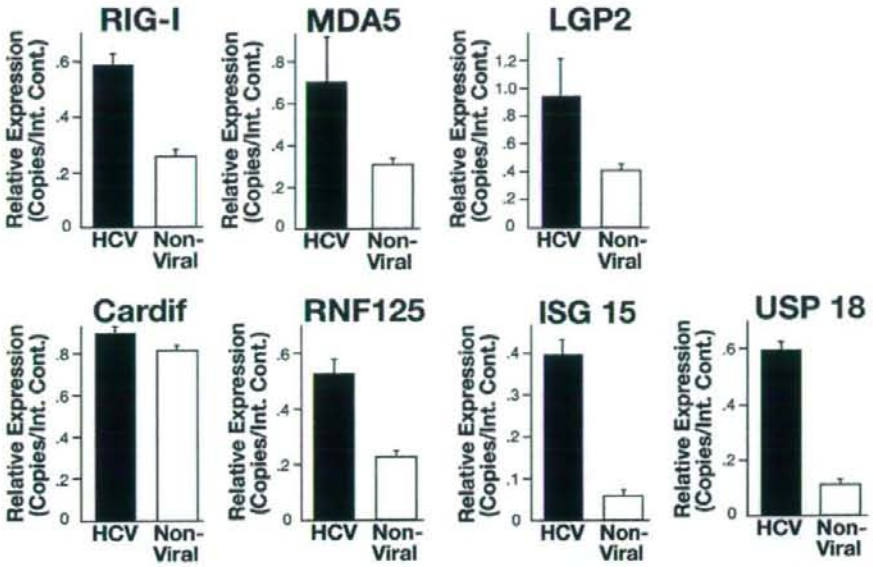

Figure 1. Comparison of hepatic gene expression levels between chronic hepatitis C patients (n = 74) and nonviral liver disease patients (n = 5). Expression levels of RIG-I, MDA5, LGP2, Cardif, RNF125, ISG15, and USP18 are shown. *Error bars* indicate the standard error. Upon HCV infection, expression of these genes except Cardif was stimulated. The *P* values determined by Mann–Whitney *U* test between 2 groups were as follows: RIG-I, *P* .02; MDA5, *P* .01; LGP2, *P* .005; Cardif, *P* .7; RNF125, *P* .06; ISG15, *P* .007; USP18, *P* .004.

Results

Patient Characteristics

According to the final virologic response, patients were classified into 3 groups: 30 were SVR, 24 were TR, and the remaining 20 were NVR, as shown in Table 1. Viral decline rates in NVR were significantly lower in both the first and second phases of HCV dynamics. It should be noted that most NVR patients exhibited no second-phase viral decline.

Data on factors that were available before starting the treatment were compared according to virologic response by univariate analysis. As shown in Table 1, only age and platelet count were associated with viral response, and no other clinical factors were predictive of NVR before initiation of the therapy.

Gene Expression Involving Innate Immunity in the Liver

First, we compared basal hepatic gene expression between the chronic hepatitis C patients (n = 74) and the nonviral liver disease patients (n = 5). As shown in Figure 1, levels of RIG-I, MDA5, LGP2, ISG15, and USP18 expression were significantly higher in the chronic hepatitis C patients than in the nonviral liver disease patients. However, there was no significant difference in levels of Cardif expression between the chronic hepatitis C and nonviral-related liver disease patients.

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, levels of gene expression were compared based on the final virologic response. As shown in Figure 2, the hepatic expression levels of RIG-I, MDA5, and LGP2 were significantly higher in NVR than in SVR and TR. In marked contrast, hepatic Cardif expression was significantly lower in the NVR group. The hepatic expression of RNF125, which is specific E3-ubiquitin ligase for RIG-I, MDA5, and Cardif, was also significantly lower in the NVR group. Because negative correlation was found between RIG-I and Cardif or RNF125 expression, we calculated the ratio of RIG-I to Cardif or RNF125 expression levels. As shown in Figure 2, the difference among the groups was conspicuous when comparison was made with the RIG-I/Cardif ratio or RIG-I/RNF125 ratio. Moreover, the RIG-I/Cardif expression ratio before treatment was negatively and significantly correlated with the exponential viral decline rate in both the first and the second phases of HCV dynamics (first phase, $r = -0.4$, $P < .0005$; second phase, $r = -0.5$, $P < .0001$). Similar correlation was found between RIG-I/RNF125 ratio and viral decline rate (first phase, $r = -0.4$, $P = .004$; second phase, $r = -0.2$, $P = .09$, data not shown).

Like RIG-I and MDA5, intrahepatic expression levels of ISG15 and USP18 were significantly higher in NVR than in SVR and TR (Figure 2). When we assessed the correlation of these 2 genes in individual patients, we found a strong and significant correlation between ISG15 and USP18 ($r^2 = 0.88$, $P < .0001$). Levels of ISG15 and USP18 expression before treatment were negatively correlated with the exponential viral decline rates calculated from

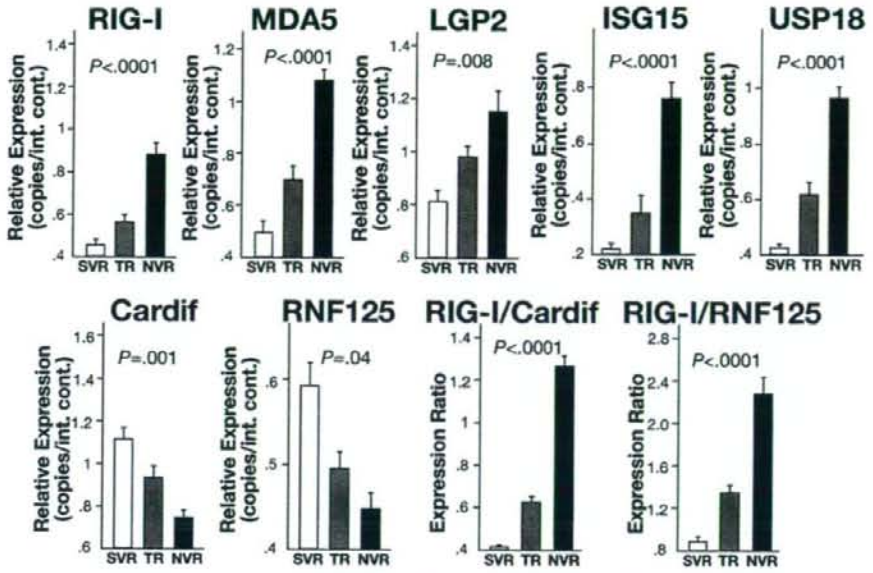

Figure 2. Comparison of hepatic gene expression levels according to final virologic outcome. Expression levels of RIG-I, MDA5, LGP2, ISG15, USP18, Cardif, RNF125, RIG-I/Cardif ratio, and RIG-I/RNF125 ratio are shown. *Open columns* indicate SVR (n = 30), *shaded columns* indicate TR (n = 24), and *solid columns* indicate NVR (n = 20). *Error bars* indicate the standard error. The *P* values were analyzed by the Kruskal–Wallis test.

the first and the second phases of HCV dynamics (ISG15, first phase, $r = -0.5$, $P < .0001$; ISG15, second phase, $r = -0.3$, $P = .02$; USP18, first phase, $r = -0.5$, $P < .0001$; USP18, second phase, $r = -0.3$, $P = .01$).

Receiver Operator Characteristic Analysis

To determine the usefulness of these gene quantifications as predictors, receiver operator characteristic (ROC) analysis was conducted (Figure 3). The area under the ROC curve for the RIG-I/Cardif ratio, ISG15, and USP18 was 0.91, 0.90, and 0.91, respectively, suggesting that quantification of these gene transcripts is of use for the prediction of NVR (Table 2). In addition, this analysis also suggested that RIG-I/Cardif ratio would be more specific for prediction of NVR, whereas ISG15 and USP18 would be more sensitive (Table 2).

Multivariate Analysis

Multivariate analysis for factors that were available before initiating therapy indicated that a higher ratio of RIG-I/Cardif and higher expression of ISG15 were independent factors that were associated with NVR (Table 3). In this analysis, USP18 was excluded because of its strong correlation with ISG15.

Protein Levels of Cardif in the Liver

Because hepatic expression of Cardif mRNA was significantly lower in NVR patients than in SVR patients,

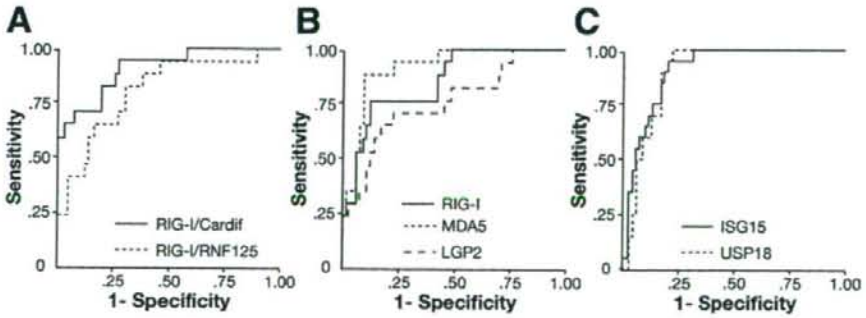

Figure 3. Receiver operator characteristic (ROC) curve for prediction of nonvirologic response. ROC curves were generated to compare (*A*) RIG-I/Cardif ratio (*solid line*) and RIG-I/RNF125 ratio (*shaded line*); (*B*) RIG-I (*solid line*), MDA5 (*shaded line*), and LGP2 (*dotted line*); and (*C*) ISG15 (*solid line*) and USP18 (*shaded line*).