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A Novel Cyclohexene Derivative, Ethyl (6*R*)-6-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), Selectively Inhibits Toll-Like Receptor 4-Mediated Cytokine Production through Suppression of Intracellular Signaling

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ABSTRACT

Proinflammatory mediators such as cytokines and NO play pivotal roles in various inflammatory diseases. To combat inflammatory diseases successfully, regulation of proinflammatory mediator production would be a critical process. In the present study, we investigated the in vitro effects of ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), a novel small molecule cytokine production inhibitor, and its mechanism of action. In RAW264.7 cells and mouse peritoneal macrophages, TAK-242 suppressed lipopolysaccharide (LPS)-induced production of NO, tumor necrosis factor-α (TNF-α), and interleukin (IL)-6, with 50% inhibitory concentration (IC_{50}) of 1.1 to 11 nM. TAK-242 also suppressed the production of these cytokines from LPS-stimulated human peripheral blood mononuclear cells (PBMCs) at IC_{50} values from 11 to 33 nM. In addition, the inhibitory effects on the LPS-induced IL-6 and IL-12 production were similar in human PBMCs, monocytes, and macrophages. TAK-242 inhibited mRNA expression of IL-6 and TNF-α induced by LPS and interferon-γ in RAW264.7 cells. The phosphorylation of mitogen-activated protein kinases induced by LPS was also inhibited in a concentration-dependent manner. However, TAK-242 did not antagonize the binding of LPS to the cells. It is noteworthy that TAK-242 suppressed the cytokine production induced by Toll-like receptor (TLR) 4 ligands, but not by ligands for TLR2, -3, and -9. In addition, IL-1β-induced IL-8 production from human PBMCs was not markedly affected by TAK-242. These data suggest that TAK-242 suppresses the production of multiple cytokines by selectively inhibiting TLR4 intracellular signaling. Finally, TAK-242 is a novel small molecule TLR4 signaling inhibitor and could be a promising therapeutic agent for inflammatory diseases, whose pathogenesis involves TLR4.

Cytokines and NO are involved in a variety of inflammatory diseases, including sepsis, rheumatoid arthritis (RA), atherosclerosis, inflammatory bowel disease (IBD), asthma, and chronic obstructive pulmonary disease. In RA, for example, interleukin (IL)-1 is considered to be a mediator. Plasma concentrations of IL-1 in patients with RA are elevated, and they correlate with disease activity (Eastgate et al., 1988). The expression of IL-1 in the bronchial epithelium of patients

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ABBREVIATIONS: RA, rheumatoid arthritis; IBD, inflammatory bowel disease; IL, interleukin; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric-oxide synthase; IFNγ interferon-γ; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TAK-242, ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate; LTA, lipoteichoic acid; GM-CSF, granulocyte macrophage–colony stimulating factor; PGN, peptidoglycan; poly(I:C), polyinosinic-polycytidylic acid; CpG DNA, nonmethylated CpG oligodeoxynucleotide; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cell; BSA, bovine serum albumin; PCR, polymerase chain reaction; TBS-T, Tris-buffered saline/Tween 20; Ab, antibody; JNK/SAPK, c-Jun NH_2-terminal kinase/stress-activated protein kinase; MAb, monoclonal antibody; CCR5, CC-chemokine receptor 5; Erk, extracellular signal-regulated kinase; LPS(S), lipopolysaccharide from *S. typhimurium*; TIR, Toll/IL-1 receptor; TICAM, Toll/IL-1 receptor domain-containing adaptor molecule-1; tyrphostin AG126, (3-hydroxy-4-nitrobenzylidene)malononitrilella-cyano-(3-hydroxy-4-nitro)cinnamonitrile.

with asthma is significantly elevated compared with healthy volunteers (Sausa et al., 1996). In some animal models of sepsis, hemodynamics and survival are improved when the actions of IL-1 are blocked by an IL-1 receptor antagonist (Fischer et al., 1992; Norman et al., 1995). Tumor necrosis factor-α (TNF-α) is also known to exhibit diverse physiologic effects and is one of the most prominent proinflammatory mediators. It can exert host-damaging effects in sepsis, fever syndromes, and cachexia as well as in autoimmune diseases such as RA, psoriasis, and IBD (Raza, 2000; Hehlgans and Pfeffer, 2005). For example, TNF-α induces the secretion of inflammatory cytokines and chemokines from stroma cells, endothelial cells, and mucosal mononuclear cells in IBD (MacDermott, 1996). NO derived from inducible NO synthase (iNOS) also seems to be a proinflammatory mediator with immunomodulatory effects (Guzik et al., 2003). The toxic properties of NO are the key in the pathogenesis of septic shock, and overproduction of NO during septicemia is considered to be responsible for irreversible arterial hypotension, vasoplegia, lactic acidosis, necrosis, and apoptosis (Parratt, 1997). Furthermore, the manifestations of allergic airway disease, including infiltration of eosinophils, microvascular leakage, and airway occlusion are markedly less severe in $iNOS^{-/-}$ animals, and iNOS promotes allergic inflammation in airways via down-regulation of interferon-γ (IFN-γ) (Ricciardolo et al., 2004).

Lipopolysaccharide (LPS), a major constituent of Gram-negative bacterial outer membrane, can cause inflammatory responses such as the release of cytokines and NO from various types of cells, including monocytes and macrophages. To activate the cells, the lipid A moiety of LPS attaches to the LPS-binding protein (LBP), and the LPS/LBP complex binds to CD14 and is then transferred to the Toll-like receptor (TLR) 4-MD-2 complex (Wright et al., 1990; da Silva Correia et al., 2001). The resulting activation of the cells induces the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) as well as release of inflammatory mediators (Guha and Mackman, 2001). TLR4 is also known to be a receptor for endogenous ligands such as fibrinogen, hyaluronic acid, and heat shock proteins (Rifkin et al., 2005). Thus, an agent that inhibits TLR4-mediated cytokine and NO production could be a promising drug for the treatment of inflammatory diseases. In fact, some synthetic lipid A analogs showed beneficial effects as TLR4 antagonists in septic shock models and IBD models in mice (Christ et al., 1995; Mullarkey et al., 2003; Fort et al., 2005).

We have discovered a novel cyclohexene derivative, TAK-242, which selectively inhibits the TLR4-mediated production of cytokines and NO. The chemical structure of TAK-242 is shown in Fig. 1. TAK-242 is the first small-molecule compound that selectively inhibits TLR4 signaling. In this study, we investigated the inhibitory effect of TAK-242 on the production of inflammatory mediators by macrophages and monocytes as well as its mode of action.

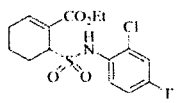

Fig. 1. Structure of TAK-242.

Materials and Methods

Materials. TAK-242 was synthesized at Takeda Pharmaceutical Company Limited (Osaka, Japan). AG126 was purchased from Calbiochem (San Diego, CA), LPS (from *Escherichia coli* serotype O111: B4) and lipoteichoic acid (LTA) (from *Staphylococcus aureus*) were from Sigma-Aldrich (St. Louis, MO), and LPS(S) (from *Salmonella typhimurium*) was from Difco (Detroit, MI). Recombinant mouse IFN-γ was purchased from Genzyme (Cambridge, MA), whereas recombinant human granulocyte macrophage–colony stimulating factor (GM-CSF) was purchased from PeproTech EC (London, UK). Peptidoglycan from *S. aureus* (PGN) was purchased from Fluka (Buchs, Switzerland); polyinosinic-polycytidylic acid [poly(I:C)] was from Amersham Biosciences Inc. (Piscataway, NJ); paclitaxel (Taxol) and recombinant human IL-1β were from Wako Pure Chemicals (Osaka, Japan), nonmethylated CpG oligodeoxynucleotide (CpG DNA) was from Hokkaido System Science (Sapporo, Japan), and 2,3-diaminonaphthalene, which an agent for the detection of nitrite (Misko et al., 1993), was from Dojindo Laboratories (Kumamoto, Japan).

Cells. The murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium (Nikken Bio Medical Laboratories, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (FCS) and 10 μg/ml kanamycin at 37°C under a humidified atmosphere with 5% CO_2. Resident mouse peritoneal macrophages were harvested from BALB/c mice (Charles River Japan, Kanagawa, Japan). The peritoneal cells were seeded at a density of 5×10^5 cells/well in 96-well culture plates (Nalge Nunc International, Naperville, IL) and cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin for 2 h. After shaking the cultures, nonadhesive cells were aspirated. The adhesive cells were washed and used as peritoneal macrophages. Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood obtained from healthy human volunteers by density gradient centrifugation using Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and suspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. To obtain monocytes, PBMCs were washed and suspended in phosphate-buffered saline containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The cells were then treated with microbeads coated with anti-CD14 monoclonal antibody (Miltenyi Biotec, Gladbach, Germany) and subjected to a magnetic cell separation system according to the manufacturer's protocol. To differentiate the monocytes into macrophages, monocytes obtained using the above-mentioned procedure were plated in 96-well culture plates (Corning Glassworks, Corning, NY) at a density of 1×10^4 cells/well and cultured for 7 days in RPMI 1640 medium supplemented with 10 ng/ml GM-CSF and 10% FCS at 37°C under a humidified atmosphere with 5% CO_2.

Treatments of the Cells. RAW264.7 cells were plated at a density of 1×10^5 cells/well in 96-well culture plates and incubated overnight. After removing cell culture supernatants, the cells were stimulated with various concentrations of TLR ligands in the presence or absence of IFN-γ for 20 h in a stimulation medium (phenol red-free RPMI 1640 medium containing 1% heat-inactivated FCS and 10 μg/ml kanamycin). Resident mouse peritoneal macrophages were stimulated with 1 ng/ml LPS and 1 U/ml IFN-γ in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin for 4 h (for TNF-α and IL-6 assay) or 20 h (for IL-1β and NO assay). For PBMC assay, PBMCs were seeded at a density of 8×10^4 cells/well in 96-well culture plates and stimulated with 1 ng/ml LPS and 1 U/ml IFN-γ for 20 h. To examine the efficacy on IL-8 production induced by LPS and IL-1β, PBMCs were stimulated with 1 ng/ml LPS or 10 ng/ml IL-1β for 20 h. For the comparison of the efficacy on PBMCs, monocytes and macrophages, the cells were stimulated with 10 ng/ml LPS in the presence of TAK-242 for 18 h. In another experiment, human macrophages were

stimulated with 10 ng/ml LPS or 20 μg/ml PGN for 18 h. For all the experiments, TAK-242 was dissolved in *N,N*-dimethylformamide, diluted with appropriate medium, and added to the cells just before the stimulation.

Measurement of the Concentrations of Nitrite and Cytokines in the Culture Supernatants. Using 2,3-diaminonaphthalene, the production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, by a fluorometric method (Misko et al., 1993). The concentration of TNF-α, IL-6, IL-1β, IL-8, and IL-12 in the culture supernatants were determined by specific enzyme-linked immunosorbent assay [Amersham Pharmacia Biotech UK (Little Chalfont, Buckinghamshire, UK), R&D Systems (Minneapolis, MN), or Genzyme Techne (Minneapolis, MN)]. Fifty percent inhibitory concentration (IC_{50}) values of TAK-242 were calculated by least-squares linear regression analysis over the descending linear portion of the log dose-response curve.

Real-Time Quantitative Polymerase Chain Reaction Analysis of TNF-α and IL-6 Expression. RAW264.7 cells were seeded at a density of 3×10^6 cells/well in six-well culture plate (BD Biosciences, Bedford, MA) and incubated overnight. After washing with RPMI 1640 medium supplemented with 1% FCS and 10 μg/ml kanamycin, the cells were stimulated with 5 ng/ml LPS and 1 U/ml IFN-γ in the presence or absence of TAK-242 (1–100 nM) for the indicated time. Culture supernatants were removed, and total RNA was isolated using the total RNA isolation reagent ISOGEN (Nippon Gene, Tokyo, Japan). Total RNA was reverse transcribed into cDNA by using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis of TNF-α and IL-6 was performed on ABI Prism 7700 (Applied Biosystems) using predeveloped TaqMan assay reagents and Universal PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Quantitation of mRNA was performed using the comparative threshold cycle method. The highest control level attained by the stimulation (without TAK-242) was regarded as 100%, and the levels of control group at other time points and TAK-242-added group were expressed as the percentage of the highest control level.

Western Blot Analysis. RAW264.7 cells were plated at a density of 5×10^5 cells/well in 24-well culture plates and incubated overnight. TAK-242 or tyrphostin AG126, a tyrosine kinase inhibitor, was added to the cells and incubated for 15 min before 30-min stimulation with LPS. After the removal of cell culture supernatants, the cells were incubated in lysis buffer [25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 30 mM NaF, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 mM (4-amidino-phenyl)-methylsulfonyl fluoride] on ice for 10 min. The cell lysates were centrifuged at 15,000 rpm (Himac CF15D2; Hitachi, Ibaragi, Japan) for 10 min. The resultant supernatant was mixed with 1/4 volume of 5× SDS sample buffer (312.5 mM Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, 25% 2-mercaptoethanol, and 0.1% bromphenol blue). The proteins in the lysates were separated by SDS-polyacrylamide gel electrophoresis (10.5% gel), and blotted onto polyvinylidene difluoride Immunobilon membranes (Millipore, Molsheim, France). After blocking the membrane in TBS-T (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 3% bovine serum albumin, membranes were washed in TBS-T and probed for 1 h with anti-phospho-p44/42 MAPK antibody (Ab) (New England Biolabs, Beverly, MA), anti-phospho-p38 MAPK Ab (New England Biolabs), anti-IκBβ (C-20) Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) Ab (New England Biolabs), or anti-$p67^{phox}$ Ab (BD Biosciences, San Jose, CA). The membranes were washed four times in TBS-T and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG Fc(ab) (Cappel, Aurora, OH). After washing the membranes four times in TBS-T, the bands were detected using enhanced luminol reagent (New England Biolabs) according to the manufacturer's instruction.

Assay for LPS Binding to PBMCs. PBMCs were suspended in BSA solution (phosphate-buffered saline containing 0.1% BSA and 0.01% sodium azide). In a total volume of 50 μl, PBMCs (3×10^5 cells) were incubated with TAK-242, anti-human CD14 monoclonal antibody (MAb) MEM-18 (Monosan, Uden, The Netherlands), or anti-human CC-chemokine receptor 5 (CCR5) MAb (2D7; BD Biosciences PharMingen, San Diego, CA) as a negative control for 30 min at 4°C. The cells were further incubated with 50 ng/ml LPS from *E. coli* serotype O55:B5 conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) per milliliter in the presence of human serum at a final concentration of 1% for 45 min at 37°C. After washing twice with BSA solution, 1×10^4 cells were analyzed by flow cytometry using CytoACE300 cytofluorometer (Jasco, Tokyo, Japan). The assays were performed in triplicate for each preparation of PBMCs obtained from four different donors. Specific LPS binding was estimated by subtracting the percentage of LPS-binding cells in the absence of LPS from that in the presence of LPS.

Results

Inhibitory Effect of TAK-242 on the Production of Inflammatory Cytokines and Nitric Oxide from LPS-Stimulated Monocytes, Macrophages, and PBMCs. Resident mouse peritoneal macrophages were stimulated with 1 ng/ml LPS and 1 U/ml IFN-γ in the presence of various concentrations of TAK-242, and the amounts of nitrite (a stable metabolite of NO), TNF-α, IL-6, and IL-1β produced in the supernatants were measured. TAK-242 inhibited the production of these proinflammatory mediators in a concentration-dependent manner, with IC_{50} values ranging from 5.7 to 11 nM (Table 1, Fig. 2). TAK-242 also suppressed the production of NO, TNF-α, and IL-6 from RAW264.7 cells stimulated with 5 ng/ml LPS and 1 U/ml IFN-γ with IC_{50} values ranging from 1.1 to 3.9 nM. In addition, TAK-242 showed similar suppressive effects on the proinflammatory mediator production when RAW264.7 cells were stimulated with a high concentration (1 μg/ml) of LPS alone. NO production from RAW264.7 cells induced by IFN-γ alone was partially suppressed only by more than several hundreds times higher concentrations of TAK-242 compared with those for sup-

TABLE 1
IC_{50} values of TAK-242 for NO, TNF-α, IL-6, and IL-1β production by mouse macrophages

Cell Type and Stimuli	IC_{50} (95% Confidence Interval)			
	NO	TNF-α	IL-6	IL-1β
		nM		
Peritoneal macrophages				
1 ng/ml LPS and 1 U/ml IFN-γ	7.7 (3.6–34)	8.7 (5.2–16)	11 (8.2–16)	5.7 (4.2–8.1)
RAW264.7				
5 ng/ml LPS and 1 U/ml IFN-γ	3.8 (2.7–5.3)	3.9 (2.5–7.6)	1.1 (0.79–1.6)	N.D.
1 μg/ml LPS	3.9 (1.1–9.8)	4.6 (1.9–10)	3.7 (2.1–6.0)	N.D.

N.D., not determined.

pressing the LPS-induced activation (data not shown). TAK-242 did not show cytotoxicity at a concentration of 10 μM by using the thiazolyl blue tetrazolium bromide method (data not shown).

TAK-242 was also effective in human cells and inhibited the production of TNF-α, IL-6, and IL-1β from PBMCs stimulated with 1 ng/ml LPS and 1 U/ml IFN-γ, with IC_{50} values of TAK-242 ranging from 5.3 to 58 nM (Table 2; Fig. 3). No marked difference in the IC_{50} values of TAK-242 was observed among PBMCs derived from four different donors. The efficacy of TAK-242 in human PBMCs was similar to but slightly lower than that in the resident mouse peritoneal macrophages under the same stimulation condition. As shown in Table 3, TAK-242 also inhibited the LPS-induced IL-12 production, with IC_{50} values similar to those for IL-6. Furthermore, it should be noted that TAK-242 showed similar effects on human PBMCs, monocytes, and GM-CSF-differentiated macrophages. These results suggest that TAK-242 could show suppressive effects on the production of various inflammatory mediators from both mouse and human monocytes and macrophages stimulated with LPS.

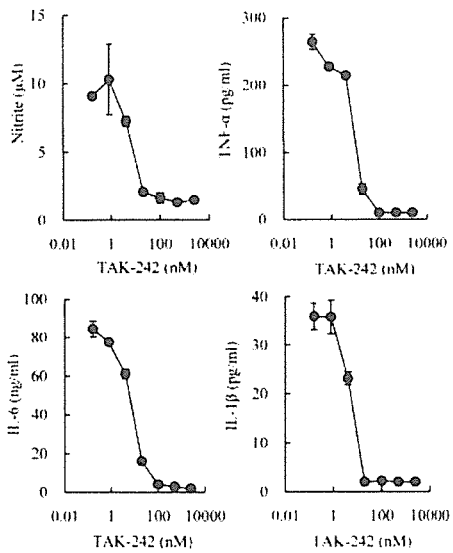

Fig. 2. Inhibitory effect of TAK-242 on NO and cytokine production from mouse peritoneal macrophages. The cells were stimulated with LPS (1 ng/ml) and IFN-γ (1 U/ml) in the presence or absence of TAK-242, and the levels of nitrite and cytokines in culture supernatants were measured as described under *Materials and Methods*. The data are expressed as mean ± S.E. (n = 3).

TABLE 2

IC_{50} values of TAK-242 for inhibition of IL-1β, TNF-α, and IL-6 production by human PBMCs

The 95% confidence intervals of the IC_{50} values have not been shown because the variance of the duplicate data in low concentrations of TAK-242 was too large for precise estimation.

Donor	IC_{50}		
	IL-1β	TNF-α	IL-6
	nM		
A	10	6.0	28
B	21	11	37
C	26	20	11
D	14	5.3	58
Mean ± S.E.	18 ± 3.5	11 ± 3.3	33 ± 9.8

Inhibitory Effect on mRNA Expression in RAW264.7 Cells. To determine whether the suppressive effect of TAK-242 on the cytokine production occurs at mRNA expression level, we used quantitative real-time PCR to examine IL-6 and TNF-α mRNA expressions in RAW264.7 cells stimulated with LPS and IFN-γ. As shown in Fig. 4, IL-6 mRNA expression was detected at 2 h after the stimulation, and the level of expression increased thereafter. On the other hand, TNF-α mRNA expression increased rapidly and reached a maximum level at 1 h after the stimulation with LPS and IFN-γ. These increases in TNF-α and IL-6 mRNA expression levels were clearly suppressed by TAK-242 at concentrations of 10 to 100 nM (Fig. 4), indicating that TAK-242 suppresses the production of cytokines by inhibiting the mRNA expression.

Inhibitory Effect on the LPS-Induced Activation of MAPK Cascades and IκB Degradation in RAW264.7 Cells. LPS activates various intracellular signaling cascades such as MAPK pathway and NF-κB pathway in monocytes and macrophages, which are required for the induction of many cytokines (Guha and Mackman, 2001). Therefore, we next examined the effect of TAK-242 on the LPS-induced phosphorylation of MAPKs and IκB degradation in RAW264.7 cells. TAK-242 markedly inhibited the LPS-in-

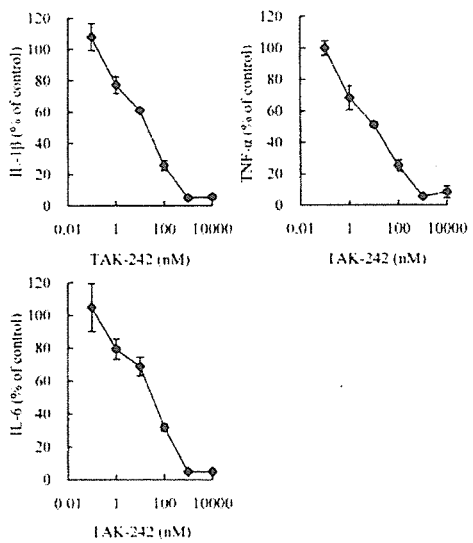

Fig. 3. Inhibitory effect of TAK-242 on IL-1β, TNF-α, and IL-6 production from human PBMCs. The cells were stimulated with LPS (1 ng/ml) and IFN-γ (1 U/ml) in the presence or absence of TAK-242, and the levels of nitrite and cytokines in culture supernatants were measured as described under *Materials and Methods*. The data are expressed as mean ± S.E. of percentage of inhibition for PBMCs prepared from four different donors. The levels of IL-1β, TNF-α, and IL-6 produced by PBMCs of each donor were 12 to 38, 34 to 120, and 450 to 740 pg/ml, respectively.

TABLE 3

IC_{50} values of TAK-242 for inhibition of IL-6 and IL-12 production by human PBMCs, monocytes, and macrophages

Cell Type	IC_{50} (95% Confidence Interval)	
	IL-6	IL-12
	nM	
PBMCs	330[a]	98 (62–160)
Monocytes	130 (71–270)	56 (22–100)
Macrophages	76 (37–190)	32 (23–47)

[a] The 95% confidence interval of the IC_{50} value has not been shown because the variance of duplicate data in low concentrations of TAK-242 was too large for precise estimation.

duced phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2), p38, and JNK/SAPK as well as degradation of IκBβ at a concentration of 100 nM (Fig. 5). Tyrosine kinase inhibitor AG126 also inhibited the LPS-induced phosphorylation of Erk1/2 and JNK/SAPK however, it did not inhibit p38 phosphorylation.

Effect of TAK-242 on LPS Binding to PBMCs. The results described above suggest that TAK-242 might target an upstream event in LPS signaling or inhibit LPS binding to the cells. It is known that LPS binds to CD14/TLR4/MD-2 complex on host cells such as monocytes and macrophages (Wright et al., 1990; da Silva Correia et al., 2001; Guha and Mackman, 2001). We conducted experiments to evaluate the effect of TAK-242 on LPS binding to the cells. Human PBMCs were used in this experiment to use a neutralizing anti-human CD14 MAb as a positive control. The cells were incubated with fluorescein-conjugated LPS, and the LPS binding was analyzed by flow cytometry. Preincubation of PBMCs with anti-CD14 MAb resulted in complete inhibition of the binding of LPS to PBMCs; however, the binding was not blocked by anti-CCR5 MAb. Thus, the binding of LPS to PBMCs was CD14-dependent. In contrast to the anti-CD14 MAb, TAK-242 did not block the binding of LPS to PBMCs even at a concentration of 10 μM (Fig. 6). However, TAK-242 at a concentration of 1 μM inhibited the production of TNF-α and IL-6 from PBMCs stimulated under conditions similar to those of the LPS binding assay (50 ng/ml LPS) by more than 85% compared with that in the absence of TAK-242 (data not shown). These results suggest that TAK-242 inhibits cytokine production without antagonizing the binding of LPS to CD14/TLR4/MD-2 complex.

Selective Inhibitory Effect on TLR4-Mediated Signaling Pathway. We investigated whether the inhibitory effect of TAK-242 is specific for LPS-induced responses. A lot of studies have revealed that TLRs are the key molecules for recognizing pathogen-associated molecular patterns to elicit inflammatory responses, and LPS is a well known TLR4 ligand. Therefore, RAW264.7 cells were stimulated with various TLR ligands, and the effect of TAK-242 on cytokine production was examined. In addition to LPS from *E. coli*, we used LPS(S) (LPS from *S. typhimurium*), LTA, and paclitaxel (a diterpene from a plant) as TLR4 ligands (Takeuchi et al., 1999; Byrd-Leifer et al., 2001). PGN, poly(I:C), and CpG DNA were used as ligands for TLR2, -3, and -9, respectively (Takeuchi et al., 1999; Hemmi et al., 2000; Alexopoulou et al., 2001). As shown in Fig. 7, TAK-242 inhibited TNF-α production from RAW264.7 cells stimulated with LPS(S), LTA, and paclitaxel in a concentration-dependent manner similar to LPS from *E. coli*. In contrast, TAK-242 did not show an inhibitory effect on the TNF-α production induced by PGN, poly(I:C), and CpG DNA. TNF-α production induced by a cell permeable ceramide-C2 was not also inhibited by TAK-242 (data not shown). In addition, similar selective inhibitory

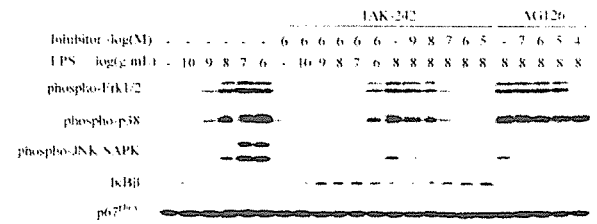

Fig. 5. Inhibitory effect of TAK-242 on the LPS-induced phosphorylation of MAPKs and IκB degradation in RAW264.7 macrophages. Cells were treated with the indicated concentrations of TAK-242 or tyrphostin AG126 for 15 min before stimulation. After the LPS stimulation for 30 min, whole cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phospho-Erk1/2, phospho-p38, phospho-JNK/SAPK, IκBβ, or $p67^{phox}$. $p67^{phox}$ was used as control.

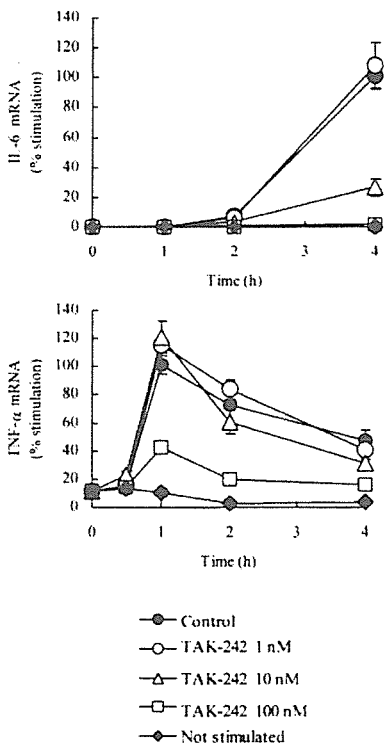

Fig. 4. Inhibitory effect of TAK-242 on mRNA expression induced by LPS and IFN-γ in RAW264.7 macrophages. Cells were incubated with LPS (5 ng/ml) and IFN-γ (1 U/ml) in the presence or absence of TAK-242. Expression levels of TNF-α and IL-6 mRNA were determined by real-time quantitative PCR and expressed as the percentage of the highest control level (the expression level at 1 h for TNF-α and at 4 h for IL-6). The data are expressed as mean ± S.E. (n = 3).

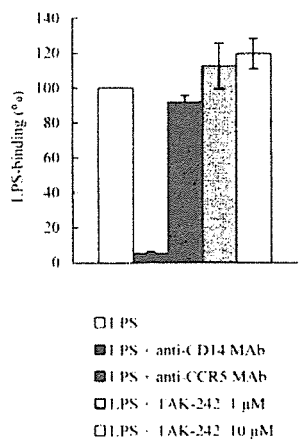

Fig. 6. Effect of TAK-242 on LPS-binding to PBMCs in the presence of human serum. PBMCs were incubated with anti-human CD14 MAb MEM-18, anti-human CCR5 MAb, or TAK-242 for 30 min at 4°C and further incubated with 50 ng/ml Alexa Fluor 488-conjugated LPS in the presence of 1% human serum for 45 min at 37°C. The cells were analyzed by flow cytometry. The data are expressed as mean ± S.E. of four independent experiments using PBMCs prepared from four different donors.

patterns were observed in IL-6 and NO production (data not shown). We confirmed the selective inhibitory effect of TAK-242 on TLR4-mediated cytokine production in human macrophages. TAK-242 inhibited IL-6 and IL-12 production in human macrophages stimulated with LPS, with IC_{50} values of 32 and 16 nM, respectively. In contrast, TAK-242 did not inhibit IL-6 and IL-12 production induced by PGN even at a concentration of 2500 nM (Table 4). Furthermore, TAK-242 markedly inhibited IL-8 production from PBMCs induced by LPS but showed only a marginal inhibitory effect on IL-1β-induced IL-8 production at higher concentrations (Fig. 8). These results suggest that TAK-242 selectively inhibits cytokine production mediated by TLR4 but not by TLR2, -3, and -9 or IL-1β.

Discussion

In this article, we have presented a novel small molecule cytokine production inhibitor, TAK-242 (Fig. 1), which selectively suppresses TLR4-mediated production of cytokines and NO from monocytes and macrophages. Some synthetic lipid A analogs have been reported as LPS antagonists or TLR4 antagonists (Christ et al., 1995; Rossignol and Lynn, 2002; Fort et al., 2005). However, TAK-242 is the first small-molecule compound that selectively suppresses TLR4-mediated cytokine production. TAK-242 suppressed the LPS-induced production of TNF-α, IL-1β, IL-6, and NO at similar concentrations (Tables 1 and 2; Figs. 2 and 3). These data suggest that TAK-242 could show suppressive effects on the production of various types of inflammatory mediators, including those examined in this study. In addition, the inhibitory effects of TAK-242 on cytokine production were similar in both mouse and human macrophages, which suggests that differences in species do not greatly affect the efficacy of TAK-242. The LPS plus IFN-γ-induced increase in mRNA expression levels of IL-6 and TNF-α was also suppressed by TAK-242 at similar concentrations (Fig. 4). These observations have led us to speculate that TAK-242 targets an event that is elicited earlier than the transcription of cytokine genes. Therefore, we examined the effect of TAK-242 on MAPK and NF-κB signaling pathways. TAK-242 inhibited the LPS-induced phosphorylation of Erk1/2, p38, and JNK/SAPK as well as IκB degradation in RAW264.7 cells to a similar extent (Fig. 5). Although we did not address the effect of TAK-242 on the direct NF-κB activation, it is suggested that TAK-242 might inhibit the early process of LPS signaling upstream of the phosphorylation of MAPKs and the IκB degradation.

The initial process in the activation of immune cells by LPS is the recognition of LPS by a receptor complex composed of CD14, TLR4, and MD-2 on the cell surface (da Silva Correia et al., 2001). However, it has been reported that LPS binding to the complex is two types, namely, LBP/CD14-dependent and -independent types. The binding of LPS to the cells is LBP/CD14-dependent for LPS concentrations up to 100 ng/ml, and at higher LPS concentrations, the binding of LPS is LBP/CD14-independent (Triantafilou et al., 2000). To determine whether TAK-242 inhibits cytokine production through both these types, we used two stimulation conditions with

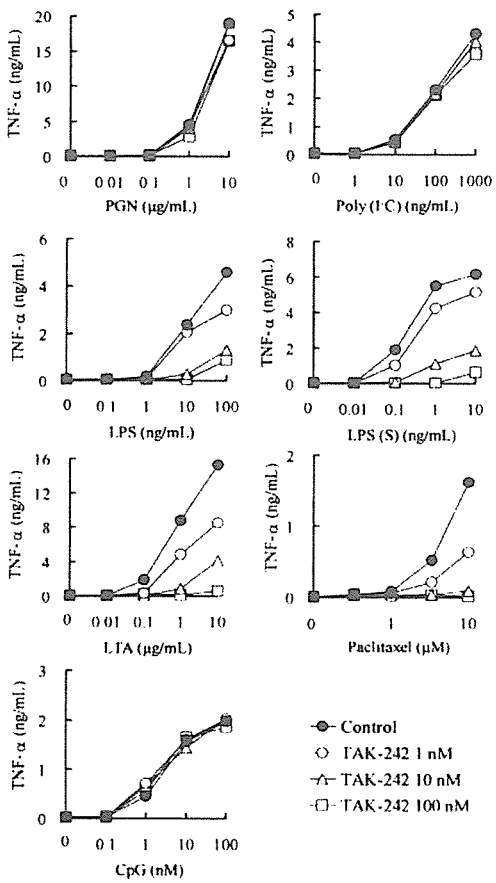

Fig. 7. Ligand specificity for the inhibitory effect of TAK-242 on TNF-α production from RAW264.7 macrophages. Cells were plated at a density of 1×10^5 cells/well in 96-well culture plates and incubated overnight. After removing the cell culture supernatants, the cells were stimulated with various TLR ligands described in the presence or absence of TAK-242 for 20 h. The concentration of TNF-α in the culture supernatant was determined in duplicate by specific enzyme-linked immunosorbent assay.

TABLE 4

IC_{50} values of TAK-242 for LPS- and PGN-induced IL-6 and IL-12 production by human macrophages

Stimuli	IC_{50} (95% Confidence Interval)	
	IL-6	IL-12
	nM	
LPS	32 (19–67)	16 (6.2–36)
PGN	>2500	>2500

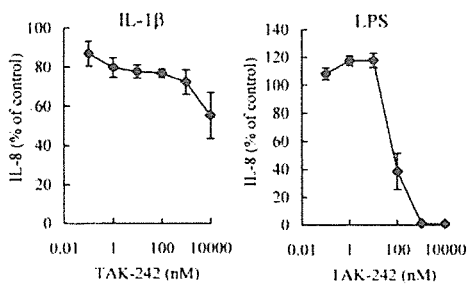

Fig. 8. Effect of TAK-242 on IL-8 production from human PBMCs stimulated with LPS or IL-1β. The cells were stimulated with IL-1β (10 ng/ml) or LPS (1 ng/ml) in the presence or absence of TAK-242, and IL-8 levels in culture supernatants were measured as described under *Materials and Methods*. The data are expressed as mean ± S.E. of percentage of inhibition for PBMCs prepared from four different donors. The IL-8 levels produced by PBMCs of each donor were from 0.85 to 1.6 ng/ml and from 7.7 to 14 ng/ml for IL-1β and LPS stimulation, respectively.

different concentrations of LPS [i.e., 5 ng/ml (plus IFN-γ) and 1 μg/ml]. Regardless of the LPS concentrations, TAK-242 showed similar suppressive effects on the production of these mediators from RAW264.7 cells (Table 1). Furthermore, TAK-242 did not block the CD14-mediated binding of LPS to PBMCs, although it suppressed the cytokine production (Fig. 6). Together, TAK-242 is not an LPS antagonist but can inhibit an LPS-induced signaling process that is elicited after binding of LPS to the receptor complex.

LPS as well as other microbial components initiate signal transduction through TLRs, resulting in the release of inflammatory cytokines. TLRs are broadly distributed on the cells of the immune system (Muzio and Mantovani, 2000) and recognize a remarkably diverse array of bacterial, viral, and fungal molecular patterns (Hopkins and Sriskandan, 2005). For example, it is well known that TLR2, -3, -4, and -9 recognize PGN, poly(I:C), LPS, and CpG DNA, respectively (Takeuchi et al., 1999; Hemmi et al., 2000; Alexopoulou et al., 2001). It is noteworthy that experiments using cell stimulation with various ligands for TLR/IL-1 receptor family showed that TAK-242 selectively suppressed TLR4-mediated cytokine production. TAK-242 inhibited cytokine production in RAW264.7 cells stimulated with TLR4 ligands; however, it did not show inhibitory effects on ligands for TLR2, -3, or -9 (Fig. 7). In addition, the TLR4-selective inhibition was also observed in human PBMCs and macrophages (Table 4; Fig. 8). Furthermore, TAK-242 showed similar inhibitory effects on cytokine production from RAW264.7 cells stimulated with not only LPS from *E. coli* but also LPS from S. *typhimurium*, LTA from *S. aureus*, and paclitaxel (Fig. 7). It should be noted that we used commercial LTA from *S. aureus* as a TLR4 ligand, as reported previously (Takeuchi et al., 1999). Although it has been reported that highly purified LTA is a TLR2 ligand (Ellingsen et al., 2002), cytokine production induced by cell walls derived from *S. aureus* is partially abolished in TLR4-deficient macrophages (Takeuchi et al., 1999). It is plausible that TAK-242 inhibits the cytokine production induced by an unknown active TLR4 ligand contaminated in commercial LTA. Thus, these data suggest that TAK-242 does not discriminate between TLR4 ligands with regard to the structural differences and could suppress the activation of cells by a wide range of TLR4 ligands. Further investigation on its precise mechanism of action is needed along with the elucidation of target molecules of TAK-242. However, based on the data reported in this study, it can be inferred that TAK-242 might target an upstream event in TLR4-mediated signaling.

Intracellular signaling of TLRs is elicited from Toll/IL-1 receptor (TIR) domain, which is conserved among the cytoplasmic regions of TLRs. After the exposure of the cells to LPS, TLR4 homodimerizes and recruits four adaptor molecules that contain TIR domain: MyD88, MyD88 adaptor-like (also known as TIRAP), TIR domain-containing adaptor molecule-1 (TICAM-1, also known as TRIF), and TICAM-2 (also known as TRAM) (Dunne and O'Neill, 2005). Two signaling pathways have been suggested downstream of TLR4, namely, MyD88-dependent and MyD88-independent pathways. MyD88-deficient mice did not show production of inflammatory cytokines induced by various TLR ligands. MyD88 adaptor-like/TIRAP has been shown to be essential for the MyD88-dependent signaling pathway via TLR2 and TLR4. TICAM-1/TRIF has been demonstrated to be essential for TLR3- and TLR4-mediated MyD88-independent pathways (Yamamoto et al., 2002; Fitzgerald et al., 2003; Oshiumi et al., 2003a). Among the four adaptors, TICAM-2/TRAM specifically interacts with TLR4 and is involved in a TLR4-mediated signaling pathway (Fitzgerald et al., 2003; Oshiumi et al., 2003b; Yamamoto et al., 2003). TICAM-2/TRAM-deficient mice show defects in cytokine production in response to TLR4 ligand but not to other TLR ligands. MD-2 is also a TLR4-specific molecule; it is a coreceptor of TLR4, which is essential for LPS signaling of TLR4 (Shimazu et al., 1999; Nagai et al., 2002). MD-2 is physically associated with the extracellular domain of TLR4 and augments TLR4-dependent LPS responses. In MD-2-deficient embryonic fibroblasts, TLR4 does not reach to the plasma membrane and predominantly resides in the Golgi apparatus; this suggests that MD-2 is also essential for appropriate intracellular distribution of TLR4. Although the target molecule of TAK-242 remained to be identified, TLR4 and its associated molecules MD-2, CD14, LBP, and TICAM-2/TRAM may be involved in its inhibitory mechanism. Among these, TLR4, MD-2, and TRAM might be the most probable candidates for the target because TAK-242 is a selective inhibitor for TLR4-mediated cytokine production. TAK-242 might directly inhibit TLR4, TRAM, or MD-2. Otherwise, TAK-242 might suppress or activate an unknown molecule that is uniquely required to regulate TLR4 signaling.

Some TLRs play an important role in the pathogenesis of infectious and inflammatory diseases such as sepsis, meningitis, atherosclerosis, inflammatory bowel disease, hepatitis, and autoimmune diseases (e.g., multiple sclerosis and systemic lupus erythematosus) (O'Neill, 2003). The involvement of TLR4 in some diseases such as sepsis and atherosclerosis was indicated based on studies on polymorphisms in the TLR4 gene (Kiechl et al., 2002; Lorenz et al., 2002). In addition, because tissue macrophages play an important role in the pathogenesis of various inflammatory diseases (Linton and Fazio, 2003; Schwacha, 2003), it is essential that drugs used to treat these diseases should act effectively on macrophages as well as on monocytes. The efficacy of TAK-242 against the LPS-induced IL-6 and IL-12 production was almost the same between human monocytes and GM-CSF-differentiated macrophages (Table 3). Thus, TAK-242 could offer a new therapeutic approach for inflammatory diseases whose pathogenesis involves TLR4. In fact, TAK-242 protected mice when tested in the endotoxin shock model and showed beneficial effects in some sepsis models (T. Sha, M. Ii, M. Sunamoto, T. Kitazaki, J. Sato, and Y. Iizawa, manuscript in preparation). Based on the beneficial effects observed in preclinical studies, a clinical trial of TAK-242 in severe sepsis is now ongoing.

In conclusion, we discovered a novel cyclohexene derivative, TAK-242, which selectively suppresses TLR4-mediated cytokine production. TAK-242 could be a promising drug for the treatment of inflammatory diseases involving TLR4, such as sepsis. The precise mechanism of action of TAK-242 is being investigated.

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ORIGINAL ARTICLE

Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype

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RNA interference (RNAi) can be used to inhibit viral replication in mammalian cells and therefore could be a powerful new antiviral therapy. Small interfering RNA (siRNA) may be effective for RNAi, but there are some technical problems that must be solved in each case, for example, predicting the effective siRNA target site and targeting heterogeneous sequences in a virus population. We show here that diced siRNA generated from long double-stranded RNA (dsRNA) is highly effective for inducing RNAi in HuH-7 cells harboring hepatitis C virus (HCV) replicons and can overcome variations in the HCV genotype. However, in mammalian cells, long dsRNA induced an interferon response and caused cell death. Here we describe an improvement of this method, U6 promoter-driven expression of long hairpin-RNA with multiple point mutations in the sense strand. This can efficiently silence HCV RNA replication and HCV protein expression without triggering the interferon response or cell death normally caused by dsRNA. In conclusion, intracellular-diced dsRNA efficiently induces RNAi, and, despite the high rate of mutation in HCV, it should be a feasible therapeutic strategy for silencing HCV RNA.

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Keywords: *RNAi; long dsRNA; dicer; heterogeneous; interferon; U6 vector; HCV*

Introduction

An estimated 170 million people worldwide are persistently infected with hepatitis C virus (HCV).[1] Although the initial infection is frequently asymptomatic, there are several subsequent clinical manifestations, including fibrosis of the liver, cirrhosis and hepatocellular carcinoma. Although combination therapy with interferon (IFN)-α and ribavirin has markedly improved the clinical outcome, less than half of the patients with chronic hepatitis C can be expected to respond favorably to currently available agents.[2] Therefore, developing a new therapy for chronic HCV is a major public health objective.

The genome of HCV, a member of Flaviviridae family, is encoded in an approximately 9.6-kb single-stranded RNA with positive polarity that includes a 5′-untranslated region (UTR)[3] and a 3′-UTR containing a 3′X terminal sequence.[4] Hepatitis C virus displays a high rate of mutation and is classified into distinct genotypes (1–6) and subtypes, whose distribution varies both geographically and between risk groups.[5] Furthermore, several distinct but closely related HCV sequences coexist within each infected individual. These are referred to as quasispecies and reflect the high replication rate of the virus and the lack of a proofreading activity of the RNA-dependent RNA polymerase.[6,7]

Gene targeting with functional nucleic acids is commonly used to determine gene function and has potential as a treatment for viral diseases. Although antisense RNA and ribozyme technologies are successful in some situations, they have been difficult to apply universally and are less effective *in vivo*.[8] A possible alternative, sequence-specific post-transcriptional gene silencing by double-stranded RNA (dsRNA), also known as RNA interference (RNAi), has been found in plants, *Caenorhabditis elegans* and mammalian cells.[9,10] As RNAi with small interfering RNA (siRNA) can inhibit the replication of several viruses, including human immunodeficiency virus type 1 (HIV-1)[11] and poliovirus,[12] it may be a powerful new antiviral therapy. Recently, it has been demonstrated that replication of HCV RNA is also receptive to RNAi machinery,[13–18] but it has been difficult to design highly effective siRNAs against HCV because of the exquisite sequence specificity of the siRNAs coupled with the variation in HCV genotypes and the enormous diversity of HCV sequences between and within infected individuals.

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In this report, we show that dicer-generated siRNAs from long dsRNA can silence the replication of HCV RNA of different genotypes. Furthermore, we employed the modified long hairpin-RNA (hRNA) expression system to inhibit HCV replication and to avoid triggering the IFN response, which is normally caused by dsRNA. We demonstrated that intracellular-diced dsRNA can be used in mammalian cells to silence HCV RNA and, therefore, that long dsRNA-mediated RNAi could be useful as a therapeutic agent for natural viral infection by HCV.

Results

Synthetic small interfering RNA inhibits hepatitis C virus replication in HuH-7 cells containing replicating hepatitis C virus RNA

As siRNA-mediated RNAi is strictly sequence specific, an siRNA targeting site was selected in regions conserved among the various HCV genotypes. Of the HCV genome sequences, the 5′-UTR and the 3′X region are the most highly conserved.[19] Therefore, we selected six sites in the 5′-UTR or core coding regions (A–F) and three sites in the 3′X regions (G–I) (see Materials and methods). HuH-7 cells carrying the HCV replicon were established as described.[20] We also modified the replicon RNA derived from the HCV genotype 1b clone (GenBank accession number AY045702) by substituting the *neo*^{r} gene with the firefly luciferase gene fused to foot-and-mouth disease virus (FMDV) 2A and the *neo*^{r} gene (named the R6FLR-N replicon). This modification enables the sensitive and precise quantification of HCV replication levels using a luciferase assay.

To examine the ability of siRNAs to inhibit HCV replication, the nine synthetic siRNAs were transfected into R6FLR-N replicon cells (Figure 1a, left). Of the siRNAs, siE (nucleotides (nt) 325–344) was the most effective and it dose-dependently inhibited HCV replication (Figure 1a, right). Moreover, continuous transfection with siE but not the negative control p53m siRNA caused a gradual decrease in the HCV replicon titer up to the 23rd day (Figure 1b). Using Northern blot analysis, we confirmed that the effects of siRNAs on the luciferase activity are associated with siRNA-directed degradation of the HCV replicon RNA (data not shown). These results indicated that siE was the most potent siRNA for inhibiting HCV replication of the selected siRNA sites.

Effect of ex-vivo *dicer-generated small interfering RNAs from long double-stranded RNA*

We found that shifting the siRNAs 5′ or 3′ from the siE target position reduced the efficacy of siRNA-mediated RNAi (Figure 2a). Therefore, to overcome site specificity of the selected siRNAs, we prepared *ex-vivo* recombinant human dicer (rhDicer)-generated siRNAs (d-siRNAs) from long dsRNAs (Figure 2b).[21] R6FLR-N replicon cells were transfected with d-siRNAs targeting the HCV genome or p53 mRNA (negative control). Luciferase reporter assays indicated that d-siRNAs generated from the 5′-UTR of HCR6 sequences (D5-357, D5-197, and D5-50) silenced the HCV RNA more efficiently than siE. In contrast, the d-siRNAs generated from the 3′-UTR of the HCR6 sequences were less effective than siE. These

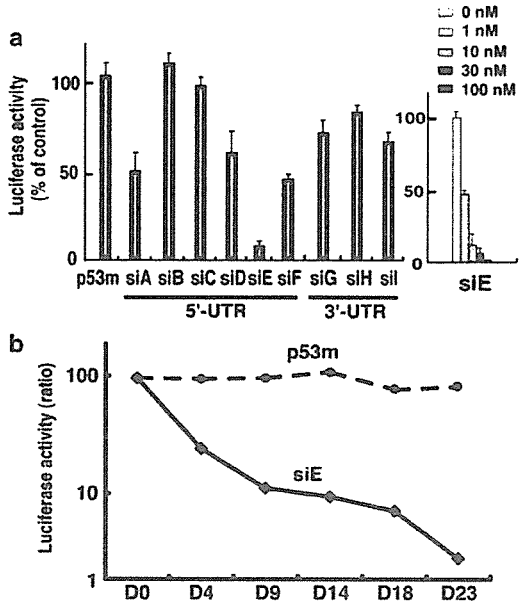

Figure 1 Effect of siRNA on HCV replication. (a) Inhibition of the HCV replicon by siRNAs in R6FLR-N replicon cells. Luciferase activity was measured 24 h after transfection using the Trans IT TKO reagent. Data represent means ± s.d. ($n = 3$) compared with mock-transfected cells. siA to siF, siRNA in the 5′-UTR or core cording regions; siG to siI, siRNA in the 3′-UTR; p53 m, negative control siRNA. (b) Long-term effect of siE. The R6FLR-N replicon cells were transfected with siRNAs every 4 days. Luciferase activity was measured on the indicated days.

results indicated that d-siRNAs generated from 5′-UTR containing the siE sequences, especially those generated from 197-bp dsRNAs, were more effective than the synthetic siE.

Dicer-generated siRNAs generated from 197-bp double-stranded RNA overcome hepatitis C virus genotype variation

Genotype 1b-derived d-siRNAs generated from the conserved sequence motifs within the NS5B sequence do not block the replication of HCV genotypes 1a and 2a.[14] To examine whether our selected d-siRNAs can overcome HCV genotype variation, we transfected genotype 2a-specific d-siRNAs into R6FLR-N replicon cells, which harbor the genotype 1b replicon. As shown in Figures 3a and b, the genotype 2a-derived d-siRNAs generated from 197-bp dsRNA efficiently inhibited genotype 1b replication, even though genotypes 2a and 1b differ by 15 bases within the 197-bp dsRNA sequences (sequence homology = 92%). In contrast, genotype 2a-derived siE, which harbors a single mutation at position 18 of the sense strand (sequence homology = 95%), showed a weak silencing activity against genotype 1b. These results demonstrated that d-siRNAs generated from the 197-bp dsRNA were highly effective for RNAi and could overcome HCV genotype variation.

Long double-stranded RNA transfection into HuH-7 replicon cells induces target-specific silencing

Dicer is a large multi-domain protein present in all eukaryotes.[22] Recently, Kim *et al*.[23] reported that syn-

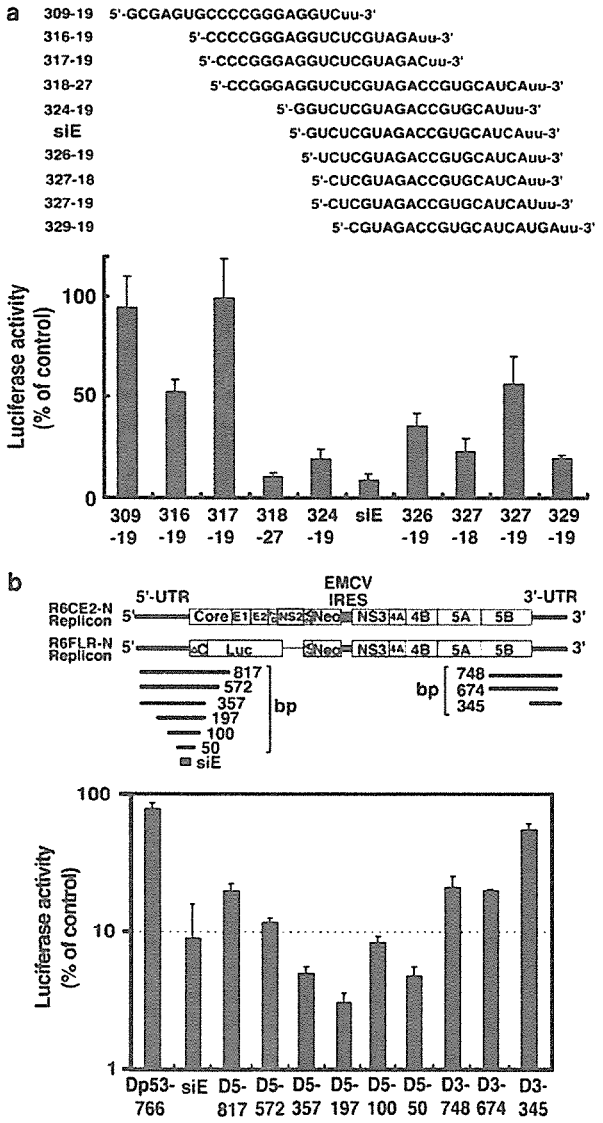

Figure 2 Small interfering RNAs cleaved by rhDicer from long dsRNA. (a) Effect of the positional variations in the siE region. R6FLR-N replicon cells were transfected using Lipofectamine 2000 with siRNAs in which the target position was shifted towards either the 5′- or 3′-end of the siE region. Luciferase activity assay measured 48 h after transfection with 1 nM siRNAs. Data represent means±s.d. compared with mock-transfected cells ($n=5$). (b) Upper panel, schematic representation of the long dsRNAs used for targeting different sites in the HCV genome RNA; lower panel, effect of d-siRNAs. The d-siRNAs were generated from the long dsRNAs by cleavage with rhDicer. R6FLR-N cells were transfected with d-siRNAs. Luciferase activity was measured after 48 h. Data represent means±s.d. compared with mock-transfected cells ($n=5$). Dp53-766, which targeted p53 mRNA (766 bp), was used as a negative control.

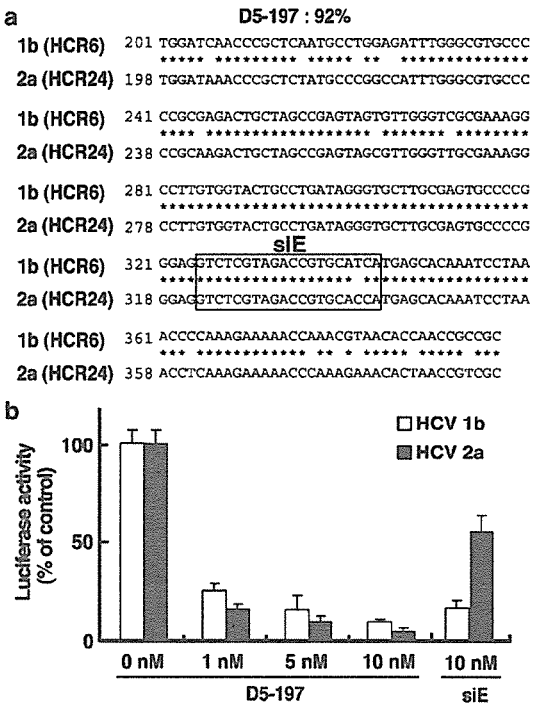

Figure 3 Dicer-generated siRNAs directed at the HCV genotype 2a can cause silencing of genotype 1b RNA. (a) The sequence homology between genotypes 1b and 2a was 92% within the 197-bp region (182/197 nt) and 95% within the 20-bp siE region (19/20 nt). (b) R6FLR-N cells harboring the genotype 1b HCV replicon RNA were transfected with the d-siRNAs generated from a 197-bp dsRNA directed at HCV genotype 2a (HCR24; accession number AY746460). Data represent means±s.d. compared with mock-transfected cells ($n=5$).

thetic RNA duplexes 25–30 nt in length are substrates of the dicer endonuclease, directly linking the production of siRNAs to incorporation in the RNA-induced silencing complex. We also expected that intracellular dsRNA duplexes longer than 50 nt would be recognized by dicer and thus induce RNAi. Therefore, we directly transfected long dsRNA into R6CE2-N replicon cells, which harbor the core to NS2 portion of the HCV genome (Figure 2b). The same amount of dsRNA was transfected into replicon cells, and the replicon copy number was determined by quantitative real-time detection (RTD)-polymerase chain reaction (PCR).[24] We found that, except for the 817-bp dsRNA, the long dsRNAs targeting sites in the HCV genome reduced the HCV RNA copy number. In contrast, an unrelated dsRNA targeting a site in endogenous p53 mRNA had no effect (Figure 4a). A luciferase assay in R6FLR-N replicon cells showed similar results for HCV-specific silencing (data not shown). On the other hand, immunoblot analysis with antibodies against p53 showed that p53-specific long dsRNA suppressed the level of p53 protein, whereas HCV-specific dsRNA had no effect on p53 expression (Figure 4b). These results indicated that in HuH-7 replicon cells, direct transfection of long dsRNA can specifically produce RNAi against HCV and reduce endogenous p53 expression.

Effect of long double-stranded RNA on the intracellular interferon response and cell death in HepG2 cells

In mammalian cells, Toll-like receptor (TLR) 3[25,26] recognizes dsRNA duplexes longer than 30 nt. This binding induces a type I IFN response, resulting in cell

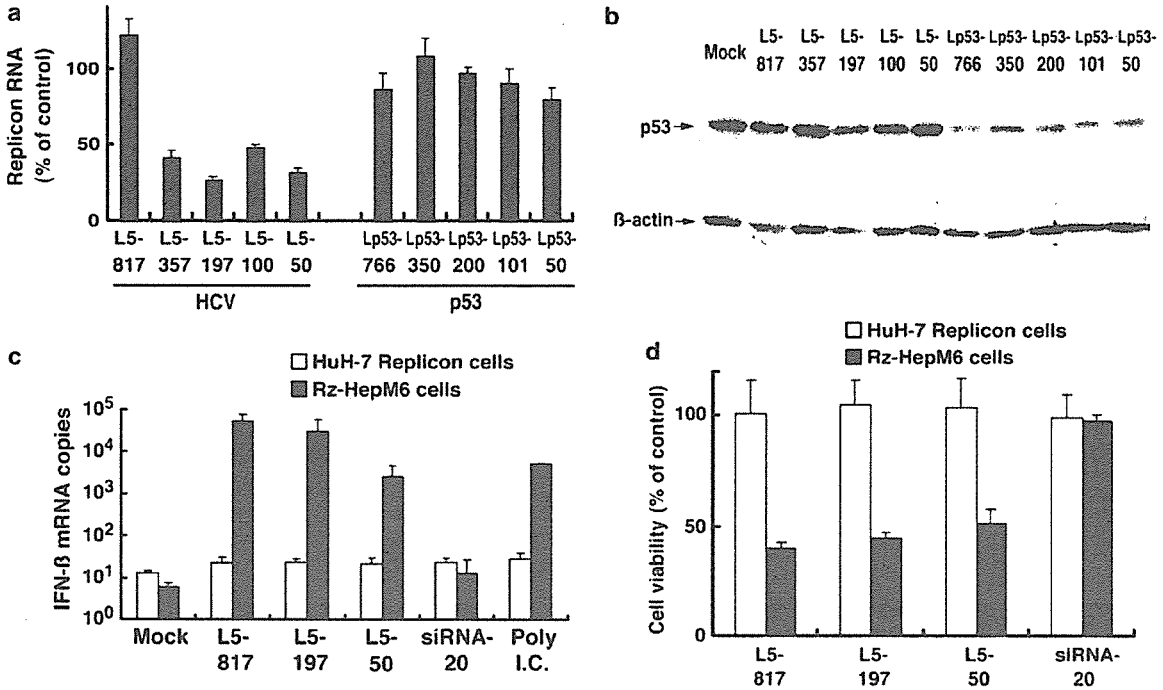

Figure 4 Transfection of long dsRNAs into HuH-7 replicon cells, which lack dsRNA-triggering IFN response, induces target-specific silencing. (a) R6CE2-N cells were transfected with long dsRNAs. Replicon RNA levels in cells transfected with 262 ng of dsRNA per 48-well dish were measured after 48 h by RTD–PCR. Data represent means ± s.d. ($n = 3$) of replicon levels compared with mock-transfected cells. (b) Immunoblot analysis of p53 and β-actin from replicon cells transfected with dsRNA targeting sites in the HCV genome or p53 mRNA. (c) Levels of human IFN-β mRNA were quantified by RTD-PCR 7 h after transfection with 50 ng of dsRNAs per 48-well dish. Values represent the mean copy number for each RNA per μg total RNA ± s.d. ($n = 5$). (d) Cell viability was determined after 48 h by WST-8 assay. Data represent means ± s.d. ($n = 3$) of WST conversion compared with mock-transfected cells.

death by apoptosis.[27] To examine the type I IFN response caused by direct transfection of dsRNA, we measured the intracellular IFN-β mRNA copy number and assessed cell viability. The IFN-β mRNA levels of R6FLR-N replicon cells (HuH-7 replicon cells) and the numbers of viable cells did not change following transfection with long dsRNAs or with the RNA duplex poly(rI):poly(rC) (Figures 4c and d). These results show that the dsRNA did not induce intracellular IFN-β mRNA or enhance apoptosis in HuH-7 replicon cells.

HuH-7 replicon cell lines are used as models for HCV replication and do not respond to the IFN signals.[28] We therefore investigated the effect of dsRNAs on the IFN-β response in another cell type. As an alternative model, we used HepG2 cells stably expressing the full genome HCV RNA (Rz-HepM6 cells).[29] Transfection with poly (rI):poly(rC) or long dsRNAs induced an IFN-β mRNA level of 10^3–10^5 copies per μg total RNA, whereas siRNA-20, a 20-nt duplex, induced only 10 copies per μg total RNA (Figure 4c). Furthermore, the number of viable Rz-HepM6 cells was reduced by transfection with long dsRNAs, but not with siRNA-20 (Figure 4d). These results indicated that direct transfection with dsRNA duplex longer than 50 nt induces IFN-β mRNA and causes cytotoxicity in Rz-HepM6 cells, but not in HuH-7 replicon cells. Therefore, to observe the knockdown efficiency of long-dsRNA against the HCV replicating model and the IFN response induced by long dsRNA, we tested the effects of RNAi in HuH-7 replicon, Rz-HepM6 and HepG2 cells.

U6 promoter-driven expression of long hairpin-RNA with mutations in the sense strand causes gene silencing without triggering an interferon response or cell death

We examined the ability of a stable hairpin-type siRNA-expression vector[30–32] to silence the HCV genome. Recently, U6 promoter-driven transcription of hRNA with mutations in the sense strand has been reported to be more effective for RNAi than hRNA containing nonmutated sense strands.[32] Therefore, we constructed vectors for U6 promoter-driven expression of hRNAs containing multiple mutations (mhRNA) and examined their ability to cause gene silencing. To confirm the RNAi effect, we transfected the long mhRNA-expression vectors into R6FLR-N replicon cells. The 50- and 197-bp mhRNA vectors against the HCV sequence reduced luciferase activity as effectively as the siE-20-bp mhRNA vector (Figure 5a). Furthermore, in Rz-HepM6 cells, the 50- and 197-bp mhRNA vectors targeted to the HCV sequence specifically suppressed HCV core protein expression (Figure 5b). To avoid the inhibition of IFN-β activation by HCV itself,[28] we next examined the IFN response in original HepG2 cells. In contrast to the direct transfection of dsRNAs targeted to the same sequences, the 50- and 197-bp mhRNA vectors did not induce the expression of IFN-β mRNA (Figure 5c). Owing to palindrome structure-specific recombination in the mammalian gene,[33] it was not possible to construct stably transformed cells expressing hRNA vectors against the

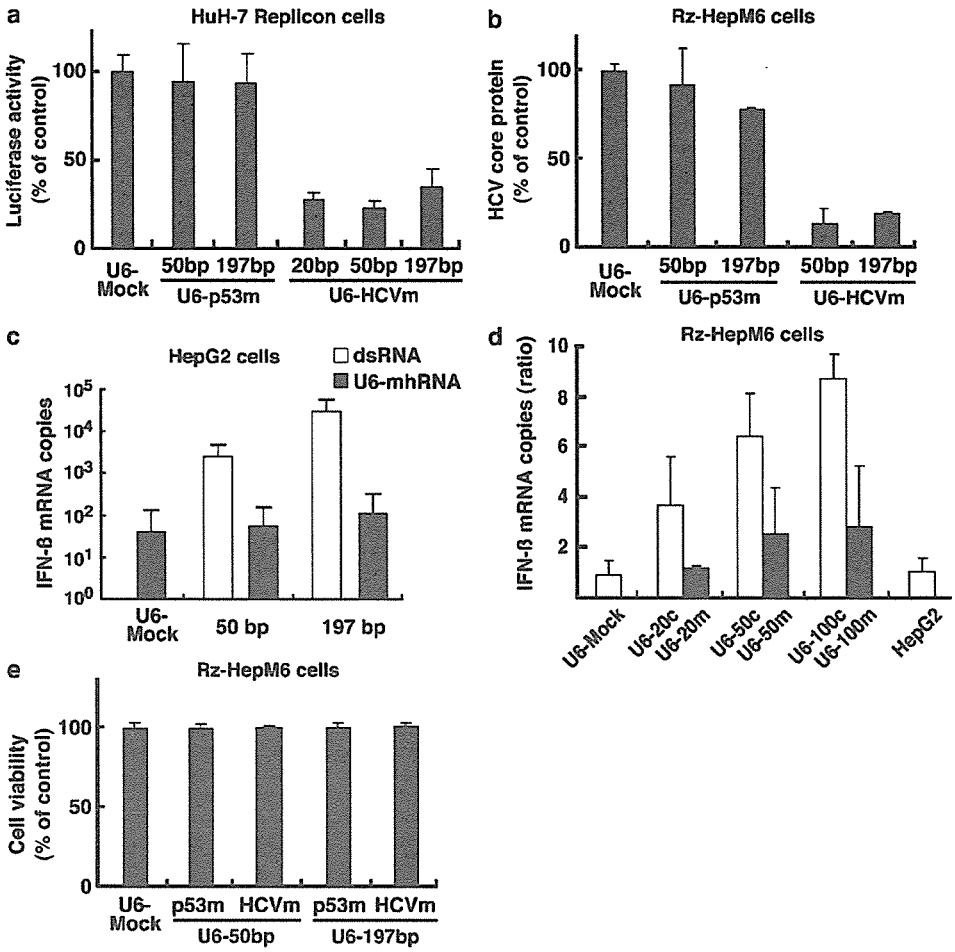

Figure 5 U6 promoter-driven expression of long mhRNA causes gene silencing without triggering an IFN response or cell death. (**a**) U6 promoter-driven transcription of long hRNAs containing multiple point mutations within the sense strand induced RNAi in R6FLR-N replicon cells. Luciferase activity was measured 96 h after transfection with 200 ng vector per 96-well dish. Data represent means ± s.d. compared with control U6 vector-transfected cells ($n = 3$). (**b**) Gene silencing for the long mhRNA-expression vector in Rz-HepM6 cells. All assays were performed 120 h after transfection with 600 ng vector per 48-well dish. Data represent means ± s.d. compared with control U6 vector-transfected cells ($n = 3$). (**c**) Interferon responses in original HepG2 cells following targeting of the same HCV sequences by direct transfection with dsRNA and U6 promoter-driven expression of mhRNA. The level of human IFN-β mRNA was measured by RTD-PCR 7 and 16 h after transfection. Values represent the mean copy numbers for each RNA per μg total RNA ± s.d. ($n = 3$). (**d**) U6 promoter-driven expression of mhRNA caused a lower IFN response in Rz-HepM6 cells than expression of hRNA containing non-mutated sense strands. The level of human IFN-β mRNA was measured by RTD-PCR 16 h after transfection. Values represent the mean ratios compared to control U6 vector-transfected cells ± s.d. ($n = 3$). U6-20c, HCVc-20bp; U6-20m, HCVm-20bp; U6-50c, U6-50m, U6-100c, and U6-100m are U6 vectors against the luciferase gene. (**e**) Cell viability of Rz-HepM6 cells was determined after 120 h by WST-8 assay. Data represent means ± s.d. ($n = 3$) of WST-8 conversion compared with control U6 vector-transfected cells.

HCV sequence containing non-mutated sense strands longer than 50 bp. Using control vectors against the luciferase gene (U6-50c, U6-50m, U6-100c and U6-100m), we confirmed an intracellular IFN response. In Rz-HepM6 cells, all of the mhRNA vectors (HCVm-20 bp, U6-50m and U6-100m) had a reduced IFN response compared to the hRNA vectors containing non-mutated sense strands (HCVc-20 bp, U6-50c and U6-100c; Figure 5d). Moreover, U6 promoter-driven expression of long mhRNAs against the HCV sequence was not cytotoxic (Figure 5e).

These results indicated that in IFN-responsive cells, U6 promoter-driven expression of modified long dsRNA, which be made by inserting multiple mutations in the sense strand of hRNA, can effectively and specifically silence HCV without triggering the IFN response or cell death.

Discussion

Previous studies have shown that HCV RNA can be suppressed by the RNAi machinery in replicon cells.[13–18] We demonstrated that there are two significant limitations for the use of siRNA-mediated RNAi as a therapy for HCV: first, it is difficult to predict which target site will be most effective for siRNA; and, second, it is difficult to target the other HCV genotypes with multiple sequences. We further examined the ability of d-siRNAs and intracellular-diced long dsRNAs to overcome these problems and inhibit HCV replication in HCV replicon

cells. We found that *ex-vivo* dicer-generated siRNAs generated from the 5′-UTR sequences are more effective for silencing than the most potent synthetic siRNA, siE. Our results further demonstrated that 50- and 197-bp dsRNA regions of the HCV genome are potential target areas for RNAi. Although dsRNA duplexes targeting the 50- to 357-bp sites in the HCV genome efficiently cause target silencing, dsRNA duplexes targeting the 817-bp HCV genome are less effective for HCV replication. This suggests that the area of the HCV genome that can be targeted by the RNAi machinery is restricted because of the formation of a complex internal ribosome entry site structure. Recently, Kim *et al.*[23] showed that 27-mer duplexes that are substrates of cellular dicer have enhanced RNAi potency and efficacy in mammalian cells. Our results also suggest that siRNAs generated by dicer from dsRNA duplexes longer than 50 nt are available in their natural form and, therefore, can have enhanced efficacy for RNAi.

In HuH-7 HCV replicon cells, which lack a long dsRNA-induced IFN response, the long dsRNAs were effective at causing RNAi of the HCV genome or endogenous p53. Therefore, we further examined the effect of dsRNA on HepG2 cells, in which dsRNA causes production of IFN-β and activates downstream signaling, including 2′–5′-oligoadenylate synthetase and protein kinase R.[29] Although transfection with dsRNA duplexes longer than 50 nt induced IFN-β and caused cell death, U6 promoter-driven expression of long hRNAs containing multiple point mutations in the sense strand (i.e., near-complementary inverted repeats) efficiently inhibited HCV replication, but was not cytotoxic. Moreover, the intracellular IFN-β mRNA titer was equivalent to that induced by the control U6 vector. The precise mechanism is now under investigation, but it is clear that this system allows intracellular-diced long dsRNA to induce RNAi without activating the IFN response in mammalian cells.

The genotype 2a-derived d-siRNAs generated from the 197-bp dsRNA were able to efficiently inhibit HCV genotype 1b replication. Thus, siRNAs generated from long dsRNA can cause silencing of heterogeneous viruses and should be able to overcome siRNA escape mutations. Long-term HIV-1 replication assays[34] revealed that, after 3–6 weeks of culture, siRNA-mediated RNAi-resistant viruses containing nucleotide substitutions or deletions in the target sequence arise. Wilson *et al.*[35] reported that HCV replicons escaped RNAi induced by subsequent treatment with the same siRNA directed against the NS5B cording region. In contrast, we also examined the long-term efficiency of long dsRNA-mediated RNAi using HCV replicon cells. When examined over 5 weeks with continuous transfection of 197-bp dsRNA, the HCV replicon RNA titer gradually decreased to a 100-fold reduction and never rebounded (data not shown). The degree of sequence conservation reflects the fact that the structural elements in the 5′- and 3′-terminal regions of the RNA are essential for viral replication.[36,37] Therefore, long dsRNA-mediated RNAi targeting a site in the 5′-UTR can avoid the problem of escape virus generation because extensive alterations in a conserved region of the viral genome would be required.

In summary, our results show that dicer-generated siRNAs from long dsRNA are highly effective for RNAi of the HCV genome and overcome genotype variations. We also showed that U6 promoter-driven expression of modified long dsRNA avoids activation of the IFN response and the induction of cell death normally caused by dsRNA. This strategy should be useful for therapy against natural viral infection by HCV and other RNA viruses, such as HIV-1, that display a high rate of mutation.

Materials and methods

Small interfering RNAs

We synthesized T7 siRNAs using the Silencer siRNA Construction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The sense sequences of siRNAs were as follows:

siA (nt 26–45), 5′-ACUCCACCAUAGAUCACUCCUU-3′;
siB (nt 53–73), 5′-GGAACUACUGUCUUCACGCAGUU-3′;
siC (nt 139–159), 5′-GCCAUAGUGGUCUGCGGAACC UU-3′;
siD (nt 278–299), 5′-AGGCCUUGUGGUACUGCCUGAU UU-3′;
siE (nt 325–344), 5′-GUCUCGUAGACCGUGCAUCAUU-3′;
siF (nt 368–387), 5′-AGAAAAACCAAACGUAACACUU-3′;
siG (nt 9517–9537), 5′-GGCUCCAUCUUAGCCCUAGU CUU-3′;
siH (nt 9540–9560), 5′-GGCUAGCUGUGAAAGGUCCG UUU-3′; and
siI (nt 9553–9572) and 5′-AGGUCCGUGAGCCGCAUGA CUU-3′.

The sense sequence of the p53 m siRNA, which contains two nucleotide mismatches in the target sequence,[38] was 5′-GACUCCAGUGAUAAUCUGCUU-3′ (nucleotide mismatches underlined).

Long double-stranded RNAs

Long dsRNAs were prepared by *in vitro* transcription of PCR-amplified DNA templates. A modified T7 promoter sequence was added to the 5′-end of each PCR primer for amplification (Table 1). The dsRNAs were produced from the purified DNA templates using an Ampliscribe T7 transcription kit (Epicenter Technologies, Madison, WI, USA). Single-stranded RNA was converted to dsRNA by allowing annealing the two strands. Purification of dsRNA was performed as described for dicer-generated siRNAs.

Dicer-generated small interfering RNAs

Digestion with rhDicer (Gene Therapy Systems, San Diego, CA, USA) was carried out according to the manufacturer's protocol. The rhDicer-cleaved siRNAs and dsRNAs were separated by electrophoresis on a nondenaturing 12% polyacrylamide gel and detected by ultraviolet shadowing on a Fluor-coated thin-layer chromatography plate (Ambion). The rhDicer-cleaved siRNAs migrating as 20- to 21-bp bands were excised from the gel and extracted at 37°C for 4 h in extraction buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS). Following buffer exchange and desalting by gel filtration with Sephadex G-25 (Amersham Biosciences, Piscataway, NJ, USA), the rhDicer-cleaved siRNAs were dissolved in TE buffer. The cleaved siRNAs were then quantified by adsorption at 260 nm and stored at −70°C.

Table 1 Templates, PCR primers and amplicons used for the generation of dsRNAs

Template (source[a])	*Primer set*	*Primer sequence (5′ to 3′)*	*Amplicon name*	*Start position*	*Stop position*	*dsRNA size (nt)*
HCR6 genotype lb (AY045702[a])	F-1	GCG TAA TAC GAC TCA CTA TAG GGA GAG AGT GCC CCG GGA GGT CTC GTA GAC	L5-50	311	360	50
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-2	GCG TAA TAC GAC TCA CTA TAG GGA GAT AGT GTT GGG TCG CGA AAG GCC TTG	L5-100	261	360	100
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-3	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TCA ACC CGC TCA ATG CCT GGA	L5-197	201	397	197
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-357	41	397	357
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-572	41	612	572
	R-3	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCT CGT TGC CAT AGA GGG GCC A				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-817	41	857	817
	R-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA ACC GGG CAA ATT CCC TGT TGC ATA				
	F-5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG GGG AGA GAT ATA TCA CAG C	L3-345	9267	9611	345
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-674	8864	9537	674
	R-7	GCG TAA TAC GAC TCA CTA TAG GGA GAG ACT AGG GCT AAG ATG GAG CCA CCA				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-748	8864	9611	748
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
HCR24 genotype 2a (AY746460[a])	F-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TAA ACC CGC TCT ATG CCC GGC	2a-197	198	394	197
	R-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGA CGG TTA GTG TTT CTT TGG G				
p53 (NM_000546[a])	F-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC ATC ACA CTG GAA GAC TCC AG	Lp53-50	1013	1062	50
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAG TGT AAC AGT TCC TGC ATG GG	Lp53-101	962	1062	101
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p3	GCG TAA TAC GAC TCA CTA TAG GGA GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA C	Lp53-200	863	1062	200
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p4	GCG TAA TAC GAC TCA CTA TAG GGA GAC ACC CGC GTC CGC GCC ATG G	Lp53-350	713	1062	350
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CAA TGG ATG ATT TGA TGC TG	Lp53-766	366	1131	766
	R-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCC TTT CTT GCG GAG ATT C				

dsRNA = double-stranded RNA; PCR = polymerase chain reaction.
[a]GenBank accession number.

Construction of U6 vectors

Plasmids containing a human U6 promoter were prepared as described previously.[30] A series of long-hairpin–RNA expression vectors was constructed by inserting a sense sequence between the U6 promoter and the corresponding antisense sequence. Sequences downstream of the U6 promoter were as follows (nucleotide substitutions underlined and loop sequence indicated in lowercase letters):

HCVc-20 bp, 5′-GTCTCGTAGACCGTGCATCAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

HCVm-20 bp, 5′-GTCTTGTAGATTGTGTATTAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

p53m-50 bp, 5′-CATTACATTGGAGGATTCCAGTGGT GATCTATTGGGGCGGAGTAGCTTTGgtgtgctgtccCA AAGCTGTTCCGTCCCAGTAGATTACCACTGGAGT CTTCCAGTGTGATGTTTTT-3′;

HCVm-50 bp, 5′-GAGTGTTCTGGGAGGTTTCGTAG ATCGTGTATCGTGAGTACAAGTTCTAAgtgtgctgtccT TAGGATTTGTGCTCATGATGCACGGTCTACGAGA CCTCCCGGGGCACTCTTTTT-3′;

p53m-197 bp, 5′-GTGTTTGGGTGATAGAGACACCTC TCGGCATGGTGTGGTGGTGTCTTATGAGTCGCT TGGGGTTGGTTCTGATTGTATCACTATCTATTACA GCTACGTGTGTGATAGTTCTTGTATGGGTGGCATG GACCGGGGGTCCATTCTCATCATTATCGCACTGG GAGATTCTAGTGGTGATCTATTGGGGCGGACGG CTTTGgtgtgctgtccCAAAGCTGTTCCGTCCCAGTAG ATTACCACTGGAGTCTTCCAGTGTGATGATGGTG AGGATGGGCCTCCGGTTCATGCCGCCCATGCAG GAACTGTTACACATGTAGTTGTAGTGGATGGTGG TACAGTCAGAGCCAACCTCAGGCGGCTCATAGG GCACCACCACACTATGTCGAGAAGTGTTTCTGTC ATCCAAATACTTTTT-3′;

HCVm-197 bp, 5′-ATGGGTCAGCTCGTTCAATGCTT GGAGGTTTGGGTGTGTCCTCGTGAGATTGCTAGT CGAGTGGTGTTGGGTTGCGGAAGGTCTTGTGGTG CTGTCTGATGGGGTGTTTGTGAGTGTCCTGGGAG GTTTCGTTGACTGTGCATTATGAGTACAGATCCTA GACCTCAGAGAAGGACCAGACGTGACATCAACT GCCGCgtgtgctgtccGCGGCGGTTGGTGTTACGTTTG GTTCTTCTTTGGGGTTTAGGATTTGTGCTCATGAT GCACGGTCTACGAGACCTCCCGGGGCACTCGCA AGCACCCTATCAGGCAGTACCACAAGGCCTTTC GCGACCCAACACTACTCGGCTAGCAGTCTCGCG GGGGCACGCCCAAATCTCCAGGCATTGAGCGGG TTGATCCATTTTT-3′;

U6-50c, 5′-GCCTTCAGGATTACAAGATTCAAAGTG CGCTGCTGGTGCCAACCCTATCTttcaagagaGAATA GGGTTGGCACCAGCAGCGCACTTTGAATCTTGTA ATCCTGAAGGCTTTTT-3′;

U6-50m, 5′- GCCTTTAGGATTATAAGGTTCAAAGTG TGCTGTTGGTGTCAACTCTATCTttcaagagaGAATAG GGTTGGCACCAGCAGCGCACTTTGAATCTTGTAA TCCTGAAGGCTTTTT -3′;

U6-100c, 5′-GATTTCGAGTCGTCTTAATGTATAGATT TGAAGAAGAGCTGTTTCTGAGGAGCCTTCAGGA TTACAAGATTCAAAGTGCGCTGCTGGTGCCAACC CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′ and

U6-100m, 5′-GATTTCGGGTTGTCTTGATGTATGGGT TTGGAGAGGAGTTGTTTCTGGGGAGTCTTTAGGA TTATAAGGTTCAAAGTGTGCTGTTGGTGTCAACT CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′.

Construction of recombinant plasmids for expressing the hepatitis C virus replicon

The HCV genotype 1b replicon pRep-R6FLR-NRz was assembled and cloned from pRep-R6Rz and the 1bneo/delS plasmid.[39] Replicon pRep-R6Rz was engineered from pHCR6-Rz[29] as described previously,[20] and replicon pRep-R6-NRz was engineered by replacing a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) in pRep-R6Rz with a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) from the 1bneo/delS plasmid. The final replicon, pRep-R6FLR-NRz, was constructed by replacing the neomycin phosphotransferase (*neo*^{r}) gene of pRep-R6-NRz with a chimeric gene encoding firefly luciferase protein fused in-frame with the 2A genes of FMDV and *neo*^{r}.

The HCV genotype 1b replicon pRep-R6CE2-NRz was assembled and cloned from pRep-R6-NRz and pHCR6-Rz. Plasmid pRep-R6CE2-NRz was engineered by replacing the HCV internal ribosome entry site gene (nt 1–389) in pRep-R6-NRz with a Core-NS2 gene (nt 1–3030; *Rsr*II site) from the pHCR6 plasmid. The pRep-R6CE2-NRz replicon was constructed by fusing the HCV NS2 protein gene in-frame with the genes for FMDV 2A protein and *neo*^{r}.

Cell culture and transfection

We maintained the human hepatoma cell line HuH-7 in complete Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA). G418 was added to a final concentration of 500 μg/ml to cell lines carrying HCV replicons.[20] Replicon cells were transfected with synthetic siRNA using Trans IT TKO reagent (Mirus, Madison, WI, USA) or with modified siE, dicer-generated siRNAs, long dsRNA and DNA vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Also, Rz-HepM6 cells[29] were transfected with various amounts of dsRNAs or DNA vector using Lipofectamine 2000.

Luciferase assays

The luciferase assay was performed using the Steady-Glo or Bright-Glo luciferase assay systems (Promega). Luciferase activities were quantified using a luminometer (Mithras LB940; Berthold Technologies, Wildbad, Germany).

Cell viability assay

To evaluate the cytotoxic effects of dsRNAs, cell viability was measured by metabolic conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) using a Cell Counting Kit-8 (Wako, Tokyo, Japan) according to the manufacturer's protocol.

Immunoblot analysis

Immunoblot analysis was performed as described previously.[29] Anti-p53 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies.

Real-time detection–polymerase chain reaction analysis

The HCV genome RNA and IFN-β mRNA were quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) as described previously.[24,29]

Quantification of hepatitis C virus core protein

Hepatitis C virus core protein was assessed in cell lysates using a fluorescent enzyme-linked immunosorbent assay.[40]

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Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model

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Abstract

Serine palmitoyltransferase (SPT) is a first-step enzyme in the sphingolipid biosynthetic pathway. Myriocin is an inhibitor of SPT and suppresses replication of the hepatitis C virus (HCV) replicon. However, it is still unknown whether this SPT inhibitor suppresses HCV replication in vivo. We investigated the anti-HCV effect of myriocin against intact HCV using chimeric mice with humanized liver infected with HCV genotype 1a or 1b. We administered myriocin into HCV infected chimeric mice and succeeded in reducing the HCV RNA levels in serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. Furthermore, combined treatment with pegylated interferon reduced the HCV RNA levels to less than 1/1000 of the control levels. We strongly suggest that suppression of SPT reduces HCV replication, and therefore that the SPT inhibitor is potentially a novel drug in the treatment of HCV infection.

Keywords: HCV; Myriocin; Serine palmitoyltransferase; Lipid raft; Chimeric mice with humanized liver

Hepatitis C virus (HCV) infection usually causes chronic hepatitis and often leads to cirrhosis of the liver or hepatocellular carcinoma [1,2]. The number of carriers now amounts to approximately 3% (~170 million) of the population worldwide. The most effective treatment against HCV infection is a combination of pegylated interferon (PEG-IFN) and ribavirin [3,4]. However, many people cannot tolerate the serious side effects and thus the number of patients able to receive the therapy is limited. The development of novel drugs to treat HCV with greater safety and better efficacy is therefore urgently required.

HCV is a single-stranded RNA virus that belongs to the Flaviviridae family [5]. The RNA genome produces at least 10 viral proteins, which include structural and non-structural (NS) proteins. The former are involved in the formation of the HCV particle. The latter play a key role in HCV genome replication [6]. It is generally accepted that a complex of NS proteins is associated with the lipid raft on the Golgi and endoplasmic reticulum membranes, where HCV replication occurs [7,8]. Thus, disruption of assembly of the lipid raft may lead to suppression of HCV replication.

Myriocin (ISP-1) is a specific inhibitor of serine palmitoyltransferase (SPT), a first-step enzyme in the sphingolipid biosynthetic pathway (Fig. 1A; [9,10]). Myriocin inhibits SPT activity due to its structural similarity to sphingosine (Fig. 1B), resulting in decreased intercellular sphingomyelin and its intermediates, dihydrosphingosine, sphingosine, ceramide, and sphingosine-1-phosphate (Fig. 1A). Inhibition of SPT by myriocin is thought to eventually lead to disruption of lipid raft assembly, as sphingomyelin is one of the major integral components of its assembly [11].

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