Dual effects of duplex RNA harboring 5′-terminal triphosphate on gene silencing and RIG-I mediated innate immune response

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Duplex RNA harboring the 5′-terminal triphosphate RNA is hypothesized to not only execute selective gene silencing via RNA interference, but also induce type I interferon (IFN) through activation of the retinoic acid inducible gene I (RIG-I). We evaluated gene silencing efficacy of the shRNA containing 5′-triphosphate (3p-shRNA) targeting the hepatitis C virus (HCV) RNA genome in hepatic cells. Gene silencing efficacy of the 3p-shRNA was diminished due to the presence of the 5′-triphosphate moiety in shRNA, whereas the shRNA counterpart without 5′-triphosphate (HO-shRNA) showed a strong antiviral activity without significant induction of type I IFN in the cells. 3p-shRNA was observed to be a better activator of the RIG-I signaling than the HO-shRNA with an elevated induction of type I IFN in cells that express RIG-I. Taken together, we suggest that competition for the duplex RNA bearing 5′-triphosphate between RIG-I and RNA interference factors may compromise efficacy of selective gene silencing.

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1. Introduction

Partially or fully duplex RNA that produces RNA interference (RNAi) in eukaryotic cells can be synthesized chemically or produced by in vitro transcription using bacteriophage promoters from linearized DNA templates [1,2]. RNA transcribed using bacteriophage RNA polymerase has 5′-terminal triphosphate, which has been shown to cause an innate immune response by triggering a cellular interferon response [3]. RNA bearing the 5′-terminal triphosphate can be recognized as a foreign RNA by retinoic acid inducible gene I (RIG-I), which is a pathogen-associated molecular pattern recognition receptor (PRR) [4,5].

RIG-I is a major detector and protector among pathogen derived nucleic acid detecting receptors such as MDA5 and TLR3, 7, and 8 [5]. Duplex RNA (dsRNA) with 5′ triphosphate produced during viral replication are known as pathogen-associated molecular pattern (PAMP) substrates, which are critical triggers for activation of antiviral immunity mediated by RIG-I [4,5]. Under RIG-I signaling, activated interferon regulatory factor 3 (IRF3) induces interferon-α/β (IFN-α/β) and interferon stimulated genes (ISGs), which are key genes involved in innate immune defense [6,7]. Numerous reports have suggested that exogenous RNAs, including double-stranded RNA (dsRNA), can be recognized as foreign RNAs and induce the expression of interferon [8–10].

Previously, Poeck et al. designed bifunctional siRNA with 5′-triphosphate ends (3p-siRNA), which activates RIG-I-mediated interferon induction and contains gene silencing potency against the anti-apoptotic protein family member, Bcl2 [11]. A similar approach has recently been applied using 3p-siRNA to evoke innate immune activation by activating RIG-I dependent type I IFN production without affecting gene silencing to inhibit hepatitis B viral infection [12]. The bifunctional 3p-siRNA was also applied to induce type I IFN and chemokine as well as gene silencing of TGF-β in pancreatic tumor tissue, resulting in systemic immune cell activation and profound tumor cell apoptosis in vivo [13]. However, the relationship between RNA-mediated innate immune response and the target gene silencing effect of siRNA has not yet been fully elucidated.

In this study, we evaluated efficacy of duplex RNAs bearing 5′-triphosphate on gene silencing and RIG-I mediated interferon
response in hepatic cells harboring the HCV genome. Short hairpin RNA containing 5′-triphosphate (3p-shRNA) targeting the HCV genome was compared with the shRNA counterpart without 5′-triphosphate (HO-shRNA). At a low dose of 3p-shRNA transfection into the cells, the efficacy of target gene silencing via RNAi was diminished as compared with HO-shRNA. We suggest that 3p-shRNA is likely sequestered by the RIG-I dependent interferon induction pathway due to the favorable binding of 3p-shRNA to the RIG-I protein. Furthermore, we demonstrated that when the 3p-siRNA was transfected into the cells, the RNA can be partitioned into both the RIG-I dependent interferon induction pathway due to the favorable binding of 3p-shRNA to the RIG-I protein. Furthermore, we demonstrated that when the 3p-siRNA was transfected into the cells, the RNA can be partitioned into both the RIG-I dependent interferon induction pathway and the gene silencing pathway via RNAi.

2. Materials and methods

2.1. RNA oligonucleotides and plasmids

5′-Triphosphate RNAs were prepared by in vitro transcription using T7 RNA polymerase as described previously [14]. Plasmid pGL3-IFN-β and pRL-TK that were previously used for the luciferase reporter assay for interferon [15] were kindly provided by Dr. Fan Xiu Zhu (Florida State University, Tallahassee, FL, USA). Human RIG-I overexpression plasmid, pUNO-hRIG-I, was purchased from Invivogen (San Diego, CA, USA), pEGFP-C2 vector and pDS-Red vector for green fluorescent protein and red fluorescent protein expression, respectively, were purchased from Clontech (Mountain View, CA, USA). pcDNA-HCV NS3/4A construct was generated by cloning the HCV NS3/4A genes that were PCR-amplified from the HCV replicon genome into the eukaryotic expression vector, pcDNA3.1/His A (Invitrogen), and the recombinant construct was confirmed by sequencing. Yeast tRNA and Poly (I:C) nucleotides were purchased from Invitrogen (S. Louis, MO, USA).

2.2. Cell culture and transfection of RNA

The carcinomic human alveolar basal epithelial cell line A549, carcinomic human liver cell line HepG2 and Huh7 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The Huh7 cells carrying the modified HCV subgenomic replicon RNA of the genotype 1b strain (GenBank accession number AJ238799) were obtained from the Mogam Biotechnology Research Institute (Yongin, Gyeonggi-do, Korea) and maintained with 0.5 mg/ml G418 (Sigma). The Huh7 cells carrying the HCV subgenomic replicon RNA harboring the luciferase reporter system (Huh-7/Luc-Neo cells) were kindly provided by Dr. Bartenslager (University of Heidelberg, Germany). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 100 U of penicillin, 100 μg of streptomycin and 10% fetal bovine serum (FBS) at 37 °C under 5% CO2. Transfection of RNA oligonucleotides was conducted by using LipoFectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. Luciferase reporter gene assay

For a luciferase reporter assay, cells were plated in a 24-well plate (1.0 × 10^5 cells) and then incubated for 12 h, after which they were transfected with the reporter constructs, pGL3-IFN-β (0.2 μg) and pRL-TK (0.1 μg) with or without expression plasmid for RIG-I (pUNO-hRIG-I, 0.5 μg). Subsequently, the cells were further incubated for 6 h to induce the expression of RIG-I, after which they were transfected with the designated amount of RNA oligonucleotides. After 18 h, the cells were disrupted with Passive Lysis buffer (Promega) and the cell extracts were subjected to the luciferase assay using a dual-luciferase assay system (Promega). The luminescences of firefly luciferase and Renilla luciferase were measured using a VICTOR X3 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA), and the ratio of firefly luciferase to Renilla luciferase was calculated. Each luminescence was normalized against EGFP fluorescence as a control of the transfection efficiency.

2.4. Western blot analysis and reverse-transcription PCR

Total cell lysates (40 μg) were separated by electrophoresis on an 8% SDS–polyacrylamide gel, after which they were transferred to a nitrocellulose membrane (Whatman, Piscataway, NJ). The blots were then blocked with PBS containing 4% nonfat milk for 1 h at room temperature and probed with monoclonal antibodies specific for HCV NS3 (ViroStat, Portland, ME), human RIG-I (Santa Cruz Biotechnology), or β-actin (Santa Cruz Biotechnology).

Total cellular RNA was extracted from the cells using easy-BLUE (Intron Biotechnology). A total of 6 μg of the total RNA was used to generate cDNA from each sample using a RT-PCR premix kit (Intron Biotechnology). RIG-I specific PCR was conducted using the following primers: RIG-I forward primer, 5′-TCTTTTATGAG-TATGTCGGCA-3′, and RIG-I reverse primer, 5′-TCGGCCACAGAA-TATCCTT-3′ and 30 cycles of (i) 60 s at 94 °C; (ii) 90 s at 60 °C; (iii) 40 s at 72 °C. HCV NS3/4A gene specific PCR was conducted using the following primers: HCV NS3/4A forward primer, 5′-TAG-GATCCCTCAGGGGCTATATA-3′; HCV NS3/4A reverse primer, 5′-ATGAAATCCACCTTCTACATCGAACC-3′. PCR was conducted by subjecting the sample to 30 cycles of (i) 30 s at 94 °C; (ii) 90 s at 60 °C; (iii) 75 s at 72 °C. The PCR products were analyzed by 2% agarose gel electrophoresis.

2.5. Fluorescent microscopic imaging of cells

A549 cells were plated onto 12-well plate (1.5 × 10^5 cells) and then incubated for 12 h. After incubation, they were co-transfected with pEGFP-C2 (3 g) and pDS-Red (3 g) with or without 10 or 100 nM of each siRNA targeting EGFP gene; conventional siRNA (siRNA), 5′-triphosphate siRNA (3p-siRNA), and 5′-dephosphorylated siRNA (HO-siRNA) by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After further incubation for 24 h, the cells were washed with phosphate buffered saline (PBS, pH 7.4) and observed by an inverted fluorescence microscopy (Axiovert200, Carl Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

Comparison of the data between experimental groups was statistically analyzed by an unpaired Student’s t-test. All data shown are the means ± S.D. Statistical significance was set at P < 0.05.

3. Results and discussion

3.1. Gene-silencing efficacy of 3p-shRNA targeting the HCV RNA genome

Firstly, to assess the antiviral efficacy of shRNAs against the HCV genome in HCV replicon-infected Huh7 cells, we designed shRNAs targeting the internal ribosomal entry site (IRES) region of HCV RNA genome (Fig. 1A), which has long been recognized as an effective anti-HCV targeting region [16]. To produce shRNA containing 5′-terminal triphosphate (3p-shRNA), shRNA was synthesized using T7 RNA polymerase, while the synthesized shRNA counterpart without 5′-terminal triphosphate (HO-shRNA) was prepared. Negative control shRNAs with scrambled sequences (sc_shRNA) were also designed and prepared as either 3p-sc_shRNA or HO-sc_shRNA (Fig. 1A).
Fig. 1. Effects of 3p-shRNA and HO-shRNA on silencing of HCV genome expression. (A) Schematic presentation of target sites for shRNA in the HCV subgenomic replicon RNA. Sequences and 5′-terminal structure of shRNAs are illustrated with notations. (B) Suppression of HCV NS3 protein expression with shRNAs in Huh7 cells carrying HCV subgenomic replicon RNA. Mock represents a transfection control (transfection reagent only). The values shown are the means ± S.D. from triplicate experiments. *P < 0.05.

Each shRNA was transfected into the HCV replicon-infected Huh7 cells and expression of the HCV genome (i.e., NS3) was measured by western blot analysis at 48 h after transfection. As shown in Fig. 1B, both 3p-shRNA and HO-shRNA were equally effective at gene silencing of HCV genome when 100 nM of shRNA was used. However, at a lower concentration (50 nM) of shRNA, HO-shRNA significantly more decreased expression of the HCV genome than 3p-shRNA (Fig. 1B). These gene silencing effects exhibited by the HCV IRES-targeting shRNAs were not observed with shRNAs containing scrambled sequences at all concentrations regardless of 5′-triphasate. To further confirm this observation, we carried out the luciferase reporter assay for monitoring expression of the HCV genome in hepatic cells. 3p-shRNA or HO-shRNA targeting the IRES of HCV at different concentrations was transfected into the HCV replicon-infected Huh7 cells harboring the luciferase reporter system (Huh-7/Luc-Neo cells). Suppression of HCV genome translation was expressed as the % change of luminescence relative to a transfection control (transfection reagent only). The values shown are the means ± S.D. from triplicate experiments. *P < 0.05.

3.2. Activation of RIG-I dependent interferon induction by 5′-triphasate RNA

The results shown above prompted us to hypothesize that exogenous 3p-shRNA be sequestered from RNA interference factors due to recognition of the 5′-triphasate motif by the intracellular RIG-I. To test the hypothesis, we investigated if the 5′-triphasate dsRNA elicits IFN production via RIG-I dependent innate immune response. We investigated several types of 5′-triphasate RNAs to determine if they induced IFN response in cells. The IFN-β activity of the interferon-stimulated response element (ISRE) in cells was measured at 24 h after transfection of each RNA. We first examined the induction of IFN production in the HCV-free naïve hepatoma cells (Fig. 2A). None of the RNAs stimulated the IFN promoter reporter gene activity regardless of the RNA transfection dosage. The naïve Huh7 cells failed to induce IFN in response to the 5′-triphasate RNA transfection at concentrations up to 100 nM. In contrast, RNAs containing the 5′-triphasate significantly increased expression of the type I IFN than the RNA counterparts without the 5′-triphasate in the A549 cells (Fig. 2B). We next examined whether the difference of the IFN induction by 5′-triphasate RNA between these two cell lines was caused by different amounts of intracellular RIG-I. The intracellular RIG-I was abundant in lung epithelial cells (A549 cells) whereas naïve Huh7 cells and other hepatoma cells (HepG2) expressed little RIG-I (Fig. 2C).
Based on these results, we hypothesized that the type I IFN induction by the 5'-triphosphate RNAs was caused by the RIG-I-dependent innate immune response pathway, in which intrinsic RIG-I level in cells is important to respond the exogenous RNAs containing 5'-triphosphate. To test this hypothesis, the naive hepatoma cells were cotransfected with the RIG-I expression plasmid (pUNO-RIG-I) plus 3p-shRNA and monitored for the IFN induction (Fig. 2D). In addition, the 3p-shRNA showed significant differences with the HO-shRNA in the activation of the IFN-β response upon overexpression of RIG-I, indicating that the 3p-shRNA was significantly better as an activator of type I IFN than the HO-shRNA (Fig. 2D).

3.3. Attenuation of RIG-I-dependent IFN induction by 3p-shRNA in HCV replicon-infected Huh7 cells

To determine if the RIG-I signaling pathway was influenced by overexpressing HCV NS3/4A in cells, type I IFN induction with 3p-shRNA in naive Huh7 cells overexpressing RIG-I was examined in cells harboring the NS3/4A expression plasmid (pcDNA-NS3/4A). The HCV NS3/4A complex is an antagonist of RIG-I signaling that functions by cleaving interferon-β promoter stimulator I (also known as Cardif, MAVS, or VISA), a key effector molecule of RIG-I functions by cleaving interferon-beta promoter. Overexpression of HCV NS3/4A in Huh7 cells eliminated the type I IFN response exerted by 3p-shRNA, despite RIG-I overexpression (Fig. 3A). Since overexpression of HCV NS3/4A did not influence the level of RIG-I expression in cells (Fig. 3A), attenuation of IFN induction was caused by the presence of HCV NS3/4A. These data indicate that 3p-shRNA-mediated IFN induction in naive Huh7 cells was indeed caused by activating the RIG-I signaling pathway, which can be abrogated by the HCV NS3/4A complex in Huh7 cells [19].

Since HCV NS3/4A that was transiently expressed in naive Huh7 cells diminished RIG-I-mediated IFN induction by 3p-shRNA, we further examined whether the shRNAs targeting the HCV RNA genome could activate the intracellular RIG-I to induce type I IFN in HCV replicon Huh7 cells that stably expressed NS3/4A. Although

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**Fig. 2.** Interferon induction by 3p-RNA transfection in Huh7 cells and A549 cells. Naive Huh7 cells or A549 cells were transfected with 3p- or HO-shRNA; 3p-sc or HO-sc shRNA; or 3p-ss or HO-ssRNA (90 nt). Each luciferase reporter assay was conducted as described in Section 2. RNA induced-activation of IFN-β, fold index

<table>
<thead>
<tr>
<th>RNA Type</th>
<th>Naive Huh7 Cells</th>
<th>A549 Cells</th>
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<tbody>
<tr>
<td>5'-OH RNA</td>
<td>10 nM</td>
<td>10 nM</td>
</tr>
<tr>
<td>5'-PPP RNA</td>
<td>++</td>
<td>++</td>
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<td>HCV IRES shRNA</td>
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<td>Scrambled shRNA</td>
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<tr>
<td>ssRNA 90 nts</td>
<td><strong>+</strong></td>
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**3p-shRNA in naive Huh7 cells overexpressing RIG-I was examined in cells harboring the NS3/4A expression plasmid (pcDNA-NS3/4A). The HCV NS3/4A complex is an antagonist of RIG-I signaling that functions by cleaving interferon-beta promoter stimulator I (also known as Cardif, MAVS, or VISA), a key effector molecule of RIG-I. Overexpression of HCV NS3/4A in Huh7 cells eliminated the type I IFN response exerted by 3p-shRNA, despite RIG-I overexpression (Fig. 3A). Since overexpression of HCV NS3/4A did not influence the level of RIG-I expression in cells (Fig. 3A), attenuation of IFN induction was caused by the presence of HCV NS3/4A. These data indicate that 3p-shRNA-mediated IFN induction in naive Huh7 cells was indeed caused by activating the RIG-I signaling pathway, which can be abrogated by the HCV NS3/4A complex in Huh7 cells [19]. Since HCV NS3/4A that was transiently expressed in naive Huh7 cells diminished RIG-I-mediated IFN induction by 3p-shRNA, we further examined whether the shRNAs targeting the HCV RNA genome could activate the intracellular RIG-I to induce type I IFN in HCV replicon Huh7 cells that stably expressed NS3/4A. Although
HCV replicon Huh7 cells were found to slightly upregulate RIG-I expression when compared to naive Huh7 cells (Fig. 2C), RIG-I was further overexpressed in the HCV replicon cells by transient transfection of RIG-I expression plasmid into the cells. The 3p-shRNA was transfected into the HCV replicon cells at two different concentrations (10 and 100 nM), after which an IFN promoter reporter assay for IFN-β induction was conducted (Fig. 3B). 3p-shRNA transfection (10 nM) induced almost no response, regardless of RIG-I overexpression, whereas slight activation of the IFN-β promoter was observed in response to 100 nM 3p-shRNA by about 2-fold greater than that of cells treated with 10 nM 3p-shRNA. The enhanced IFN induction was not observed in response to 100 nM HO-shRNA, which was not affected by the overexpression of RIG-I in HCV replicon cells (Fig. 3B).

3.4. Effects of 5′-triphosphate within siRNA on gene silencing activity

The results described above indicated that duplex RNA containing 5′-triphosphate was an effective ligand recognized by RIG-I to induce IFN production in cells. Therefore, we hypothesized that small interfering RNAs (siRNAs) with 5′-triphosphate would be also recognized as ligand by RIG-I, and activating IFN induction. To assess the effects of the 5′-triphosphate within siRNA on gene silencing activity as well as IFN induction via RIG-I activation, we designed a set of siRNAs which target the same site on the enhanced-green fluorescence protein (EGFP) gene (Fig. 4A); (1) conventional siRNA with 2-nt 3′ RNA overhangs (siRNA), (2) siRNA with 5′-triphosphate with blunt end (3p-siRNA), and (3) 3p-siRNA counterpart treated with phosphatase (HO-siRNA). It is well characterized that the presence of 2-nt 3′ overhang within duplex RNA precludes activation of the RIG-I signaling in cells; whereas the blunt end within duplex is required to stimulate the RIG-I signaling pathway [8,20]. Each siRNA was transfected into the A549 cells and the IFN-β induction was measured at 24 h post-transfection. 3p-siRNA and Poly (I:C) as a positive control induced the IFN-β induction was greatly enhanced even at low dosage (10 nM) of transfection, whereas the IFN induction was less increased with 10 nM of the conventional siRNA and HO-siRNA than that with 3p-siRNA (Fig. 4B).

Next, we assessed gene silencing efficacy of the modified 3p-siRNA against EGFp expression in the A549 cells. To evaluate the gene silencing potencies of the each siRNA, RNA was co-transfected with expression vectors pEGFP-C2 (an EGFP transient expression construct) and pDS-Red (a RFP transient expression construct as a negative control) into the A549 cells, and fluorescence was monitored and quantified at 24 h post-transfection (Fig. 4C). The cells transfected with 3p-siRNA at low dosage (10 nM) exhibited attenuated gene silencing efficacy in A549 cells with an inhibition rate of less than 30%, as compared with the conventional siRNA or HO-siRNA that showed an inhibition rate of more than 50%. In contrast, at moderate concentration of siRNA (100 nM), 3p-siRNA exhibited a comparable EGFP silencing activity to the one observed with conventional siRNA or HO-siRNA. As observed with the siRNAs targeting HCV viral genome (100 nM transfection dosage in Fig. 1), both 3p-siRNA and HO-siRNA were equally effective in suppression of the target gene when 100 nM of siRNA was used (Fig. 4C). These results suggest that the presence of 5′-triphosphate moiety within the siRNA may attenuate the selective gene silencing potency.

In conclusion, present study support a model for dual function of 5′-triphosphate shRNA capable of both gene silencing and binding to the immune dsRNA sensor RIG-I, which results in sequestration of the 3p-shRNA from RNA interference bona fide factors such as Dicer and RNA-induced silencing complex (RISC) in cells. Because the RIG-I and RNA interference factors coexist in cytoplasm, the exogenous 3p-shRNAs can compete for binding in cells. We found that our designed anti-HCV 3p-shRNA displayed marginal interferon response in HCV replicon-infected Huh7 cells when compared to naive Huh7 cells. Due to the immune-evasion mechanism of HCV proteins, activated RIG-I signaling seemed to be suppressed. If activating RIG-I is necessary and shown to be clinically safe in the treatment of viral diseases, it may be preferable to use the bifunctional RNAs for gene silencing and immune stimulation. Thus, as a synergistic antiviral agent, application of 5′-triphosphate shRNA or siRNA at a moderate dosage may serve to induce both the innate immune response and the RNA interference, achieving more profound therapeutic efficacy.
Acknowledgments

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References


Fig. 4. Effects of 5'-triphosphate within siRNA targeting EGFP gene on gene silencing activity. (A) Schematic presentation of a set of the siRNAs to target the enhanced green fluorescence protein (EGFP) gene: conventional EGFP-targeting siRNA with 2 nt-3' overhang (siRNA), 5'-triphosphate EGFP siRNA (3p-siRNA), and 5'-dephosphorylated EGFP siRNA (HO-siRNA). (B) The A549 cells were co-transfected with pGL3-INFβ and pRL-TK with or without pUNO-hRIG-I. The cells were transfected with each siRNA (10 or 100 nM), yeast tRNA as negative control, or Poly I:C as positive control at 6 h post-transfection, and further incubated for 18 h. Each luciferase reporter assay for measuring the IFN induction was conducted as described in Section 2. RNA induced-activation of IFN-β promoter was represented as the fold-index relative to the mock transfection control. The values are the means ± S.D. from triplicate experiments, *P < 0.05, asterisks. (C) Fluorescent microscopic imaging of cells transfected with siRNAs. The A549 cells were co-transfected with 10 or 100 nM of each siRNA together with plasmid constructs; pEGFP-C2 and pDs-Red, the EGFP transient expression construct and the RFP transient expression construct (as a transfection control), respectively. After incubation for 24 h, both of EGFP and RFP expression in the cells were imaged (left) and quantified (graph in right panel). EGFP expression was represented as the % fluorescence change relative to the Mock transfection control (transfection reagent only).