High doses of siRNAs induce *eri-1* and *adar-1* gene expression and reduce the efficiency of RNA interference in the mouse

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RNAi (RNA interference) is a gene-silencing mechanism that is conserved in evolution from worm to human and has been a powerful tool for gene functional research. It has been clear that the RNAi effect triggered by endogenous or exogenous siRNAs (small interfering RNAs) is transient and dose-dependent. However, there is little information on the regulation of RNAi. Recently, some proteins that regulate the RNA-silencing machinery have been identified. We have observed in previous work that the expression of target genes rebounds after being suppressed for a period of time by siRNAs. In the present study, we used secretory hepatitis B virus surface antigen gene as a reporter and compared its expression level in cell culture and mice challenged by different doses of siRNAs. A quicker and higher rebound of gene expression was observed in mice tail-vein-injected with higher doses of siRNA, and the rebound was associated with an increase in the mRNA level of *meri-1* (mouse enhanced RNAi) and *adar-1* (adenosine deaminase acting on RNA) genes encoding an exonuclease and RNA-specific adenosine deaminase respectively. Down-regulation of *meri-1* by RNAi enhanced the sensitivity and efficiency of siRNA in inhibiting the expression of hepatitis B virus surface antigen. These results indicate that RNAi machinery may be under negative regulation, through the induction of a series of genes coding for destabilizing enzymes, by siRNAs introduced into the cell, and also suggest that a suitable amount of siRNA should be used for research or therapeutic applications.

Key words: *Escherichia-coli*-expressed and enzyme-digested RNA (esiRNA), hydrodynamic injection, mouse enhanced RNA interference gene (*meri-1*), RNA interference (RNAi), RNA interference regulation, small interfering RNA (siRNA).

INTRODUCTION

RNAi (RNA interference) is a widely conserved phenomenon of specific gene silencing among nearly all eukaryotes [1–4], in which dsRNA (double-stranded RNA) induces the sequence-dependent degradation of cognate mRNA in the cytoplasm. Once introduced into the cell or transcribed from a transgene, dsRNA is first cleaved by Dicer, a member of the RNase III family, into siRNAs (small interfering RNAs) approx. 21–23 nucleotides in length, containing a two-nucleotide overhang at the 3′ end of each strand [5]. Then, R2D2 and Dicer complex delivers siRNA into a multi-component RISC (RNA-induced silencing complex), where its cognate mRNA degrades [6]. RNAi has been proven to be a powerful tool to knock down specific genes *in vitro* and *in vivo*, and clinical trials have showed it to be a potential technology to validate new drug targets and to treat diseases [7–9].

RNAi regulates the expression of downstream target genes, but the interference itself is also under regulation by unknown mechanisms. In *Caenorhabditis elegans* and plants, it has been found that the silencing effect can be amplified through the spread of silencing signals by enzymes called RDRPs (RNA-dependent RNA polymerases), which synthesize dsRNA from target mRNA. Several negative regulators of RNAi have been identified [10]. Protein p19, a viral suppressor protein encoded by plant tombusvirus, for example, has been found to bind a duplex of 21-nucleotide siRNAs with a two-nucleotide 3′-overhanging end(s). The binding allows p19 to discriminate siRNA duplexes from longer or shorter dsRNAs, thus interfering with the host cell's RNA silencing machinery. This is believed to be a defence strategy to thwart the host cell's immune response against RNA viruses [11–13]. In another example, ADARs (adenosine deaminases acting on RNA) in mammalian cells convert adenosine into inosine, eliminating the complementarity between the dsRNA and the target mRNA, and also destabilize the edited dsRNA, making it a poor substrate for Dicer and limiting siRNA efficacy in the cell [14–16]. However, the mechanisms of the regulation of RNAi have not been fully understood.

Studies of the regulation of gene function *in vivo* by RNAi can be performed in cell culture and/or whole model animals. Typically, *in vivo*, siRNA is introduced at a dose of approx. 1 μg for 5×10^5 cells (dozens to 100 nmol/l) or 1 nmol (approx. 10 μg) for 6–8-weekold mice to give rise to a satisfactory silencing effect. However, in previous work performed in our laboratory, we observed a striking phenomenon that a relatively higher dose of purified esiRNAs (*Escherichia-coli*-expressed and enzyme-digested siRNAs) had an inhibitory effect of shorter duration than a lower dose of esiRNAs, in both cell culture and animal models (Z. Qian, B. Xuan and J. Hong, unpublished work). The quicker and higher rebound of gene expression for a high dose of siRNA has never been investigated before, although similar phenomena may have been observed in some studies [17,18]. It seems that a repelling mechanism in the cell might be triggered by the introduction of a large amount of siRNAs. Recently, a highly conserved exonuclease-activity-containing protein ERI-1 (enhanced RNAi) was discovered through genetic screens in *C. elegans* [19]. Genetically dysfunctional *eri-1* mutants show increased RNAi in neurons. Biochemical analysis suggests that ERI-1 recognizes and blocks siRNA. In the present work, we try to find out whether this new member of the RNAi machinery plays a regulatory role in RNAi in mammals. We identified a

Abbreviations used: Adar, adenosine deaminase acting on RNA; CHO, Chinese-hamster ovary; dsRNA, double-stranded RNA; eri, enhanced RNA interference; esiRNA, *Escherichia-coli*-expressed and enzyme-digested siRNA; HBsAg, type B hepatitis virus surface antigen; HBVP, type B hepatitis virus polymerase; meri-1, mouse eri-1; NP, nucleoprotein; RDRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; RT, reverse transcription; siRNA, small interfering RNA.

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mouse homologue of the *C. elegans eri-1* gene (named *meri-1*) and prepared esiRNA from bacterial-cell-expressed dsRNA of the DNA fragment. Our results showed that exogenous siRNA caused an increase of *meri-1* and *adar-1* mRNAs, and that silencing of *meri-1* rescued RNAi effectiveness, and indicated that the RNAi machinery may be under negative regulation, through the induction of a series of genes coding for destabilizing enzymes, by siRNAs introduced into the cell.

MATERIALS AND METHODS

Construction of dsRNA-expressing plasmids

To express dsRNA in *E. coli*, a pET2P plasmid was constructed by positioning a tac promoter into pET-22b [20] in an opposite direction to the T7 promoter separated by multiple cloning sites. The DNA fragment encoding the second half of the HBVP (type B hepatitis virus, subtype adr, polymerase) gene was PCR-amplified with the following primers: HBVP-sense 5′-GGAATTCGTC-TTGGGTATACATTTGACC-3′ (the introduced EcoRI site is underlined) and HBVP-antisense 5′-GGGGTACCAGAGGAC-AACAGAGTTG-3′ (the introduced KpnI site is underlined). The DNA fragment containing exon 2 and exon 3 of seven mouse *eri-1* exons (MERI-1) (GenBank® accession number NM_026067) was amplified from mouse cDNA with following primers: eri-1-sense 5′-CGGAATTCGCAGACTTGAT-3′ (the introduced EcoRI site is underlined) and eri-1-antisense 5′-CC-GGTACCTGGCCTCACATA-3′ (the introduced KpnI site is underlined). The DNA fragment encoding part of the avian influenza virus NP (nucleoprotein) was amplified from cDNA with following primers: np-sense 5′-GCGAATTCTCTGCACTCATCC-TGAGAGG-3′ (the introduced EcoRI site is underlined) and np-antisense 5′-CGGGTACCTACTCCTCTGCATTGTCTCC-3′ (the introduced KpnI site is underlined). The DNA fragments obtained above were first cloned into pBluescript II KS (Stratagene) with the introduced restriction enzyme recognition sites and sequence-verified, then subcloned into pET-2P between EcoRI and KpnI sites to obtain pET2P-HBVP, pET2P-MERI-1 and pET2P-NP respectively.

Reporter plasmid pCMV-iHBS was constructed as described in [21] containing an HBsAg (type B hepatitis virus surface antigen)-coding sequence placed downstream of mouse Igκ-chain leader sequence, which enables the expressed protein to secrete to the outside the cell. The secretory plasmid was used for both cell culture assay and animal testing.

Expression of dsRNA in *E. coli* and esiRNA preparation

The dsRNA expression vectors obtained above were transformed into *E. coli* strain BL21(DE3) (Stratagene). Single colonies were inoculated into 2 ml of LB (Luria–Bertani) medium supplemented with 100 μg/ml ampicillin and cultured with shaking (250 rev./min) at 37 °C overnight. The seed culture was inoculated into 200 ml of fresh medium and continued growing until the D_{550} reached 0.6, and IPTG (isopropyl β-D-thiogalactoside) was added to final concentration of 0.25 mmol/l to induce for 3 h before cells were harvested by centrifugation at 3800 ***g*** for 15 min. The dsRNA purification and esiRNA preparation were manipulated with the method described previously [18]. Briefly, RNA-containing cell lysate in 20 % ethanol was passed through a Whatman® fibrous cellulose CF-11 column equilibrated with 20 % ethanol containing 1× STE (10 mM Tris/HCl, 100 mM NaCl and 1 mM EDTA, pH 8.0). After washing with 5 column vol. of 1× STE containing 18 % ethanol, dsRNA was then eluted out of the column with 20 ml of 1× STE, ethanol-precipitated and resuspended in 500 μl of RNase-free water. The concentration and purity of the dsRNA preparation was determined by measuring the absorbance at 260 nm and 280 nm.

To prepare siRNAs, 100 μg of purified long dsRNA was digested with 100 μg of recombinant RNase III (Ambion) in a 400 μl reaction mixture containing 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 10 mM $MnCl_2$ and 1 mM DTT (dithiothreitol) at 37 °C for 1 h. The digestion mixture was separated on a 15 % non-denaturing polyacrylamide gel, and the esiRNAs of approx. 21–23 bp in length were recovered from gel with the method described by Yang et al. [22].

Cell culture and transfection

CHO (Chinese-hamster ovary) cells were grown at 37 °C in an atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum (Biological Industries, Kibutz Beit Haemek, Israel), streptomycin (100 μg/ml) and penicillin (100 units/ml). To establish a cell line that constitutively expresses HBsAg, we transfected 600 ng/ml pCMV-iHBS plasmid DNA into CHO cells in a 24-well plate (70 % confluence) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. The level of HBsAg in the medium was measured 72 h after transfection, and G418 was added to cells to a concentration of 800 μg/ml. G418-resistant cells were then serially diluted to make constitutively expressive clonal HBsAg strains named CHO-iHBS cell strains.

For esiRNA dose–response experiments, CHO-iHBS cells from six-well plates (70 % confluence, approx. 5×10^6 cells) were transfected with 4–10 μg of esiHBVP using Gene Pulser Xcell™ system (Bio-Rad) according to the manufacturer's instructions. Cells were immediately seeded into new six-well plates with fresh medium. Every 24 h, medium was removed for analysis, and the cells were replenished with fresh medium. Secretory HBsAg in the medium was analysed using an ELISA.

Animal care and treatment

Male ICR mice (6–8 weeks old, 18–20 g; Shanghai Laboratory Animal Center, Shanghai, China) were used for hydrodynamic injection (high-volume intravenous injection). For dose–response experiments, appropriate amounts of esiRNAs and reporter plasmid pCMV-iHBS in 2 ml of Ringer's buffer were injected into mice within 5 s. Control mice were injected with 2 ml of Ringer's buffer. The procedure was performed in accordance with the requirements of the Shanghai Laboratory Animal Center of Shanghai, which proved the procedure to be safe for animals. Mouse blood was obtained on the indicated days after injection, and the titre of HBsAg in sera was measured using the ELISA.

RT (reverse transcription)–PCR analysis

Total RNA was isolated from freshly harvested livers of mice injected with pCMV-iHBS plasmid DNA and siRNAs using a Qiagen RNA isolation kit. RT was performed from total RNA using RNase-free MMLV (Moloney murine leukaemia virus) reverse transcriptase (Takara, Osaka, Japan). To correct the amplification process for tube-to-tube variability in amplification efficiency, β-actin mRNA was used as an internal standard for the semiquantification of the RT–PCR. The primers for β-actin were 5′-TGATGGACTCCGGTGACGG-3′ (forward) and 5′-TGTC-ACGCACGATTTCCCGC-3′ (reverse). After normalization with β-actin amplicon (179 bp), the same amount of cDNA was used as a template to amplify *meri-1* and *adar-1* genes using the following primers: *meri-1*-sense primer 5′-CGGAATT-CGCAGACTTGAT-3′ and *meri-1*-antisense primer 5′-CCGG-TACCTGGCCTCACATA-3′; *adar-1*-sense primer 5′-GCTCT-AGAGTTCCAGTACTGTGTAGCAGT-3′ and *adar-1*-antisense

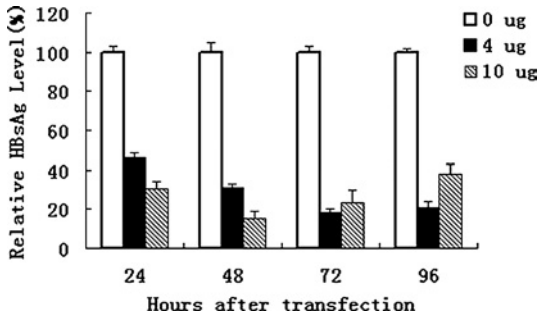

Figure 1 Suppression of HBsAg expression by esiHBVP in CHO-iHBS cells

CHO-iHBS cells (5×10^6) were transfected with the indicated amounts of esiHBVP or PBS as a control. Amounts of HBsAg secreted into the medium were measured at 24, 48, 72 and 96 h post-transfection. Data are presented as percentages of the amounts of HBsAg in transfected cells compared with those in untransfected CHO-iHBS cells (control). Results are means ± S.D. for three independent experiments.

primer 5′-ATGCGAATTCGGATCCTTGGGTTCGTGAGGAG-GTCC-3′. The PCR program was set up as follows: denaturing at 94 °C for 1 min, annealing at 52 °C for 0.5 min and extension at 72 °C for 1 min. The number of amplification cycles was 30 for *meri-1* and *adar-1* genes and 25 for β-actin. Then 15, 20, 25, 30, 35, 37 and 40 cycles of each kind of RT–PCR were performed to verify that under the described conditions the PCR-amplification of each fragment was still in the linear range. Samples were analysed on a 2 % agarose gel stained with ethidium bromide. The density of bands was quantified by using a Molecular Imager FX Pro Fluorescent Imager (Bio-Rad).

RESULTS

Higher doses of esiRNA induced stronger rebound of HBsAg expression after a period of suppression in CHO-iHBS cells

It is clear that inhibition of gene expression is sequence-specific and dose-dependent, and that the RNAi effect is transient and usually lasts 3–4 days. Previous work in our laboratory suggested that the expression of homologous gene rebounds after 3–4 days of suppression by esiRNAs and that the rebound effect was stronger in the cells or in animals challenged with higher doses of siRNAs than those challenged by lower doses of siRNAs (Z. Qian, B. Xuan and J. Hong, unpublished work).

To examine inhibitory effects of esiRNA of HBVP, we first linearized HBsAg-expressing plasmid pCMV-iHBS and integrated it into the genome of CHO cells to establish a cell line in which HBsAg is constitutively expressed (see the Materials and methods section). We then transfected these CHO-iHBS cells with 4 μg or 10 μg of esiHBVP dissolved in PBS. Approx. 5×10^6 cells/well were used for transfection and the same volume of PBS without any DNA was used as a negative control. We measured the concentration of HBsAg secreted into the medium at various time points after transfection and normalized the expression of secretory HBsAg relative to the negative control. The results showed a continuous increase of inhibition of HBsAg expression in cells transfected with 4 μg of esiHBVP from 24 h to 72 h before a slight rebound at 96 h post-transfection, while cells given 10 μg of esiHBVP elicited a better inhibitory effect at an earlier stage and began to rebound at 72 h post-transfection (Figure 1). It seems that the inhibitory effect of RNAi began to be lost at later time points and the overall expression level of the gene in the cells began to rise. Interestingly, the cells given higher doses of siRNA showed a much higher rebound at 96 h after transfection. To explain this phenomenon, it might be possible that some sort of repelling mechanism is triggered in the cell when large amounts of siRNA were introduced into cells to protect cells from RNA viral infection.

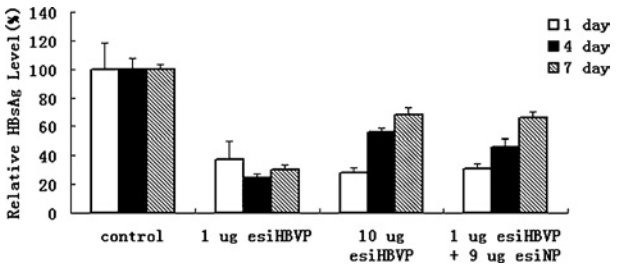

Figure 2 Relative expression of HBsAg in serum of mice injected with pCMV-iHBS and esiHBVP/esiNP

ICR mice were randomly assigned to four groups with six mice in each group. Reporter plasmid (10 μg) pCMV-iHBS and 1 μg of esiHBVP, 10 μg of esiHBVP or 1 μg of esiHBVP plus 9 μg of unrelated esiNP were administered to each group of mice by hydrodynamic intravenous injection. Mouse blood was obtained 1, 4 and 7 days after injection, and the amount of HBsAg was measured by ELISA. Results are mean percentages ± S.D. of the amounts of secreted HBsAg in the serum of mice injected with siRNAs compared with those of control mice for three independent experiments.

Higher doses of esiRNA reduced the RNAi effect faster than lower doses in mice

We then tested the inhibitory effect of esiHBVP in mice by hydrodynamic injection (high-volume injection in tail vein), an effective method to transfer nucleic acid to up to 40 % of mouse liver cells [23,24]. We treated separate groups of mice with 10 μg of reporter plasmid pCMV-iHBS, together with indicated amount of siRNAs. A group of mice was given additional esiNP as non-specific control. At days 1, 4 and 7 after injection, we measured the amount of HBsAg in serum. Similar to the findings obtained in cell experiments, all challenged mice revealed a reduced level of expression by siRNAs (Figure 2). However, on day 4 after injection the mice given 10 μg of siRNA showed 58 % of the HBsAg level in the serum of uninjected mice, compared with approx. 22 % in mice given 1 μg of siRNA. On day 7 after injection, the HBsAg level of the group given 10 μg of siRNA came back to 70 % of the control, and those with the lower dose of siRNA also showed a slight increase of secretory level compared with 3 days before. Remarkably, mice injected with 1 μg of esiHBVP and 9 μg of unrelated esiNP displayed a much stronger rebound of secretion level, a quite similar pattern to that of mice given 10 μg of esiRNA. These results suggest that the RNAi machinery may be down-regulated at some point after large amounts of siRNAs are introduced into the cells.

Silencing of *meri-1* gene could make RNAi more effective in mouse liver

Kennedy et al. [19] identified an exonuclease activity encoded by gene *eri-1* in *C. elegans*, whose normal function suppresses RNAi in most tissues. The *eri-1* gene encodes an evolutionarily conserved protein with domains homologous with nucleic-acid-binding and exonuclease proteins. Mouse genome database searches revealed a mouse orthologue of *eri-1* (named *meri-1*) with seven exons. We amplified a fragment overlapping and including the whole of exon 2 and the N-terminal part of exon 3 from mouse cDNA and made its esiRNA as described in the Materials and methods section. We treated mice with reporter plasmid pCMV-iHBS and different amounts of esiHBVP together with or without esiMERI-1 using hydrodynamic injection. The amount of HBsAg in serum after injection was assayed to monitor

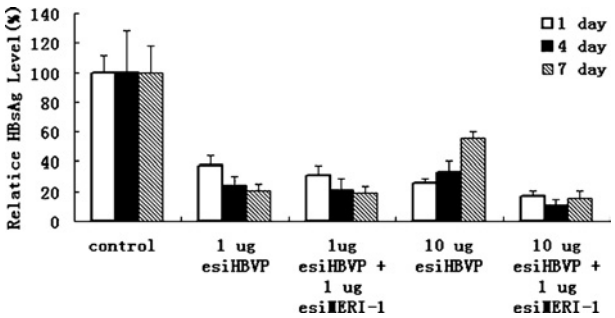

Figure 3 Expression of HBsAg in serum of mice injected with pCMV-iHBS and esiHBVP/esiMERI-1

ICR mice were randomly assigned to five groups with six mice in each group. Reporter plasmid (10 μg) pCMV-iHBS and different doses of siRNAs as indicated were given to each group of mice by hydrodynamic intravenous injection. Mouse blood was obtained 1, 4 and 7 days after injection, and secretory HBsAg was measured. Results are mean percentages $\pm$ S.D. of the amounts of secreted HBsAg in serum of mice injected with siRNAs compared with those of control mice for three independent experiments.

RNAi efficacy. The mice injected with 1 μg or 10 μg of esiHBVP accompanied by 1 μg of esiMERI-1 displayed a much lower secretion level of HBsAg, compared with those not simultaneously given 1 μg of esiMERI-1 (Figure 3), suggesting that the decline (if not the loss) of the function of exonuclease MERI-1 may increase the efficacy of the RNAi machinery in mouse liver; this enhancement started the first day of injection and lasted for at least 7 days.

RT–PCR analysis of the expression level of ERI-1 in mice

In *C. elegans*, the *eri-1* gene was identified to be an exonuclease that negatively regulates RNAi machinery. We then examined whether or not the expression level of *meri-1* in the liver changed when siRNA was introduced into the body. Various amounts of esiHBVP or non-related control esiNP were injected into mice by using hydrodynamic injection method. At 4 days after administration, total RNA was extracted from livers and RT–PCR was performed using *meri-1* and *adar-1* gene-specific primers. All reactions were normalized with β-actin. As shown in Figure 4, the mRNA levels of *meri-1* and *adar-1* genes were increased markedly by the introduction of exogenous siRNAs. The group injected with 10 μg of esiHBVP showed a near 3-fold increase of mRNA level with the *meri-1* gene and over 4-fold increase with the *adar-1* gene than the uninjected group. The increase was also observed in the group injected with 1 μg esiHBVP plus 9 μg of non-specific esiNP. However when 1 μg of esiERI-1 was injected into mice together with 10 μg of esiHBVP, the mRNA levels of both *meri-1* and *adar-1* were reduced. In particular, the *meri-1* mRNA showed a level close to that of mice injected with only 1 μg of esiHBVP. It seems that the administration of high doses of exogenous siRNAs, either 10 μg of esiHBVP or 1 μg of esiHBVP plus 9 μg of esiNP, induced the expression of *meri-1* and *adar-1* genes, and the addition of 1 μg of esiERI-1 offset, to some extent, the increase of *meri-1* mRNA.

DISCUSSION

In most organisms, short interfering dsRNAs can reduce the accumulation of a sequence-related mRNA, often resulting in a loss-of-function phenotype, a process known as RNAi. Although RNAi is an extremely powerful tool for gene silencing and has therefore been widely investigated in gene functional studies and therapy trials, a complete understanding of its mechanisms has not yet been reached. Many components of the interference machinery still remain to be identified.

Several lines of evidence have shown that RNAi appears to be suppressed by certain cellular and viral factors. For instance, p19 protein encoded by plant viruses and ADAR-1 by mammalian cells have been identified as being able to suppress RNAi efficacy by binding to siRNA and competing with RNAi machinery [12–16].

The loss of function of a putative RDRP of *C. elegans*, RRF-3, makes *C. elegans* supersensitive to RNAi in diverse tissues [25]. One hypothesis is that the RRF-3 protein may compete with RRF-1 and EGO-1 for components of intermediates in the RNAi reaction. These data indicate that RNAi in the cell is under physiologically negative modulation [26,27].

A genetic screen for mutants of *C. elegans* with enhanced sensitivity in the nervous system to dsRNA has identified an

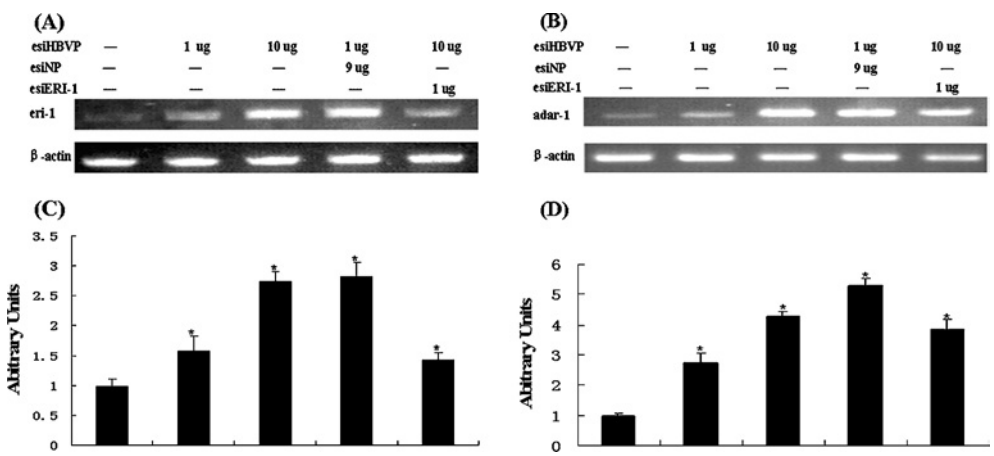

Figure 4 RT-PCR analysis of *meri-1* and *adar-1* gene expression in livers of mice injected with different doses of siRNAs

Each group of mice was injected with 10 μg of pCMV-iHBS plasmid DNA and esiRNAs as indicated. At 4 days after injection, total RNA of mouse liver was extracted and subjected to semi-quantitative RT–PCR. RNA samples were normalized with β-actin before the detection of expression levels of *meri-1* and *adar-1* genes. RT–PCR samples were analysed on 2 % agarose gels stained with ethidium bromide. The density of bands was quantified by using Molecular Imager FX Pro Fluorescent Imager (Bio-Rad). (**A**, **B**) Typical electrophoretic profiles of *meri-1* and *adar-1* amplification products on agarose gels respectively. (**C**, **D**) Statistical analysis of mRNA levels of *meri-1* and *adar-1* determined by densitometric analysis of respective bands in three independent experiments. Each bar represents an average of measurements from more than six mice. Results are means $\pm$ S.E.M. $^*P < 0.05$, significantly different from the corresponding controls.

evolutionarily conserved gene *eri-1* encoding a protein with nucleic acid binding and exonuclease activity, loss of which increased RNAi in neurons and other tissues. ERI-1 was suggested to inhibit RNAi by degrading 3′ overhangs of siRNAs, and the resulting siRNAs lacking 3′ overhangs may be non-functional because they may fail to enter the RISC. The human orthologue of ERI-1 (3′hExo) can participate in processing a histone mRNA with stem–loop structure. It is a major regulator of histone mRNA biogenesis and metabolism [28]. However, the function of the mouse orthologue of ERI-1 is not yet clear.

In the present work, we tested the inhibition of expression of transgene of HBsAg using different amounts of siRNA in the presence or absence of *meri-1*. It has been accepted that the inhibition of target gene expression by siRNA is dose-dependent when the usual concentrations of siRNA were tested [29–31]. Our dose-dependence analyses carried out in cell culture and animal experiments using esiRNA confirmed this effect. However, our results revealed an unexpected decrease of the inhibitory effect that was associated with the introduction of higher doses of esiHBVP, compared with lower doses of esiHBVP. The reduction of the RNAi effect may be caused by the induction of exonuclease ERI-1 and ADAR, as revealed by RT–PCR analysis. It appeared that, while triggering RNAi, the introduction of higher doses of esiRNAs might induce the expression of a series of enzymes, such as exonuclease ERI-1 and ADAR, to limit or completely degrade the exogenous dsRNAs. This may act as a protective mechanism in the cell against viral infection. This phenomenon could be triggered by non-specific siRNAs and suppressed by siRNA of *meri-1*. This notion was suggested for the first time and was supported by experimental data from RT–PCR analysis in the present work and data obtained from microarray analyses [32,33]. Our results from the present study indicate that RNAi machinery may be under negative regulation through the induction of a series of genes coding for destabilizing enzymes by siRNAs introduced into the cell. They also indicated that suitable amounts of siRNA should be used for research or therapeutic application. However, more studies need to be done to elucidate the negative regulation mechanism that underlies this phenomenon.

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Received 21 April 2005/17 June 2005; accepted 11 July 2005
Published as BJ Immediate Publication 11 July 2005, doi:10.1042/BJ20050647