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Generation of stable 3′-mRNA cleavage fragments induced by siRNA in cells with high-levels of duck hepatitis B virus replication

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ABSTRACT

Therapeutic small interfering RNAs (siRNAs) have attracted a lot of interest both in basic biomedical sciences as well as in translational medicine. Apart from their therapeutic efficacy adverse effects of siRNAs must be addressed. The generation of stable mRNA cleavage fragments and the translation of N-truncated proteins induced by antisense oligodeoxynucleotides (ASOs) have been reported. Similar to ASOs, siRNAs are considered to function via an antisense mechanism that promotes the cleavage of the target mRNA. To further investigate whether the stable mRNA cleavage fragments also occur in siRNA we constructed a short hairpin RNA (shRNA) expression plasmid, pshRNA794, containing the same sequence reported in experiments using ASOs which directly targeted the overlapping region of the pre-genomic mRNA (pgmRNA) and sub-genomic mRNA (sgmRNA) of duck hepatitis B virus (DHBV). The shRNA resulted in a 70.9% and 69.9% reduction of the DHBV mRNAs in LMH and HuH-7 cells, respectively. In addition a 70% inhibition of the DHBV DNA level was observed. Interestingly, 3′-mRNA cleavage fragments were detected in LMH but not in HuH-7 cells. Taken together, our findings demonstrate that the ASO sequence was also effective in siRNA. Importantly, our results provide direct evidence that stable 3′-mRNA fragments were generated by siRNA in cells with high levels of DHBV replication. Whether these can cause adverse RNAi effects needs to be explored further.

1. Introduction

RNAi is a naturally occurring, highly conserved post-transcriptional gene silencing mechanism triggered by double-stranded microRNAs (miRNAs) [1]. The gene silencing ability of RNAi provides a mechanism that can be exploited for novel therapeutic applications. More than 30 clinical trials have been conducted to date involving 21 different siRNAs [2].

HBV is an excellent candidate for siRNA therapy because it has an unusually compact genome that lacks redundancy, resulting in a limited sequence plasticity and a low risk viral mutations that may result in evasion from the siRNAs action. Therefore, a single siRNA would ideally target multiple viral transcripts simultaneously, and because HBV is amplified through an RNA intermediate, viral DNA replication would also be inhibited [3]. A phase I clinical trial on the use of siRNA NUC B1000 in patients with HBV infection has been reported, indicating that it is safe and well-tolerated. However, all study participants reported pharyngitis, chills, fever, and increases in inflammatory cytokine levels [4]. Side effects of siRNAs include off-target effects, activation of IFNs and pro-inflammatory cytokines, saturation of the endogenous miRNA pathway, blood and lymphatic vessel endothelial cell toxicity, and induction of retinal degeneration, among others [5–10]. Although research involving therapeutic siRNAs has significantly progressed, the side effects of siRNAs may be more wide-ranging than previously thought. Therefore, the need to more carefully probe the possible side effects of siRNAs remains.

Hasselblatt reported that treatment with ASOs directed against DHBV mRNAs resulted in the generation of stable 3′-mRNA cleavage fragments in human and avian cells that are efficiently translated into N-terminal truncated proteins via a cap-independent mechanism [11]. ASOs are very similar to siRNAs and are used to inhibit gene expression by inducing endonucleolytic cleavage of target mRNAs [12]. A recent analysis revealed that Argonaute has a similar catalytic domain structure and activity to RNase H

Abbreviations: siRNA, small interfering RNA; ASOs, antisense oligodeoxynucleotides; shRNA, short hairpin RNA; DHBV, Duck hepatitis B virus; pgmRNA, pre-genomic mRNA; sgmRNA, sub-genomic mRNA; RISC, RNA-induced silencing complex; miRNA, microRNA; POL, polymerase; RC, relaxed circular; SS, single stranded.

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[13]. Therefore, we were interested to see whether stable RNA cleavage fragments are also generated using siRNAs.

In this study, we constructed a shRNA expression vector. To accurately compare the effects of ASO and siRNA cleavage and degradation on DHBV mRNA sequences, an isosequential siRNA directed against the same DHBV target site sequences as described by Hasselblatt was employed [11]. Our results showed that the ASO sequence present in the siRNA was equally effective. More importantly, we demonstrated that stable 3′-mRNA cleavage fragments were generated in cells with high levels of viral replication through the action of the siRNA.

2. Materials and methods

2.1. Construction of shRNA-expression plasmids targeting DHBV

The siRNA-ready pSIREN-shuttle expression plasmid containing the human U6 promoter was obtained from the Knockout Adenoviral RNAi System kit (Clontech, Palo Alto, CA, USA). The shRNA duplexes were directed against DHBV nucleotides 794–812, simultaneously targeting the polymerase region of the pgmRNA and the pre-S/S antigen region of the sgmRNA, as detailed in previous reports [11] (Fig. 1). Briefly, to construct the shRNA expression plasmid (pshRNA794), overlapping top and bottom strands of shRNA oligonucleotides were chemically synthesized (Microsynth, Windisch, Switzerland), annealed, and ligated into the siRNA-ready pSIREN-shuttle plasmid using BamH I and EcoR I. The negative control plasmid (pNeg), which contained a mock shRNA sequence, was similarly constructed.

2.2. Cell culture and transfections

LMH, an avian hepatoma cell line, supports the replication of DHBV. LMH cells were cultured in Iscove's Modified Dulbecco's Medium (GIBCO-BRL, Eggenstein, Germany), and the human hepatoma cell line HuH-7 was cultured in DMEM (PAN Biotech, Aidenbach, Germany). The media contained 8% fetal bovine serum (Biochrom, Berlin, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL, Eggenstein, Germany), and 1% nonessential amino acids (GIBCO-BRL, Eggenstein, Germany). DHBV RNA was generated using the pGemDHBV plasmid, which is a replication-competent head-to-tail dimer construct encoding DHBV F16 [14]. Cells were co-transfected using the Calphos kit (Clontech, Palo Alto, CA, USA). In a typical co-transfection experiment, LMH cells and HuH-7 cells seeded into 10-cm dishes (2.1×10^6 cells) were co-transfected with 5 μg of pGemDHBV and 5 μg of pshRNA794, respectively. Negative control transfections using 5 μg of pGemDHBV and 5 μg of pNeg were also performed. The cells were harvested after 72 h for further analysis. Meanwhile, the LMH and HuH-7 cells were transfected with 10 μg of the red fluorescent expression plasmid pcDNA3-mRFP (Addgene plasmid 13032) using the same method. The transfection efficiencies of the plasmids in LMH and HuH-7 cells were compared 72 h later under a fluorescence microscope.

2.3. Northern blot analysis

Total cellular RNAs were prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The samples were treated with 5 U of DNase I (GIBCO-BRL, Eggenstein, Germany) for 30 min to eliminate plasmid DNA contamination. Two oligonucleotide probes were designed using the DHBV sequence available in GenBank (Accession No. X12798). These probes recognize sites located upstream and downstream of the siRNA cleavage site. The sequences for the two probes were as follows: probe 762, 5′-TGACAAGGTGTTGCTGTTCCC-3′ (nt. 762–782); probe 1708, 5′-GGTGGCAGAGGAAGTCA-3′ (nt. 1708–1727). The oligonucleotide probes were chemically synthesized and purified via high-performance liquid chromatography (Microsynth, Windisch, Switzerland). Total cellular RNAs were separated using MOPS (morpholinopropanesulfonic acid)-buffered formaldehyde 1% agarose gels. The RNAs were blotted onto nylon membranes (Hybond N, References Amersham Pharmacia Biotech, Freiburg, Germany) via capillary transfer using 1.5 M NaCl and 150 mM sodium citrate. Prehybridization was performed using 5 × SSC, 5 × Denhardt's, 10% dextran sulfate, and 0.1 mg/ml denatured salmon sperm DNA at 65 °C for 1 h. Hybridization was subsequently conducted using the ^{32}P-labeled 762 probe at 65 °C for 2 h. Following hybridization, the blots were washed three times at 65 °C in 2 × SSC containing 0.1% SDS for 5 min and washed twice in 0.1 × SSC and 0.1% SDS at 65 °C for 15 min, then subjected to autoradiography at −80 °C. The blots were stripped via incubation them for 10 min in 0.1 × SSC and 0.5% SDS at 95 °C. The membrane was re-probed with the ^{32}P-labeled 1708 probe using the same experimental procedure.

2.4. Southern blot analysis

LMH cells were co-transfected with 5 μg of pGemDHBV and 5 μg of pshRNA794 or 5 μg of pGemDHBV and 5 μg of pNeg, as described in the cell culture and transfection section. The cells were then detached from the culture dish through trypsin treatment and lysed in chilled buffer containing 140 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0), and 0.5% Nonidet P-40. The cell nuclei and debris were subsequently removed via centrifugation, and the plasmid DNA was digested with 30 U of micrococcal nuclease for 2 h at 37 °C. EDTA was finally added to inactivate the nuclease, and the viral DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and visualized through Southern blot hybridization using the full-length DHBV genome as a probe.

2.5. Statistical analysis

The obtained Northern blot images were scanned, and the signals were analyzed quantitatively using Image J (NIH). The levels of remaining mRNA were expressed as a percentage of the negative

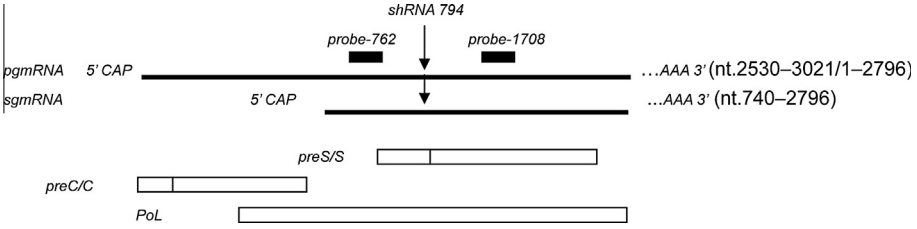

Fig. 1. Schematic representation of the DHBV open reading frame and pgmRNA and sgmRNA transcripts. The arrow indicates the location of the siRNA target site. The black bar indicates the 5′- and 3′-probe sites.

control. Data are expressed as the mean ± SEM. Each experiment was performed at least three independent times. The results were compared using Student's *t* test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Construction of the shRNA expression plasmid

To compare the experimental data reported by Hasselblatt with the data obtained in this study, the identical target site located in the DHBV genome was selected. The DHBV794 siRNA simultaneously targets both the sgmRNA and pgmRNA. We then cloned the synthetic DNA oligonucleotides into the mammalian siRNA-ready pSIREN-shuttle expression vector, which contains the human U6 promoter, to produce shRNA. The recombinant plasmids were verified by restriction digestion and sequence analysis.

3.2. Different levels of DHBV replication in LMH and HuH-7 cells

Human HuH-7 cells can support DHBV replication, but at a lower level than avian LMH cells, *in vitro* [15]. In order to determine whether the level of DHBV replication influences the action of siRNAs, high-replication and low-replication DHBV experimental models were established by transfection of LMH and HuH-7 cells, respectively with the DHBV replicon. Northern blot analysis of RNA isolated from transfected LMH and HuH-7 cells, respectively, showed that the avian LMH cells produced approximately six times more DHBV pgmRNA than the human HuH-7 cells (Fig. 2A and C). To determine whether different transfection efficiencies exist between the two cell lines, HuH-7 and LMH cells were transfected with the red fluorescence expression plasmid pcDNA3-mRFP using the same conditions as described for the DHBV replicon. No significant difference in transfection efficiency was observed between the two cell lines (Fig. 3). These results the avian LMH cells supported a significantly higher level of DHBV RNA (sgmRNA and pgmRNA) than the human HuH-7 cells, as reported previously [15,16].

3.3. Generation of an effective shRNA

Due to the effect of mRNA target-site nucleic acid sequences and secondary structures, usually only a few siRNAs are effective [17]. In our shRNA expression vector, the human U6 promoter that belongs to the RNA polymerase III promoter family, was employed to drive shRNA expression. To analyze the silencing effect, LMH cells and HuH-7 cells were co-transfected with pGemDHBV and pshRNA794 or pGemDHBV and pNeg, respectively, followed by Northern blot analysis of DHBV mRNA. These analyses revealed that siRNA significantly reduced DHBV mRNA levels in LMH and HuH-7 cells. While the viral pgmRNAs and sgmRNAs were almost completely degraded in HuH-7 cells transfected with pshRNA794 LMH cells remained higher viral pgmRNA and sgmRNA levels. Due to the different transcription levels of DHBV mRNAs in LMH and HuH-7 cells, respectively, the relative DHBV gene knockdown by pshRNA794 was compared. Relative to the negative control, the knockdown rate of the viral pgmRNAs was 70.9% in LMH vs. 69.9% in HuH-7 cells (Fig. 2D). Therefore, the silencing efficiency of pshRNA794 with respect to DHBV gene expression was not significantly different between LMH and HuH-7 cells.

In addition, pgmRNA is also an intermediate of DHBV replication and acts as a reverse transcription template for DHBV DNA synthesis. To evaluate the inhibition efficiency regarding DHBV DNA replication, the viral DNA levels in the LMH cell cytoplasm were determined by Southern blot analysis 72 h after transfection. Cytoplasmic DHBV DNA copy numbers were significantly reduced

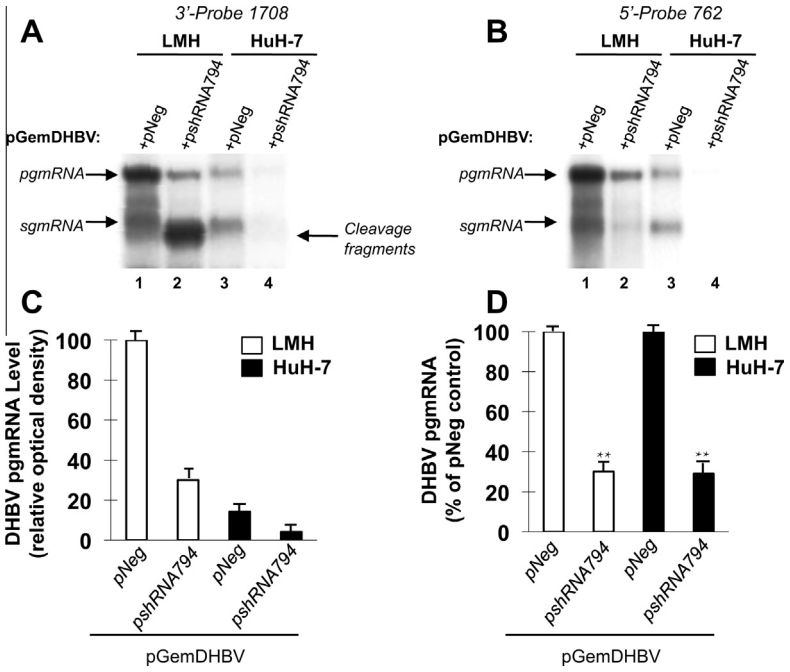

Fig. 2. DHBV replication and gene knockdown induced by pshRNA794 in LMH and HuH-7 cells, respectively. (A) Northern blot analysis of DHBV mRNAs isolated from LMH and HuH-7 cells co-transfected with pshRNA794 and pGemDHBV or pNeg and pGemDHBV using a 3′-oligonucleotide probe. (B) Northern blot analysis of viral mRNAs isolated from LMH and HuH-7 cells co-transfected with pshRNA794 and pGemDHBV or pNeg and pGemDHBV using a 5′-oligonucleotide probe. The levels of DHBV transcripts in LMH cells (lane 1) were compared to HuH-7 cells (lane 3). The effect of pshRNA794-mediated gene knockdown in LMH cells (lane 2) was compared to HuH-7 cells (lane 4). (C) Northern blots were quantitatively analyzed by image J software (NIH) based on the pgmRNA signals. (D) The degree of pgmRNA knock-down in LMH cells was compared to HuH-7 cells. The residual amount of DHBV pgmRNA was expressed as percentage of the pNeg shRNA control. The results represent three independent experiments. The average DHBV pgmRNA levels were analyzed using Student's *t*-test. $^{**}P < 0.05$ vs. the pNeg control.

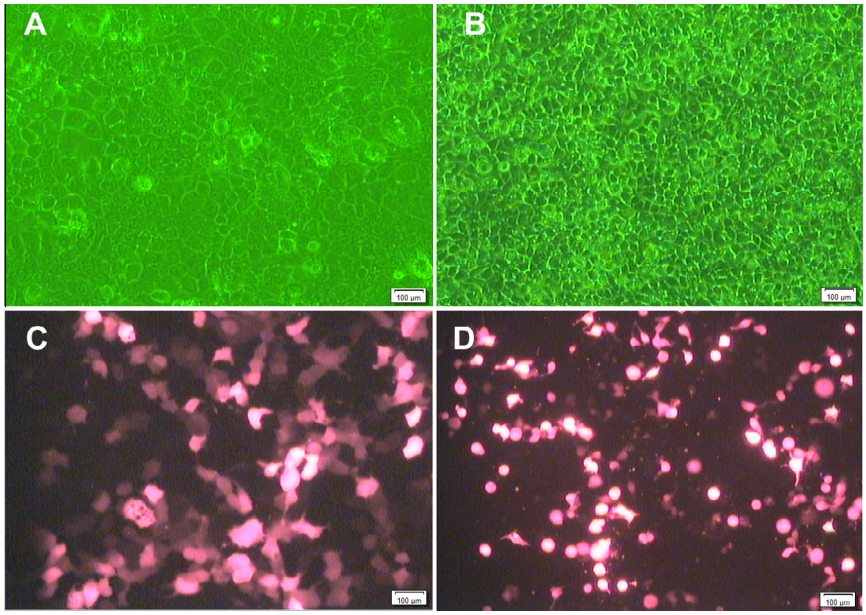

Fig. 3. The transfection efficiencies of plasmids in LMH and HuH-7 cells were compared by fluorescence microscopy. LMH and HuH-7 hepatoma cells were transfected with 10 μg of the red fluorescence expression plasmid pcDNA3-mRFP per 10 cm dish (~2.1 × 10^6 cells). Photographs were taken using bright-field and specific fluorescence filters for red fluorescence protein. (A and C) HuH-7 cells, (B and D) LMH cells. Original magnification 200×.

in LMH cells following transfection with pshRNA794 (~70%) (Fig. 4). Taken together, the analyses showed that the constructed shRNA expression vector was effective in LMH and HuH-7 cell lines, reducing target transcription and inhibiting viral DNA replication.

3.4. A significant number of 3′-mRNA fragments in LMH but not in HuH-7 cells

The resulting mRNA cleavage fragments induced by siRNA are usually rapidly degraded by cellular exonucleases makes the message permanently untranslatable. However, previous studies have shown that ASO-induced cleavage of target mRNAs often results in stable 3′-cleavage fragments rather than in rapid degradation of cleavage products [11,18]. In this study, a target site identical to that of ASO DHBV794 reported by Hasselblatt was selected. Both LMH and HuH-7 cells were cotransfected with pGemDHBV and pshRNA794, followed by Northern blot analysis DHBV mRNAs, using probes complementary to 5′ and 3′ regions of the cleavage site. Interestingly, an intense signal that was slightly smaller than the sgmRNA species was detected using a 3′-probe in the DHBV high-replication LMH cells. By contrast, this band was not observed in the DHBV low-replication HuH-7 cells even after overexposure of the blot (Fig. 2A). Different from 3′-cleavage fragment, 5′-cleavage fragments were not consistently observed in either cell line (Fig. 2B). These results were reproduced in triplicate experiments (data not shown).

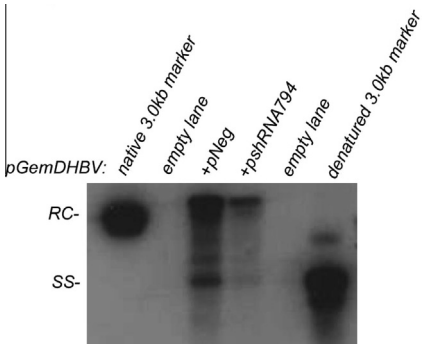

Fig. 4. Southern blot analysis of DHBV DNA isolated from the cytoplasm of LMH cells co-transfected with pGemDHBV and pshRNA794 or pGemDHBV and pNeg. Note the relative abundance of DHBV DNA in the cytoplasm of cells treated with pNeg or pshRNA794.

4. Discussion

siRNAs inhibit gene expression by degrading target mRNAs in a sequence-specific manner, and a new class of drugs designed to silence disease-causing genes in the body or to disarm an invading virus via gene knockdown has been developed using siRNAs [19,20]. However, the safety of treatment with siRNAs remains an important issue, and a more detailed investigation of their possible side effects is needed [21,22]. In the present study, we built an effective shRNA expression construct that cleaved DHBV pgmRNAs and sgmRNAs simultaneously and inhibited DHBV replication. Importantly, we showed, for the first time, that siRNAs generate stable 3′-mRNA fragments, similar to ASOs, which also give rise to several previously unknown side effects.

There are numerous methods for inducing RNAi to achieve gene knockdown. One of the most commonly used methods involves vector-based shRNAs. Compared to other methods, vector-based shRNA expression achieves a more sustained effect. ShRNA expression is commonly driven by an RNA polymerase III promoter, most often the U6 promoter. The U6 promoter is widely used to direct the expression of shRNAs because it is active in all cell types and efficiently directs the synthesis of shRNA transcripts with well-defined ends [23,24]. In this study, a commercial shRNA expression vector containing the human U6 promoter was used. The results showed that DHBV mRNAs were significantly reduced, and the rates of DHBV mRNA knockdown achieved by the siRNA relative to the negative control were equivalent in HuH-7 and LMH cells. In addition, the results of viral DNA analysis were consistent with those of the RNA analysis. Therefore, we constructed a shRNA expression vector that effectively inhibited DHBV gene expression

and replication and showed equivalent gene-silencing efficiencies in LMH and HuH-7 cells.

DHBV is a hepadnavirus that shows similarities to human HBV. Infection of the natural hosts of DHBV has been employed to simulate the conditions of human HBV infection. *In vitro*, DHBV replication is commonly studied in LMH cell line derived from chicken liver tumors. However, the HuH-7 cell line, which is derived from human hepatomas, is also competent for supporting DHBV gene expression and virus production. Nevertheless, the efficiency of DHBV mRNA transcription in HuH-7 cells is much lower than in LMH cells. Our results were consistent with previous studies [15,16]. The differential DHBV replication level between HuH-7 and LMH cells provided an ideal method for comparing the effects of viral target mRNA abundance on siRNA activity. Significant amounts of the 3′-mRNA cleavage fragments were found only in extracts from LMH cells. Therefore, siRNAs may induce the generation of stable 3′-mRNA fragments in high-virus replication-competent cells.

Hasselblatt reported that stable3′-mRNA cleavage fragments were observed when gene silencing was induced by ASOs, but not by siRNAs [11]. The siRNA sequence used in this study was identical to the ASO sequence reported by Hasselblatt, being specific for the same DHBV target site. However, our study found that a large number of 3′-mRNA fragments were generated following siRNA cleavage. Furthermore, these fragments were only limited to the transfected LMH cells and absent in the HuH-7 cells. In addition,5′-mRNA cleavage fragments were not detected by the 5′-probe. First, the results showed that the isosequences employed in both the ASO and siRNA experiments were effective. The ASO and siRNA were both designed to hybridize to a target RNA sequence via Watson–Crick base pairing rules. We postulate that this effect is due to the secondary structure of the mRNA target site, which is appropriate for the complementary corresponding ASO chain and might be also appropriate for the siRNA. Second, a large number of 3′-mRNA cleavage fragments can be generated through both the ASO/RNase H pathway and siRNA/RISC pathway. Mechanistic studies examining siRNA/RISC-mediated cleavage have revealed that Argonaute proteins are essential protein components of RISCs. This conserved family of proteins is characterized by two homologous domains, PAZ and PIWI. The PIWI domain resembles RNaseH [25]. In addition, P-bodies are involved in the normal process of intracellular mRNA turnover. Strong evidence now connects the RNA interference machinery with P-body formation [26]. In addition, Stein reported that delivered antisense oligonucleotides also apparently localize to P-bodies [27]. Indeed, ASOs and siRNAs share numerous common characteristics. We speculate that the mRNA cleavage fragments induced by the action of siRNAs or ASOs are not degraded through a dedicated pathway. For both the siRNA/RISC and ASO/RNase H pathways, following the first step of cleavage, the cleaved mRNA fragments are shuttled into the common second step decay pathway and degraded by 3′-5′ or 5′-3′ exonucleases. Therefore, it was not surprising that 3′-fragments were generated through both the ASO/RNase H and siRNA/RISC pathways.

Holen reported that 3′-mRNA cleavage fragments appeared only in the presence of a high rate of siRNA production and speculated that the limitation of the rate of mRNA decay was dependent on the degradation of mRNA cleavage fragments by exonucleases [17]. In this study, significant levels of DHBV mRNAs were transcribed in LMH cells, and pshRNA794 simultaneously targeted sgmRNA and pgmRNA. Uniform accumulation of 3′-cleavage fragments derived from pgmRNA and sgmRNA was produced following siRNA/RISC treatment, which exceeded the processing ability of the second step of the decay pathway. These fragments accumulated in the cytoplasm and were detected. By contrast, despite the fact that the same silencing efficiency of siRNA was observed in the HuH-7 cells, the quantity of DHBV mRNA transcripts was lower in these cells than in LMH cells. Thus, the resulting 3′-mRNA cleavage fragments did not enough saturate the second step of the decay pathway, and consequently, the 3′-fragments were quickly degraded in the HuH-7 cells. Therefore, the high rate of cleavage and superabundance of target mRNA produced the amount of 3′-mRNA cleavage fragments whether either ASO or siRNA was utilized, resulting from the limited processing ability of the second step of the mRNA decay pathway. This result shows that the generation of stable3′-mRNA cleavage fragments may be induced by siRNAs in cells with high levels of viral replication. The translation of truncated proteins from 3′-mRNA cleavage fragments via a cap-independent or recapped process has been reported [28]. Therefore, our findings could suggest that an unforeseen side effect associated with siRNAs results from the generation of novel polypeptides of unknown biological activity following siRNA-mediated cleavage of the target mRNA. Furthermore, the programmed degradation of normal cellular mRNAs occurred primarily in the P-body. Therefore, the siRNA saturates the inherent intracellular miRNA processing pathway as well as saturating the mRNA degradation pathway in P-bodies. This process could interfere with normal cellular mRNA turnover, which is important for some mRNAs for regulatory molecules which play a key role in the signal transduction pathway.

Collectively, one of the key challenges in developing RNAi as a therapy is avoiding potential side effects. The potential primary risk regarding side effects of siRNAs lies in a possible interference with the cellular machinery. Using siRNAs to target specific cellular or viral transcripts essentially hijacks the endogenous RNAi and normal mRNA degradation machinery. Indeed, the potential for saturating the RNAi pathway in primary cells has been described [29].

Acknowledgments

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