NEW METHODS AND TECHNOLOGIES

Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles

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ABSTRACT

Allele-specific gene silencing by RNA interference (RNAi) is therapeutically useful for specifically suppressing the expression of alleles associated with disease. To realize such allele-specific RNAi (ASP-RNAi), the design and assessment of small interfering RNA (siRNA) duplexes conferring ASP-RNAi is vital, but is also difficult. Here, we show ASP-RNAi against the Swedish- and London-type amyloid precursor protein (APP) variants related to familial Alzheimer’s disease using two reporter alleles encoding the Photinus and Renilla luciferase genes and carrying mutant and wild-type allelic sequences in their 3'-untranslated regions. We examined the effects of siRNA duplexes against the mutant alleles in allele-specific gene silencing and off-target silencing against the wild-type allele under heterozygous conditions, which were generated by cotransfecting the reporter alleles and siRNA duplexes into cultured human cells. Consistently, the siRNA duplexes determined to confer ASP-RNAi also inhibited the expression of the bona fide mutant APP and the production of either amyloid β 40- or 42-peptide in Cos-7 cells expressing both the full-length Swedish- and wild-type APP alleles. The present data suggest that the system with reporter alleles may permit the preclinical assessment of siRNA duplexes conferring ASP-RNAi, and thus contribute to the design and selection of the most suitable of such siRNA duplexes.

KEYWORDS: RNAi, allele-specific gene silencing, amyloid precursor protein, Swedish mutation, London mutation, reporter allele

INTRODUCTION

RNA interference (RNAi) is a powerful tool for suppressing the expression of a gene of interest (Dykxhoorn et al, 2003; Meister and Tuschl, 2004; Mello and Conte, 2004). In mammals, RNAi can be induced by direct introduction of synthetic small interfering RNA (siRNA) duplexes into cells or generation of siRNA duplexes using short-hairpin RNA expression vectors and its application is expanding to various fields of science; therapeutic use of RNAi in medical science and pharmacogenesiis is particularly promising (Caplen, 2004; Dykxhoorn et al, 2003; Hannon and Rossi, 2004; Karagiannis and El-Osta, 2005; Wood et al, 2003). Allele-specific gene silencing by RNAi (allele-specific RNAi: ASP-RNAi) is an advanced application of RNAi techniques, by which the expression of an allele of interest can be inhibited (Victor et al, 2002). Accordingly, ASP-RNAi is thought to be therapeutically useful, i.e., it can specifically suppress the expression of alleles causing disease without inhibiting the expression of corresponding wild-type alleles. To realize and control such ASP-RNAi, the following issues must be addressed: selection of competent siRNA duplexes that strongly induce ASP-RNAi; and qualitative and quantitative evaluation of allele-specific gene silencing.

In this article, we describe an easy assay system for assessment of ASP-RNAi with mutant and wild-type reporter alleles encoding the Photinus and Renilla luciferase genes. Using the amyloid precursor protein
(APP) variants (the Swedish- and London-type variants) related to familial Alzheimer’s disease (Goate et al, 1991; Mullan et al, 1992) as model mutant alleles, we determined the effects of siRNA duplexes against the mutant APP on allele-specific silencing as well as off-target silencing against the wild-type allele. The siRNA duplexes having the potential to specifically suppress the expression of the mutant reporter allele consistently inhibited the expression of the bona fide mutant APP as well as amyloid β 40- and 42-peptides in Cos-7 cells expressing both the full-length Swedish- and wild-type APP alleles. These observations suggest that the present system could permit the selection of siRNA duplexes having the potential to confer ASP-RNAi.

MATERIALS AND METHODS

Preparation of oligonucleotides
DNA and RNA oligonucleotides were obtained from INVITROGEN and TAKARA, respectively. For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 µM each) were mixed and annealed as described previously (Hohjoh, 2002). The sequences of synthesized oligonucleotides are shown in Tables 1 and 2. Non-silencing siRNA duplex (siControl; Qiagen) was used as a negative control.

Cell culture
HeLa, T98G and Cos-7 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (Wako) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) in 5% CO₂-humidified chamber. T98G cells (Registry No. IFO50295) were obtained from the Health Science Research Resources Bank.

Construction of reporter and expression plasmids
In order to construct plasmids carrying reporter alleles, the phRL-TK (Promega) and pG3-TK (Ohnishi et al, 2005) plasmids encoding the Renilla and Photinus luciferase genes, respectively, both of which were driven by the same herpes simplex virus thymidine kinase (TK) promoter, were digested with Xba I and Not I, and were subjected to ligation with synthetic oligonucleotide duplexes corresponding to the Swedish-, London- and wild-type APP alleles (sequences of the oligonucleotides used are indicated in Table 1). The resultant plasmids carry allelic APP sequences in the 3’-untranslated regions (UTRs) of the luciferase genes (Figure 1A). Expression plasmids, pAPP695WT and pAPP695Sw encoding full-length cDNAs of the wild- and Swedish-type APP alleles, respectively, were kindly provided by Dr Tanahashi (Tanahashi and Tabira, 2001).

Transfection and reporter assay
The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5 × 10⁵ cells/well). Cotransfection of synthetic siRNA duplexes with reporter plasmids was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions, and to each well, 0.24 µg (40 nM) of siRNA duplexes, 0.2 µg of pG3-TK-backbone plasmid, 0.05 µg of phRL-TK-backbone plasmid and 0.1 µg of pSV-β-Galactosidase control vector (Promega) were applied. Twenty-four hours after transfection, cell lysate was prepared and expression levels of luciferase and β-Galactosidase were examined by the Dual-Luciferase reporter assay system (Promega) and Beta-Glo assay system (Promega), respectively, according to the manufacturer’s instructions. In the case of transfection of siRNA duplexes and expression plasmids (pAPP695WT and pAPP695Sw) into Cos-7 cells, 0.4 µg of each plasmid and 0.24 µg of siRNA duplexes were applied. Forty-eight hours after transfection, culture media was collected and cell lysate was prepared.

Western blotting and ELISA
Culture media and cell lysate prepared from transfected Cos-7 cells were examined by western blotting as described previously (Lesne et al., 2003). Equal amounts of proteins were separated by SDS-PAGE and electrophoretically blotted onto PVDF membranes (Millipore). Membranes were blocked for 1 h in blocking solution (5% (v/v) fat-free milk and 0.05 % (v/v) Tween-20 in PBS) and

Table 1. Synthetic DNA oligonucleotides

| Name          | Sequence (5’---------3’)                          |
|---------------|--------------------------------------------------|
| ssAPPwt(Sw)   | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| asAPPwt(Sw)   | GCCCTGTCGGAATTCCTGCAATCTTTCACTGGAGATCTCTCCATG  |
| ssAPP(K670N-M671L) asAPP(K670N-M671L) | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| ssAPPwt(Lo)   | GCCCTGTCGGAATTCCTGCAATCTTTCACTGGAGATCTCTCCATG  |
| asAPPwt(Lo)   | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| ssAPP(V717I)  | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| asAPP(V717I)  | GCCCTGTCGGAATTCCTGCAATCTTTCACTGGAGATCTCTCCATG  |
| ssAPP(V717F)  | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| asAPP(V717F)  | GCCCTGTCGGAATTCCTGCAATCTTTCACTGGAGATCTCTCCATG  |
| ssAPP(V717G)  | CTAGCATGCGGAGATCTTCTGAAGTGAGATGGAGCATGGCAATCGCA |
| asAPP(V717G)  | GCCCTGTCGGAATTCCTGCAATCTTTCACTGGAGATCTCTCCATG  |
Table 2. Synthetic siRNAs used in this study. Sense- and antisense-stranded siRNA elements are indicated by ‘-ss’ and ‘-as’, respectively.

| Name             | Sequence (5’-------------3’)                        |
|------------------|---------------------------------------------------|
| si(T7/C8)-as     | AGUGAUCAUCUGAUCAGACUUU                            |
| si(T7/C8)-ss     | AUUGUCAUCUGAUCACACUUU                            |
| si(T8/C9)-as     | AAGUGAUCAUCUGAUCAGACUUU                           |
| si(T8/C9)-ss     | UUCUGAUCUGAUCACACUUU                             |
| si(T9/C10)-as    | GAAGUGAUCAUCUGAUCAGACUU                           |
| si(T9/C10)-ss    | UUCUGAUCUGAUCACACUUU                             |
| si(T10/C11)-as   | UGAUGUAUGAUGAUCAGACUU                            |
| si(T10/C11)-ss   | CUGAUCUGAUCACACUUU                               |
| si(T11/C12)-as   | CUGAUCUGAUCACACUUU                               |
| si(T11/C12)-ss   | UUCUGAUCUGAUCACACUUU                             |
| si(T12/C13)-as   | UGCUGAUCUGAUCACACUUU                             |
| si(T12/C13)-ss   | GCAUCUGAUCACACACACUUU                            |

RT-PCR

Total RNA extraction, including treatment with DNase I (Ambion) twice followed by reverse transcription, was carried out as described previously (Sago et al., 2004). The resultant cDNAs were examined by real-time (RT)-PCR using the ABI PRISM 7300 sequence detection system (Applied Biosystems) with a SYBER green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions. PCR primers used were as follows:

For detection of the Renilla luciferase transcript:

renilla-F; 5’-GGTTCTTTTCCAACGGATATTG-3’
renilla-R; 5’-GAAGCTCTTGATGTACTTAC-3’

For detection of the Photinus luciferase transcript:

photinus-F; 5’-TGTGGATGTGGATTTCGAG-3’
photinus-R; 5’-ATCGTATTTGTCAATCAGAG-3’

RESULTS

Assessment of siRNAs in heterozygous model system

In this study, the Swedish- and London-type mutants of the APP gene, which are involved in familial Alzheimer’s disease, were used as model mutant alleles. The Swedish- and London-type APP mutants carry double and single nucleotide substitutions, respectively, which are followed by amino acid substitutions (K670N-M671L in the Swedish APP, V717I, V717F or V717G in the London APP) (Goate et al., 1991; Mullan et al., 1992). The resultant amino acid sequences in the Swedish and London-type APPs are preferably digested by β- and γ-secretase, respectively, resulting in accumulation of Aβ40 and Aβ42 peptides, which are the key factors of Alzheimer’s disease (Cai et al., 1993; Citron et al., 1992; Mattson, 2004; Suzuki et al., 1994).

Mutant and wild-type reporter alleles were constructed as described in Materials and Methods. The resultant reporter alleles (Figure 1A), synthetic siRNA duplex against the mutant allele and the β-galactosidase gene (control) were cotransfected into human cells. Note that the transfected cells are artificially heterozygous with the mutant and wild-type APP reporter alleles; thus, the effects of test siRNA duplexes on suppression of both the mutant and wild-type alleles can be simultaneously examined.

ASP-RNAi against the Swedish-type APP allele

When the Renilla and Photinus luciferase genes were regarded as the Swedish and wild-type reporter alleles, respectively, the effects of the si(T7/C8) - si(T12/C13) duplexes against the Swedish mutant on allele-specific gene silencing were examined in HeLa cells. The results

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Figure 1. Assessment of ASP-RNAi with reporter alleles. (A) Schematic drawing of reporter alleles. Reporter alleles were constructed based on the *Photinus* and *Renilla* luciferase reporter genes driven by the same TK promoter, and allelic sequences of the reporter genes, i.e., the reporter alleles encode luciferase reporter genes carrying artificially inserted allele sequences of interest. Assessment of siRNA duplexes on the induction of ASP-RNAi against the Swedish *APP* mutant (B) and against the London *APP* mutants (C–E) was carried out. Synthetic siRNA duplexes against the mutants indicated were cotransfected with the mutant and wild-type reporter alleles and the *β*-galactosidase gene (control) into HeLa cells. The *Photinus* and *Renilla* luciferase genes carry the mutant and wild-type allelic sequences, respectively. Twenty-four hours after transfection, dual-luciferase and *β*-galactosidase assays were carried out. The levels of either *Photinus* (blue boxes) or *Renilla* (pink boxes) luciferase activity was normalized against the levels of *β*-galactosidase activity, and the ratios of mutant and wild-type luciferase activities in the presence of siRNA duplexes were normalized against the control ratio obtained in the presence of the siControl duplex (siCont). Data are averages of at least three independent determinations. Error bars represent standard deviations.

(A) Inserted allelic sequences

(B) N670N-M671L

(C) V717I

(D) V717F

(E) V717G

Western blot analyses of wild-type and Swedish *APP* in ASP-RNAi

We further investigated ASP-RNAi of si*APP* duplexes against the Swedish mutant with full-length cDNAs of the Swedish and wild-type *APP* alleles, which were transiently
expressed in Cos-7 cells. The pAPP695SWE and/or pAPP695WT expression plasmids encoding full-length cDNAs of the Swedish and wild-type APP alleles, respectively, and siRNA duplexes targeting the Swedish mutant were cotransfected into Cos-7 cells, and expression of wild-type APP (APPWT) and Swedish APP (APPsWE) was examined by Western blotting. As shown in Figure 2, under homo( or hemi)zygous-like conditions, in which either APPWT or APPsWE was expressed, the signal intensity of sAPPsWE (secreted APP) and cAPPsWE (cellular APP) was apparently decreased in the presence of the si(T8/C9), si(T9/C10) and si(T11/C12) duplexes. In contrast, signals for either sAPPWT or cAPPWT were detected in the presence of any of the siRNA duplexes examined, which is consistent with the data for the reporter alleles described above. When APPsWE and APPWT were both expressed in the cells (heterozygous-like conditions), signals for APP were seen in the presence of any of the siRNA duplexes. Based on the results under homozygous-like conditions, signals for APP in the presence of the si(T8/C9), si(T9/C10) and si(T11/C12) duplexes were most likely derived from APPWT.

The utility of ASP-RNAi using the siRNA duplexes assessed here in medical treatment can be demonstrated by confirming a significant decrease in Aβ peptides, which are a key factor in the development of Alzheimer’s disease under heterozygous conditions expressing both APPsWE and APPWT. We thus determined the production levels of Aβ40 and Aβ42 peptides by means of ELISA. As shown in Figure 3, significant decreases in the production of either Aβ40 or Aβ42 peptide by RNAi (Figure 3A-C) and ASP-RNAi (Figure 3D-F) with the evaluated siRNA duplexes, particularly si(T8/C9), si(T9/C10) and si(T11/C12), was confirmed under homozygous and heterozygous conditions, respectively. Therefore, these results suggest the potential utility of such siRNA duplexes as therapeutic agents.

DISCUSSION

While ASP-RNAi is believed to be a useful technique, to realize and control ASP-RNAi, it is vital to design and select competent siRNA duplexes conferring ASP-RNAi; however, this is rather difficult without a procedure for assessing such siRNA duplexes. The system we present here could allow assessment, if designed siRNA duplexes have the potential for specifically inhibiting the expression of target alleles without suppressing the expression of other alleles. From a series of experiments with the Swedish- and London-type APP variants as model mutant alleles, we were able to determine potential siRNA duplexes for inducing ASP-RNAi. With regard to siRNA duplexes targeting the Swedish mutant, we further demonstrated that the si(T8/C9), si(T9/C10) and si(T11/C12) siRNA duplexes were able to significantly decrease the production of either Aβ40 or Aβ42 peptide in Cos-7 cells expressing both the full-length Swedish- and wild-type APP alleles. Accordingly, such competent siRNA duplexes conferring ASP-RNAi against mutant alleles likely hold utility as therapeutic agents.

In contrast to the Swedish mutant, there were difficulties in suppressing the London-type mutants carrying single nucleotide substitutions from the wild-type allele by ASP-RNAi. The difference between ASP-RNAi activities against the Swedish- and London-type mutants may have been caused by the number of base substitutions: the former and latter mutants carry double and single base substitutions, respectively. Another important point to note in the results for the London-type mutant is that different substitutions showed different ASP-RNAi activities, suggesting that the type of base change between the mutant and wild-type alleles could influence ASP-RNAi. With regard to the V717I (Figure 1C) and V717G (Figure 1E) mutants, a possible wobble base pair between siRNA and the wild-type mRNA (Du et al, 2005) and high GC content of siRNA used (Ui-Tei et al, 2004), respectively, might have negatively influenced the induction of ASP-RNAi; these possibilities require further examination in the future.

To further progress ASP-RNAi, it is necessary to design competent siRNA duplexes conferring strong allele-specific gene silencing. Chemical modifications (Chiu and Rana, 2003; Hall et al, 2004) and structural devices in siRNAs are considered to be applicable for improving
ASP-RNAi, and assessment of such siRNAs is feasible using the system we presented here. Altogether, it is suggested that the present assay system may contribute to the design and selection of the most suitable of siRNA duplexes conferring ASP-RNAi.

Finally, we add data indicating the possible inhibition of wild-type allele translation by the present siRNA duplexes. Because si(T9/C10) and si(T11/C12) exhibited moderate levels of inhibition of the expression of wild-type reporter allele (Figure 1B), we further investigated RNA levels of the wild-type allele by RT (real-time)-PCR. As shown in Figure 4, the levels of RNA expression of the wild-type allele in the presence of si(T9/C10) were similar to those in the presence of siControl, suggesting the possible inhibition of translation of the wild-type allele by the si(T9/C10) duplex. This may be due to a microRNA-like effect (Poy et al, 2004; Tang, 2005), and further study into this possibility remains necessary. With regard to the si(T11/C12) duplex, because a decrease trend in the levels of wild-type allele transcript was seen, it is possible that off-target gene silencing (Jackson et al, 2003) of the wild-type allele may occur in the presence of the duplex. Consequently, it is conceivable that the present system could further contribute to studies into off-target gene silencing and the function of microRNAs.

**Figure 3.** Production of Aβ40 and Aβ42 peptides under ASP-RNAi. The pAPP695WT (A–C) plasmid and both the pAPP695SW and pAPP695WT (D–F) plasmids together with the indicated siRNA duplexes against the Swedish mutant were cotransfected into Cos-7 cells, and expressed sAPP polypeptide and Aβ40 and Aβ42 peptides in culture media were examined by western blotting (A, D) and ELISA (B, C, E, F), respectively. “Vector” indicates cells transfected with only plasmid(s). Endogenous and exogenous (expressed) sAPPs are indicated by asterisks and arrow heads, respectively. ELISA data are averages of three independent determinations. Error bars represent standard deviations.

**Figure 4.** Possible translation inhibition and off-target silencing of wild-type reporter allele by siAPP duplexes. The si(T9/C10) or si(T11/C12) duplexes against the Swedish mutant allele together with either wild or mutant reporter allele plasmid carrying Photinus luciferase and the phRL-TK plasmid encoding Renilla luciferase (control) were introduced into HeLa cells. Twenty-four hours after transfection, dual-luciferase assay and isolation of total RNA were carried out. Off-target (to wild-type reporter allele) (A) and on-target (RNAi; to mutant reporter allele) (C) gene silencing were assessed based on luciferase activities. Ratios of normalized target (Photinus) luciferase activity to control (Renilla) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the si(T9/C10) or si(T11/C12) duplexes were normalized against the ratios obtained in the presence of the siControl duplex (siCont). Isolated RNAs in (B) and (D) corresponding to (A) and (C), respectively, were subjected to reverse transcription to
CONCLUSIONS

The present assay system with wild-type- and mutant-reporter alleles could permit assessment of siRNA duplexes having the potential for specifically inhibiting the expression of the mutant allele without inhibiting the expression of the wild-type allele, and thus contribute to the design and selection of siRNA duplexes suitable for allele-specific gene silencing.

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STATEMENT OF COMPETING INTERESTS

Corresponding author has a pending patent on the method of this paper.

LIST OF ABBREVIATIONS

ASP-RNAi; Allele-specific RNA interference
APP; Amyloid precursor protein
TK; Thymidine kinase
UTR; Untranslated region
sAPP; Secreted APP
cAPP; Cellular APP
Aβ; Amyloid β

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