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Note

Fusogenic peptides enhance endosomal escape improving siRNA-induced silencing of oncogenes

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Abstract

Small interfering RNA (siRNA) molecules are the functional mediators of a post-transcriptional gene silencing process known as RNA interference (RNAi). The silencing of genes involved in diseases, using siRNA, is considered a very promising therapeutic strategy. However, as for all the nucleic acid based therapeutics, these negatively charged and hydrophilic molecules do not readily cross biological membranes. The use of cationic carriers generally results in positively charged complexes which are taken up by cells through endocytosis. Still, for gene silencing, these complexes need to escape through the endosomal membrane, thereby reaching the cytosol where all the RNAi machinery is present. One of the strategies developed to facilitate endosomal escape mimics the fusion of viral envelopes with host cell endosomal membranes, which occurs during viral infections. Several synthetic fusogenic peptides have been synthesized based on the fusion domain of the influenza virus. In this study we evaluated the effects of the influenza-derived fusogenic peptide diINF-7 on gene silencing efficiency of siRNA targeting the epidermal growth factor receptor (EGFR) and the K-ras oncogenes. For both targets, strong enhancement of gene silencing activity was noted after addition of diINF-7 fusogenic peptide, identifying endosomal escape as a limiting factor for siRNA silencing efficiency.

Keywords: Endosomal escape; Small interfering RNA; RNA interference; Fusogenic peptides; EGFR; K-ras

1. Introduction

RNA interference (RNAi) is regarded as an attractive and potent mechanism for silencing gene expression in a sequence-specific manner. Either used for understanding the function of genes, or interfering therapeutically with aberrant gene expressions, this technique has captured the interest of many researchers.

Short double-stranded RNA molecules, known as small interfering RNA (siRNA) are the functional mediators of RNAi. For RNAi to occur, siRNA molecules need to be present in the cytoplasm, where the post-transcriptional RNAi-silencing machinery is available. When siRNA assembles into the RNA-induced silencing complex (RISC), it can interact with and degrade complementary mRNA sequences, thereby interrupting the translation of specific proteins. However, the physicochemical properties of siRNA molecules, such as the relative large size (approximately 14 kDa), the negative charge and hydrophilicity, make it difficult for siRNA molecules to cross cellular membranes and to reach the cytoplasm. As a consequence, several viral and non-viral carrier systems have been developed in order to deliver chemically synthesized siRNA molecules into the cells. Non-viral systems are usually based on electrostatic complexation of negatively charged siRNA with positively charged polymers or lipids. The resulting complexes generally have a net positive charge, which facilitates the interaction with the negatively charged cellular membrane, and are likely taken up by cells through endocytosis. Once inside the endosomes, these complexes or their siRNA should be able to escape through the endosomal membrane, in order to avoid degradation and to allow RNAi to occur (Schiffelers et al., 2004).

A number of strategies have been proposed to facilitate endosomal escape: pore forming peptides, flip-flop of phospholipids, pH-buffering capacity by protonable groups (proton sponge),

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and photochemical internalization are some of the examples (Berg et al., 1999; Cho et al., 2003; Medina-Kauwe et al., 2005). This present research employs another strategy to facilitate endosomal escape: fusogenic peptides.

Since the early 1980s, many studies have been performed on membrane fusion activity of animal viruses and, as a result, many viral fusogenic peptide sequences have been identified (Wagner, 1999; White et al., 1982). The functional role of fusogenic peptides lies in the fusion process occurring between the viral envelope and host cell endosomal membrane, to transport the viral genome into the cytoplasm, after receptor-mediated endocytosis. The influenza virus hemagglutinin protein has an N-terminal fusion domain on the HA2 subunit which becomes protonated upon acidification of the endosomes. This hydrophobic fusion peptide domain, as a result, changes its conformation, and moves to the outside of the protein where it will interact with the endosomal membrane, causing its destabilization (Stegmann, 2000). Several synthetic fusogenic peptides have been synthesized based on the fusion domain of the influenza virus. Among those, the INF-7 peptide has demonstrated its fusogenic capacity by improving the transfection efficiency of non-viral gene delivery systems (Plank et al., 1994, 1998) and the corresponding dimeric peptide, diINF-7, proved to be able to enhance cytosolic delivery of macromolecules entrapped in immunoliposomes (Fretz et al., 2005; Mastrobattista et al., 2002).

In this study, we evaluated the effects of the influenza-derived fusogenic peptide diINF-7 on gene silencing efficiency of siRNA targeting the epidermal growth factor receptor (EGFR) and the K-ras oncogenes.

2. Silencing the EGFR oncogene

Human epidermoid carcinoma cells A431 were subcultured in Dulbecco's modified Eagle's medium (DMEM) containing 3.7 g/l sodium bicarbonate and 4.5 g/l glucose, supplemented with antimicrobial agents, 2 mM L-glutamine and 7.5% (v/v) foetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2. One day after seeding 4×10^4 cells/well, complexes of anti-EGFR siRNA (Eurogentec) and Lipofectamine 2000 (LF) were prepared as recommended by the manufacturer (Invitrogen). These anti-EGFR siRNA/LF complexes had an average size of 120 nm. Immediately after preparing the complexes, the diINF-7 peptide, which had been synthesized as previously described (Mastrobattista et al., 2002), was added to the particles at a concentration of 12 μg/μl LF, forming by electrostatic interactions the anti-EGFR siRNA/LF/diINF-7 complexes. The addition of diINF-7 fusogenic peptide had no effect on particle size, and a slight effect on surface charge (a consistent increase of 5 mV), compared to the anti-EGFR siRNA/LF complexes. Both the complexes were added to the cells and incubated for 5 h, after which the medium was refreshed. After 48 h, cells were detached, incubated with an anti-EGFR monoclonal antibody labelled with FITC (Santa Cruz Biotechnology Inc.), and the expression of EGFR was accessed by flow cytometry, using a FACScalibur (Becton & Dickinson).

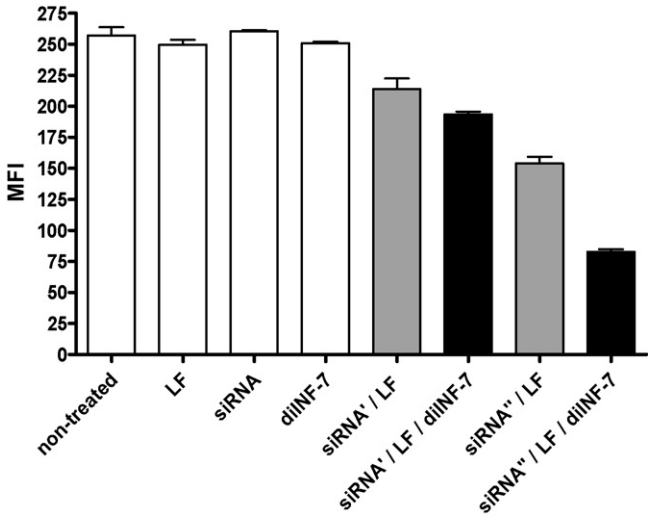

Fig. 1. Expression of EGFR. A431 cells were incubated with different complexes – the anti-EGFR siRNA/LF and the anti-EGFR siRNA/LF/diINF-7, both in 20 and 40 pmol/well doses (siRNA′ and siRNA″, respectively) – and with each element separately (Lipofectamine; anti-EGFR siRNA; diINF-7 fusogenic peptide), in 24-well plates. The values of mean fluorescence intensity (MFI) correspond to the mean of three different measurements.

Fig. 1 presents the expression of EGFR determined by flow cytometry for cells treated with both complexes (anti-EGFR siRNA/LF and anti-EGFR siRNA/LF/diINF-7) and with each component separately, as controls. Fig. 2 shows the percentages of knockdown of EGFR expression for cells treated with both complexes relatively to the controls. There is a clear increase in knockdown of EGFR expression, higher than two-fold, for the complexes which contained diINF-7 fusogenic peptide compared to the ones lacking the fusogenic peptide. Each of the elements alone showed no effect on EGFR expression.

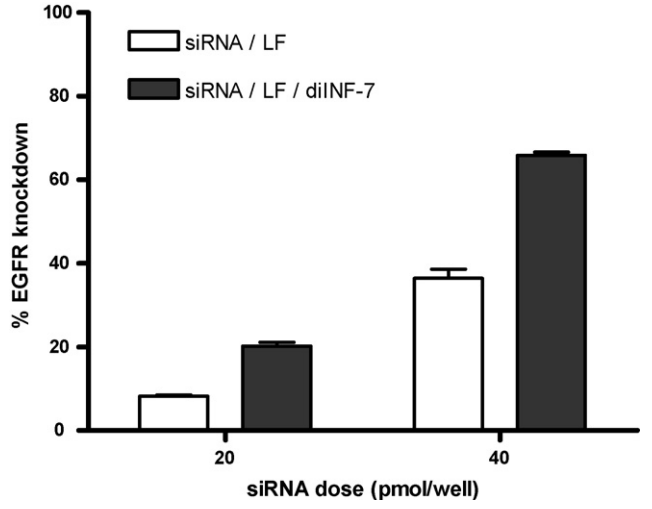

Fig. 2. Silencing EGFR protein expression. The percentage of EGFR expression is calculated using the mean fluorescence intensity (Fig. 1) determined by flow cytometry. Controls were set to 100% and the silencing efficiency of the samples was calculated by determining the ratio of fluorescence.

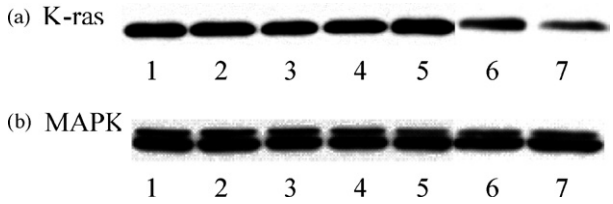

Fig. 3. Silencing K-ras protein expression. (a) Western blot of seven samples in which C26 cells had been incubated with (1) anti-K-ras siRNA/LF (450 pmol/well); (2) anti-K-ras siRNA/LF (600 pmol/well); (3) anti-NS siRNA/LF (450 pmol/well); (4) anti-NS siRNA/LF (600 pmol/well); (5) buffer; (6) anti-K-ras siRNA/LF/diINF-7 (450 pmol/well); (7) anti-K-ras siRNA/LF/diINF-7 (600 pmol/well) complexes, in six-well plates (LF: Lipofectamine; NS: non-specific); (b) MAP kinase was used as a loading control.

3. Silencing the K-ras oncogene

Murine colon carcinoma cells C26 were cultured in DMEM medium containing 3.7 g/l sodium bicarbonate and 4.5 g/l glucose, supplemented with antimicrobial agents, 2 mM L-glutamine and 10% (v/v) foetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2. One day after seeding 3×10^5 cells/well, complexes of anti-K-ras siRNA (Eurogentec) and Lipofectamine 2000 (LF) were prepared as recommended by the manufacturer (Invitrogen) and using two different siRNA concentrations (450 and 600 pmol/well). The size of these anti-K-ras siRNA/LF complexes was in the range of 110–120 nm. The anti-K-ras siRNA/LF/diINF-7 complexes were prepared as described in Section 2 and, in a similar way, only the charge was slightly affected, being more positive than for the anti-K-ras siRNA/LF complexes. Both the complexes were added to the cells and incubated for 5 h after which the medium was refreshed. Forty-eight hours after, cells were detached, the K-ras protein expression was assessed by Western blotting as previously described (Kranenburg et al., 2001). Enhanced chemiluminescence was used for detection and the intensity of the bands, on the resulting film, was analyzed and quantified with Gel-Pro Analyzer software (INTAS).

The analysis of the films (Fig. 3) revealed a decrease of K-ras protein expression of approximately 25% by the anti-K-ras siRNA/LF complexes and a decrease of approximately 80% by the anti-K-ras siRNA/LF/diINF-7 complexes, for the highest siRNA concentration (600 pmol siRNA/well) (Fig. 4). In this experiment a 3.5-fold enhancement of siRNA silencing efficiency has been obtained when diINF-7 peptide is associated with the complexes. Interestingly, the anti-K-ras siRNA/LF/diINF-7 (450 pmol/well) complexes induced a better silencing of K-ras (approximately 55%) than the complexes with the higher dose (600 pmol/well) without fusogenic peptide (approximately 25%). Complexes of control siRNA and LF, with or without the fusogenic peptide, showed no effect on K-ras knockdown (Fig. 3).

4. Discussion

In this study we demonstrate that the synthetic influenza-based diINF-7 peptide can enhance the endosomal escape of complexes composed of siRNA and Lipofectamine. The

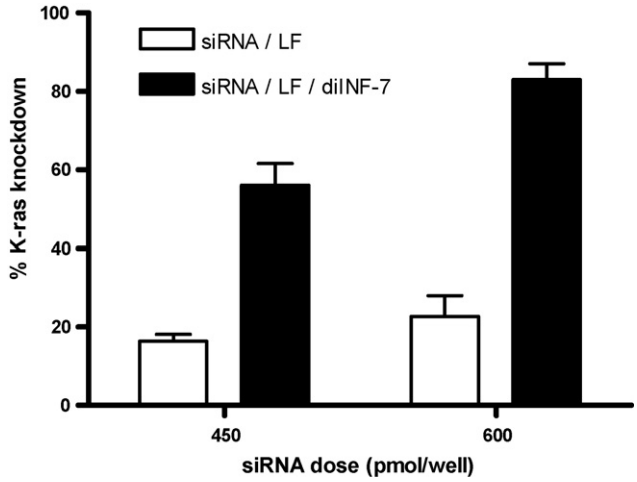

Fig. 4. Silencing K-ras protein expression. Knockdown of K-ras protein (in %) after analyses of three different Western blot films, using the Gel-Pro Analyzer software. Two different complexes were incubated with C26 cells: the anti-K-ras siRNA/LF and the anti-K-ras siRNA/LF/diINF-7. Both complexes were tested in two doses, 450 and 600 pmol siRNA/well.

improvement of the silencing efficiency that we observed is certainly related to the cationic carrier used. Here, Lipofectamine, a commercially available transfectant for nucleic acids, is capable of promoting the escape from the endosomes to a certain extent without additional helper molecules, such as fusogenic peptides. However, our data show that improvement is clearly possible. Other carriers, unable to escape from the endosomes on their own, could particularly benefit from this fusogenic peptide, and as a result become attractive carriers for siRNA. Furthermore, such enhancement of siRNA silencing efficiency, would allow the use of lower concentrations of siRNA which has been shown to reduce the non-specific effects of siRNA treatment (Uprichard, 2005). It would also prevent the saturation of the RNAi machinery, which has been co-related with a reduction of RNAi efficiency and the disturbance of the endogenous miRNA pathways (Hong et al., 2005).

Our studies focus on silencing the expression of proteins which are known to be involved with tumor progression. The EGFR is known to be overexpressed in many tumors (e.g. lung, head and neck, colorectal, prostate, and ovarian carcinoma) and to be involved in cellular proliferation, angiogenesis, and the inhibition of apoptosis (Oliveira et al., 2006). As for K-ras, mutations in this gene are associated with one third of all human cancers and 35% of colorectal cancers (Smakman et al., 2005). Enhancing the silencing efficiency of siRNA targeting these two genes certainly improves therapeutic applications of siRNA for silencing these oncogenes. Current studies are focused on *in vivo* application of endosomal escape enhancers to improve the therapeutic effects of siRNA.

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