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Enhanced gene silencing by the application of multiple specific small interfering RNAs

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Abstract Small interfering RNA duplexes (siRNA) induce gene silencing in various eukaryotic cells, although usually in an incomplete manner. Using chemically synthesized siRNAs targeting the HIV-1 co-receptor CXCR4 or the apoptosis-inducing Fas-ligand (FasL), co-transfection of cells with two or more siRNA duplexes targeting different sites on the same mRNA resulted in an enhanced gene silencing compared with each single siRNA. This was shown in the down-regulation of protein and mRNA expression, and functionally in the inhibition of CXCR4-mediated HIV infection and of FasL-mediated cell apoptosis. Transfection efficiency determined for the FasL-specific siRNAs was dose-dependent and varied among the siRNAs tested, but was not the main reason for the enhanced gene silencing.

Key words: RNA interference; Small interfering RNA; Gene silencing; CXCR4; Fas-ligand; HIV infection

1. Introduction

In 1998, Fire and coworkers reported that double-stranded RNA (dsRNA) introduced into *C. elegans* blocked gene expression by a process of sequence-specific, post-transcriptional gene silencing (PTGS), which they termed RNA interference (RNAi) [1]. Since then, gene silencing by dsRNA has been established in plants, invertebrates, vertebrates and in mammals [2].

The long dsRNA introduced into cells is cut by the RNase III-related nuclease Dicer into short (21–25 nucleotides (nt)) interfering RNAs (siRNA). These siRNAs are incorporated into a multiprotein RNA-induced silencing complex (RISC), where the siRNA duplex is unwound, leaving the antisense strand to guide RISC to its homologous mRNA targets for endonucleolytic cleavage [3–5]. The discovery of RNAi and siRNA, as well as its successful application, provides a powerful tool to target genes for de-activation. Recently, chemically synthesized siRNA duplexes with a length of 21–23 nt were shown to down-regulate the expression of endogenous and heterologous genes in various cells including cells of human origin [6].

We used specific siRNAs to down-regulate CXCR4 in HeLa cells and Fas-ligand (FasL) in HEK293 cells. CXCR4, a co-receptor for HIV-1, is necessary for HIV-1 (X4 or R5X4 isolates) to enter the T cells [7], while FasL, a member of the TNF superfamily, plays an important immune-regulatory role by inducing apoptosis [8]. We provide evidence that in both systems the use of two or more specific siRNAs significantly improved the gene silencing effect induced by a single siRNA. Investigating the mechanism we found that the transfection efficiency varied among the different siRNAs, was siRNA dose-dependent but not the main reason for the enhanced gene silencing effect observed.

2. Materials and methods

2.1. siRNA preparation

21-nt sense and antisense RNA oligomers targeting against CXCR4 mRNA or the FasL mRNA were designed based on the optimal regions found with phosphorothioate-modified antisense oligonucleotides; their locations and sequences are shown in Table 1. All siRNA strands were synthesized and IE-HPLC-purified by Qiagen AG (Basel, Switzerland), mixed in equimolar ratios and annealed [6]. To evaluate the transfection efficiency, 3′-rhodamine-labeled FasL-specific siRNAs were used.

2.2. Cells and transfection

The HeLa CD4- and CXCR4-expressing reporter cell line SX22-1 (stably transfected CD4 expressing plasmid and carrying a *lacZ* gene under the control of HIV-1 LTR), Hut/4-3 (constitutively producing HIV-1), the FasL-expressing human embryonic kidney 293 cells, HEK293-005, and BALB/c B-cell lymphoma A20 GFP, were cultured as described elsewhere [9,10]. SX22-1 or HEK293 cells were transfected for 48 h with siRNA duplexes, alone or in combinations, in the presence of the carrier LipofectAMINE 2000 Reagent (Gibco) according to the manufacturer's protocol. Concentrations of siRNAs were expressed as final concentrations in the cell culture medium.

2.3. Flow cytometry

At 2 days post-transfection, CXCR4 or FasL protein expression was stained with mouse anti-human CXCR4 (12G5) or mouse anti-human FasL (G247-4) monoclonal antibody (BD Bioscience, Basel, Switzerland) and analyzed with a FACSCalibur flow cytometer (BD Bioscience). 10 000–20 000 cells were investigated. Data were processed with the Cell Quest software [10].

2.4. TaqMan real-time reverse-transcription polymerase chain reaction (RT-PCR)

Down-regulation of FasL mRNA was determined by quantitative real-time RT-PCR, standardized by the corresponding GAPDH internal control [10]. For CXCR4 mRNA, the following primers and TaqMan probes were applied: CXCR4 sense 5′-TGATG TGTGT CTAGG CAGGA CCT-3′, CXCR4 antisense 5′-CACTA CACGC TCTGG AATGT TCA-3′, CXCR4 probe 5′-(FAM) TTCTT AGTTG CTGTA TGTCT CGT (TAMRA)-3′.

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Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; FasL, Fas-ligand

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2.5. Prevention of HIV infection

At 2 days post-transfection with 20 nM siRNA-A, a combination of siRNA duplexes or control siRNA-1, HeLa SX22-1 reporter cells were cultured with 300 μl of high-titer virus Hut/4-3 supernatant (500 000 HIV copies/ml) for 1.5 days, which permits cellular HIV-1 infection and *LacZ* reporter gene expression. After fixation with glutaraldehyde/formaldehyde (0.1%/0.7%) in phosphate-buffered saline (PBS) for 5 min, cells were stained by X-Gal for β-galactosidase enzyme [9].

2.6. Apoptosis induction by FasL

At 2 days post-transfection with FasL-specific siRNA duplexes or their combination, FasL-expressing HEK293 cells were washed three times with PBS and then co-cultured with the Fas-apoptosis reporter cells A20 GFP at a ratio of 1:2 (effector: reporter cells) in FACS tubes overnight. After washing with PBS, the percentage of apoptotic A20 GFP cells was determined by flow cytometry [10].

3. Results

3.1. Down-regulation of CXCR4 and FasL with sequence-specific siRNA duplexes

CXCR4-expressing HeLa SX22-1 cells were separately transfected with either 50 nM of siRNA-A, siRNA-B, siRNA-C or the control (FasL) siRNA-1. The inhibitory effect on CXCR4 expression was determined by flow cytometry on the protein level and by TaqMan real-time RT-PCR on the mRNA level. As shown in Fig. 1a,b, all three specific siRNA duplexes down-regulated CXCR4 gene expression (siRNA-A evoked the highest inhibition, 73% at the protein, 79% at the mRNA level), whereas the control siRNA-1 did not. Similarly, FasL-expressing HEK293 cells were transfected with 20 nM of siRNA-1, siRNA-2, siRNA-3 or the control (CXCR4) siRNA-A. Fig. 1a,c shows that all three FasL-specific siRNA duplexes inhibited FasL expression at the protein and mRNA level, whereas the control siRNA-A did not. These data show that the inhibition induced by these siRNAs is sequence-specific.

3.2. Dose-dependent down-regulation of CXCR4/FasL expression by siRNA duplexes

Titration experiments with graded concentrations of CXCR4-specific siRNA duplexes (siRNA-A, -B and -C, Fig. 2a) or FasL-specific siRNA duplexes (siRNA-1, -2 and -3, Fig. 2b) demonstrate that siRNA-mediated down-regulation of CXCR4 or FasL expression was dose-dependent. The maximal CXCR4 inhibitory effect was observed at 20 nM for siRNA-A, at 40 nM for siRNA-B and at 80 nM for siRNA-C (Fig. 2a). Further increasing the concentration did not increase the inhibitory potential of all three CXCR4-specific siRNA duplexes tested. The situation for FasL is similar (Fig. 2b). All three FasL-specific siRNA duplexes reached their optimal activities at ~20–50 nM, but siRNA-1 and siRNA-2 were less potent than siRNA-3. It was tested whether the lower inhibition by siRNA-1 and -2 was due to a lower transfection efficiency. Using rhodamine-labeled FasL-specific siRNAs, it was found that transfection efficiency was also dose-dependent and to some extent, but consistently, varied among the different siRNAs (Fig. 2c). Although there was some correlation between inhibition and transfection efficiency concerning the siRNA concentration, there was no di-

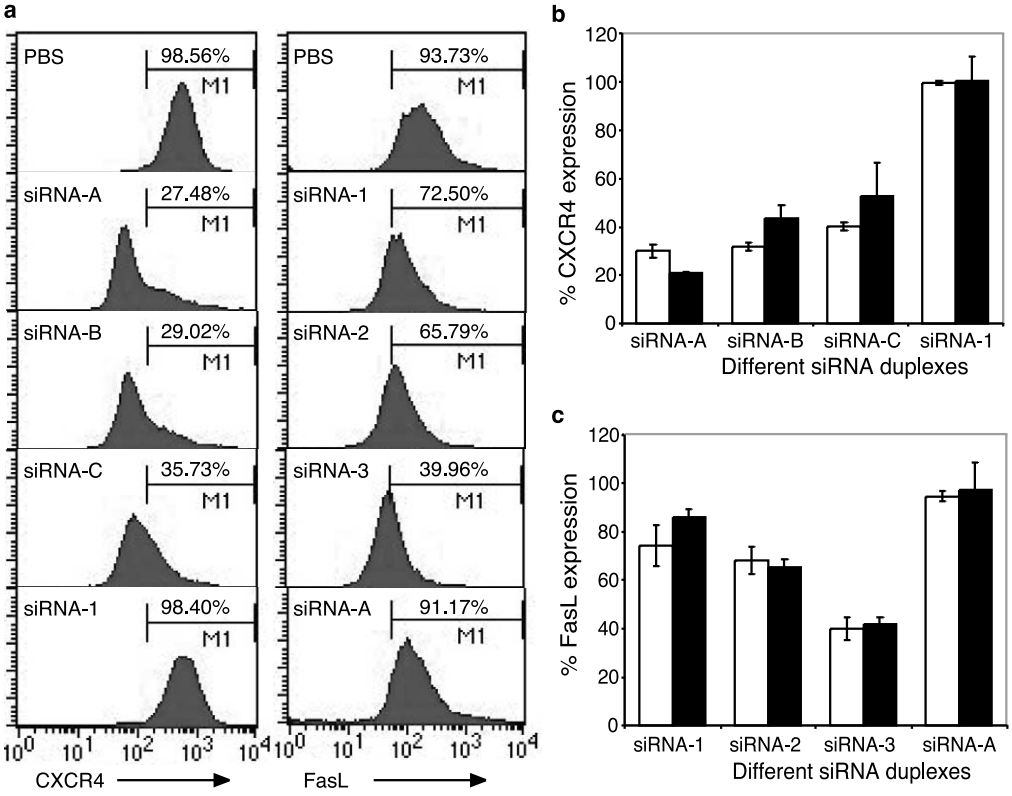

Fig. 1. Down-regulation of CXCR4 and FasL expression by siRNA. a: FACS histograms of HeLa cells separately transfected with 50 nM of CXCR4-specific siRNA-A, -B, -C and siRNA-1 as control (left column), and HEK293 cells transfected with 20 nM of FasL-specific siRNA-1, -2, -3 and the control siRNA-A (right column). CXCR4 (panel b) and FasL (panel c) mRNA expression was quantified by TaqMan real-time RT-PCR (black bar) and compared with protein expression measured by FACS (white bar). All results represent the mean of triplicate experiments including standard deviations (S.D.).

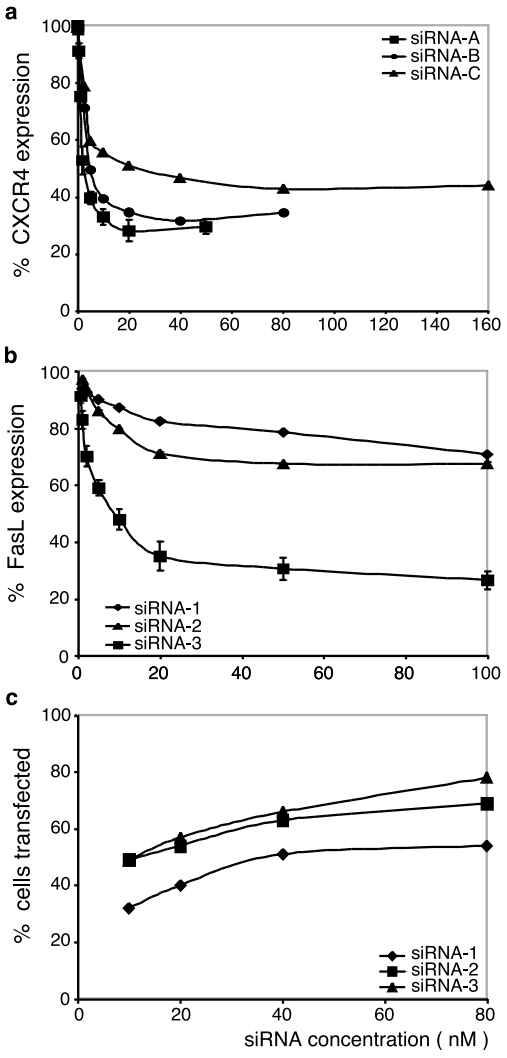

Fig. 2. Dose-dependent down-regulation of CXCR4 and FasL expression by siRNA. HeLa cells (panel a) or HEK293 cells (panel b) were separately transfected with graded concentrations of the siRNA duplexes indicated for 48 h. c: Transfection efficiency was determined by FACS analysis of HEK293 cells transfected with rhodamine-labeled siRNA-1, -2, -3 at the FL-2 channel. Down-regulation of CXCR4/FasL expression was measured by FACS at the FL-1 channel. The results of siRNA-A and siRNA-3 represent the mean of triplicate experiments including standard deviations (S.D.).

rect correlation between the number of transfected cells and the gene silencing potency of the different siRNAs used. For example, although siRNA-2 and -3 showed similar transfection efficiency, they greatly differed in their gene silencing activity.

3.3. Enhanced effect of multiple specific siRNA duplexes

The above data confirm published results that gene silencing with a single chemically synthesized siRNA is incomplete [6,11]. Therefore, it was further tested whether co-transfection of cells with a combination of two or more specific siRNA duplexes targeting different regions of mRNA could increase the inhibitory effect induced by any single siRNA duplex.

As 20 nM siRNA-A demonstrated the highest inhibitory effect (~70%) on CXCR4 expression, this concentration was taken as a reference for the combination experiments. Indeed, higher inhibition was observed when cells were treated with combinations of specific siRNA duplexes (Fig. 3a). Up to 90% inhibition of CXCR4 protein expression was reached when cells were co-transfected by a combination of siRNA-A, siRNA-B and siRNA-C. This higher inhibition is not due to an increase of the total concentration of siRNAs used (see Fig. 2a, or A+A in Fig. 3a). As shown in Fig. 3a, A plus B expressed a higher inhibitory activity than A plus C in accordance with the fact that B alone had a higher activity than C. Moreover, A plus 60 nM B had a higher activity than A plus 40 nM B or A plus 20 nM B. Finally, a combination of three siRNA duplexes exhibited a higher inhibitory activity than two. These data clearly suggest that each siRNA duplex contributes to the total activity in the combination. The enhanced inhibitory effect of multiple specific siRNA duplexes was confirmed at the mRNA level by TaqMan real-time RT-PCR and was also detected at lower concentrations of CXCR4-specific siRNA duplexes (data not shown). Of interest, control si-

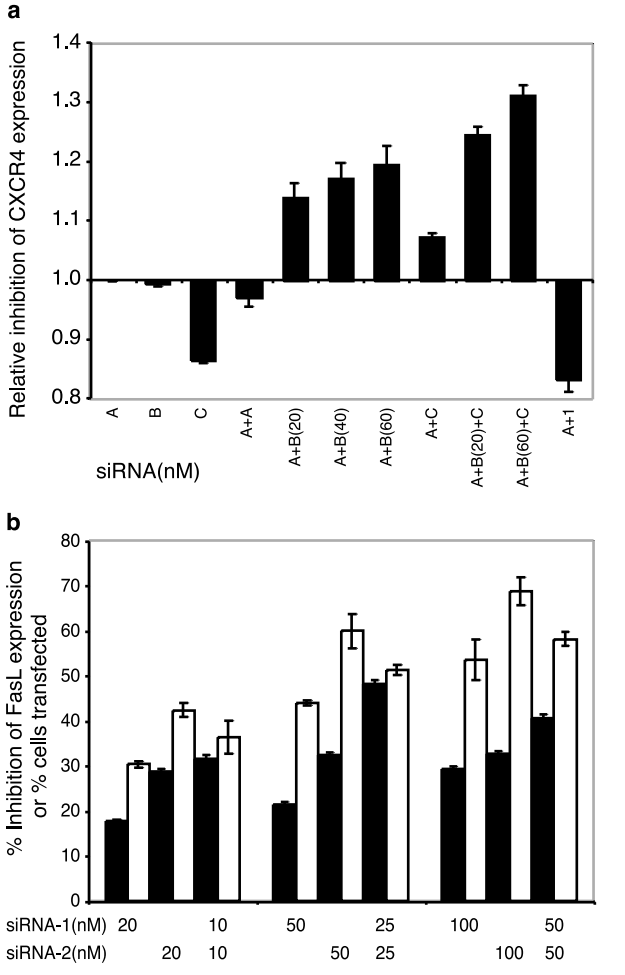

Fig. 3. Enhanced gene silencing induced by a combination of specific siRNA duplexes. a: HeLa cells were transfected with 20 nM or the indicated concentrations of siRNA duplexes alone or in combinations and CXCR4 down-regulation was measured by FACS. The relative inhibition was normalized to that of 20 nM siRNA-A alone, which was set to 1. The inhibition of >1 reflects its higher inhibitory activity than 20 nM siRNA-A. b: FasL down-regulation (black bars) and transfection efficiency (white bars) in HEK293 cells was measured as described in the legend to Fig. 2. All results represent the mean of triplicate experiments including standard deviations (S.D.).

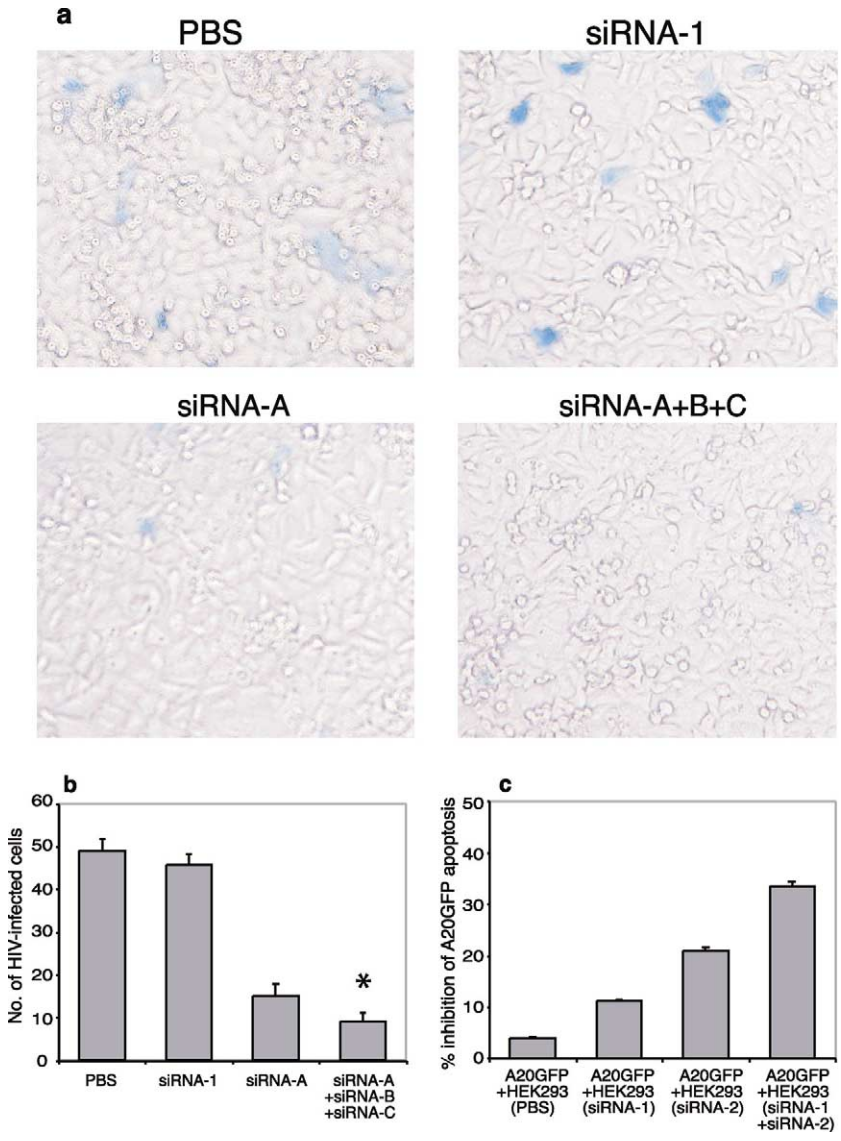

Fig. 4. Functional assay for CXCR4 and FasL. a: Inhibition of CXCR4-mediated HIV-1 infection in vitro: Photomicrograph of β-gal-stained HeLa SX22-1 cells, which were treated with 20 nM siRNA-A, a combination of specific siRNAs (A+B+C), control siRNA-1 or PBS, followed by infection with high-titer HIV-1. b: Quantification of HIV-infected cells: For each condition three non-overlapping cell areas were randomly chosen and the number of blue cells was counted. The number of infected cells is significantly different between siRNA-A and the siRNA combination treatment (*$P = 0.026$; two-sample t-test). c: Inhibition of the apoptosis-inducing function of FasL: A20 GFP cells were cultured overnight with HEK293 cells untransfected or transfected with 20 nM siRNA-1, siRNA-2 or their combination. The percentage of apoptotic A20 cells was determined by FACS analysis. All results represent the mean of triplicate experiments including standard deviations (S.D.).

RNA-1 co-transfected with siRNA-A resulted in a reduced gene silencing effect of the specific siRNA-A (Fig. 3a). This competing effect is highly reproducible and also valid for other siRNA combinations (data not shown). The mechanism of this competition is not yet known, but other groups have made similar observations [11,12].

To exclude that the enhanced inhibition by a combination of siRNAs was a CXCR4-specific effect, similar experiments

Table 1
Sequences and locations of siRNAs

Name	Sequence	Location
siRNA-A	5′-GCGGCAGCAGGUAGCAAAGTTTTCGCCGUCGUCCAUCGUUUC-5′	CXCR4 5′UTR[a]
siRNA-B	5′-AUGGAGGGGAUCAGUAUAUTTTTUACCUCCCCUAGUCAUAUA-5′	CXCR4 start codon[b]
siRNA-C	5′-CUGGGCAGUUGAUGCCGUGTTTTGACCCGUCAACUACGGCAC-5′	CXCR4 codon region[b]
siRNA-1	5′-AGUGGCCCAUUUAACAGGCTTTTUCACCGGGUAAAUUGUCCG-5′	FasL splicing site[c]
siRNA-2	5′-CAGGCAAGUCCAACTCAAGTTTTGUCCGUUCAGGUUGAGUUC-5′	FasL splicing site[c]
siRNA-3	5′-GGGCUGUACUUUGUAUAUUTTTTCCCGACAUGAAACAUAUAA-5′	FasL splicing site[c]

[a]According to *Homo sapiens CXCR4* gene, Accession No. AF005058.
[b]According to *Homo sapiens* CXCR4 mRNA, Accession No. NM-003467.
[c]According to *Homo sapiens* FasL mRNA, Accession No. D38122.

were performed with siRNAs targeting FasL. HEK293 cells were transfected with different concentrations of FasL-specific siRNA-1 and siRNA-2, either alone or in combination; both of them singly induce a suboptimal inhibition. Co-transfection of cells with both siRNA-1 and siRNA-2 evoked an enhanced inhibition of FasL expression at optimal concentrations (each 25 nM) (Fig. 3b), whereas at lower or higher doses the enhanced inhibitory effect was less marked (Fig. 3b). In analogy to CXCR4, the enhanced FasL gene silencing detected was not due to the increase of the doses used, as the inhibition of the corresponding concentrations of 20, 50 or 100 nM siRNA-1 or siRNA-2 alone was below that of the combined concentrations. Transfection efficiency was not a major reason for the enhanced inhibition, as the percentages of transfected cells by the combination of siRNA-1 and -2 lay between the single siRNAs (Fig. 3b).

3.4. *Enhanced inhibition of HIV-1 infection in vitro by multiple specific siRNA duplexes*

It was tested whether the enhanced effect of multiple specific siRNA duplexes could also be detected in the prevention of HIV-1 infection. At 2 days post-transfection with siRNA-A, a combination of three CXCR4-specific siRNA duplexes or control siRNA-1, cell-free high-titered HIV-1 culture supernatant was added to SX22-1 cells. *LacZ* reporter gene expression (as an indicator of HIV-1 infection) was measured 1.5 days later. As illustrated in Fig. 4a and confirmed by quantification of the infected cells by counting (Fig. 4b), the combination of three siRNAs prevented the in vitro infection of SX22-1 cells by HIV-1 more efficiently than siRNA-A alone. Moreover, the number of infected cells (blue stain by X-gal) not only decreased, but the few infected cells also showed a weaker blue staining, which is concordant with a lower level of Tat expression and a delayed infection [9].

3.5. *Enhanced inhibition of the apoptosis-inducing function of FasL*

FasL-expressing HEK293 cells, transfected with 20 nM siRNA-1 or siRNA-2 or their combination for 2 days, were co-cultured overnight with the apoptosis reporter A20GFP cells, followed by the determination of apoptosis by flow cytometry. Fig. 4c demonstrates that the siRNA combination inhibited FasL-mediated apoptosis better than each of the siRNA duplexes alone, confirming the enhanced inhibitory effect between specific siRNA duplexes in a functional assay.

4. Discussion

SiRNA duplexes targeting distinct sites on the same mRNA usually express different silencing efficiency [2,11]. Recently, Holen et al. reported that only a few siRNAs were effective in gene silencing and siRNA efficacy was highly dependent on target position, suggesting that accessible siRNA target sites may be rare in some human mRNAs [11]. In agreement with the above observations, we found different inhibitory activities of our siRNAs targeting CXCR4 or FasL mRNA.

Titration experiments demonstrated that the siRNA duplexes tested did not completely silence the gene of interest, which is consistent with observations made by other groups [6,11]. Indeed, each siRNA duplex tested (CXCR4- and FasL-specific siRNA duplexes) exhibited a maximal gene silencing ranging from about 20% to 70%. Possible reasons for the incomplete inhibition could be the efficiency of siRNA transfection and/or the half-life of the target protein. Using rhodamine-labeled FasL-specific siRNAs, transfection efficiency was determined and found to be dose-dependent and to some extent different among the siRNAs tested. Whereas the number of transfected cells and the inhibition partially correlated regarding the siRNA concentrations used, there was no clear correlation between transfection efficiency and gene silencing among the siRNAs tested. For example, siRNAs expressing a similar transfection efficiency substantially differed in their gene silencing capacity.

As a single siRNA inhibits expression of the target gene only incompletely, it is important to find ways to increase the inhibition. When two or more specific siRNA duplexes were co-transfected into the reporter cells, higher inhibition of gene expression was observed in two entirely different systems, i.e. CXCR4 and FasL. Holen et al. [11] did not see synergism between two or three siRNAs in another system, but no data were provided. Apart from the different gene target utilized, it is possible that the concentrations used are critical, as in our hands the cooperation of siRNAs is only optimal within a certain dose range (Fig. 3b).

The underlying mechanism of the enhanced gene silencing with multiple specific siRNA duplexes is unknown. Transfection efficiency can be excluded as the main reason (Fig. 3b). Possible but still unproven explanations are: (a) SiRNA antisense binding may change the secondary structure of mRNA [13,14], opening new sites accessible for other siRNAs; (b) two or more siRNA duplexes may increase the overall possibility of target mRNA to be accessed and digested.

Our data demonstrate an enhanced gene silencing with multiple specific siRNAs not only at the protein or mRNA expression level, but also at the functional level, evoking a higher inhibition of HIV infection and a stronger reduction of FasL-mediated apoptosis than single siRNA. RNAi and siRNA have provided new promises to treat gene-mediated disorders or viral infections. From our results, siRNA can efficiently inhibit HIV-1 infection of cells in vitro through down-regulating CXCR4 expression, supporting observations made by other groups [15–17]. CXCR4 is an ideal target, as it not only prevents viral entry but is also far less sensitive to mutation compared to the viral gene and is independent of drug pressure [7,18]. FasL plays an important immune-regulatory role, and it is also discussed as a critical molecule for immune evasion of some tumors [8]. Currently, studies try to apply expression of siRNA in vivo [15,17,19]. Therefore, the stable expression of multiple siRNA duplexes in vivo may not only make the FasL immune evasion hypothesis testable but may also help to pave the way for a clinical application to prevent HIV-1 infection [20,21]. Moreover, the same strategy could be applied to other human diseases.

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