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Inhibition of the human chemokine receptor CXCR4 by antisense phosphorothioate oligodeoxyribonucleotides

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Abstract The CXC chemokine receptor CXCR4/fusion, a major coreceptor for the T-cell line T-tropic (X4) HIV-1 virus, plays a critical role in T-tropic virus fusion and entry into permissive cells. In the present study, we describe the effects of an antisense phosphorothioate oligodeoxyribonucleotide (anti-S-ODN) on the inhibition of CXCR4 gene expression in X4 HIV-1 infected HeLa-CD4 cells, to find more efficacious therapeutic possibilities for human immunodeficiency virus type 1 (HIV-1) infection. The naked antisense phosphorothioate oligodeoxyribonucleotide (anti-S-ODN-1), containing the AUG initiation codon at the center of the oligodeoxyribonucleotide, showed a slightly higher inhibitory effect on HIV-1 gag p24 production among all sequences tested. We also examined the concomitant use of a basic peptide transfection reagent, nucleosomal histone proteins (RNP), for the delivery of the anti-S-ODN-1. The anti-S-ODN-1 encapsulated with RNP had higher inhibitory effects on p24 products than the naked anti-S-ODN-1. When the anti-S-ODN-1 encapsulated with RNP was incubated with HeLa-CD4 cells, the surface levels of this chemokine receptor showed high suppression, indicating sequence-specific inhibition. The activities of unmodified oligodeoxyribonucleotide are effectively enhanced by using a basic peptide, RNP.

Key words: Antisense oligodeoxyribonucleotide; Transfection reagent; Chemokine receptor CXCR4; HeLa-CD4 cell; HIV-1

1. Introduction

HIV-1 requires coreceptor molecules in addition to CD4 for entry and fusion into target cells [1–3]. These coreceptors include the chemokine receptors, which are 7-transmembrane domain G-protein coupled receptors. Although the list of possible coreceptors is continuously expanding, the major coreceptors are CCR5 and CXCR4, which facilitate the cell entry of the macrophage-tropic (R5 viruses, based on the new classification) and X4 (appear late in the disease) strains of HIV-1, respectively [1–6]. RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β are ligands for CCR5 and block R5 HIV-1 infection [7], whereas stromal cell-derived factor-1 (SDF-1) is a ligand for CXCR4 and blocks X4 HIV-1 infection [8,9]. Chemokines, modified chemokines, peptides, and non-peptide receptor antagonists or agonists have become important agents for the treatment of HIV-1 infection [7,10,11]. Recently, two groups have reported that the horseshoe crab blood cell-derived peptide, T22 [12], and the non-peptide, TAK-779 [13], act as CXCR4 and CCR5 antagonists that can inhibit infection by X4 and R5 HIV-1, respectively. Furthermore, a hammer-head ribozyme and a DNA enzyme can also inhibit the coreceptor CCR5 function [14,15]. Based on these findings, we proposed that down-regulation of CXCR4 expression by antisense oligodeoxyribonucleotides (anti-ODNs) may be an effective and harmless way to inhibit HIV-1 infection [16]. The use of antisense ODNs as therapeutic tools in modulating gene expression represents a newly established strategy for treating diseases [17–21]. Such antisense ODNs are designed to complement the respective target mRNAs, whereby they inhibit the translation of the specific mRNA. The applications of antisense ODNs for anti-HIV-1 gene therapy are based on the use of antisense ODNs targeted to viral transcripts [22–25].

In this study, we analyzed the down-regulation of the functional expression of the HIV-1 coreceptor, CXCR4, by RNP (basic peptide transfection reagent) [26,27] encapsulated antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-1–3), which contains the AUG initiation codon at its center and the downstream coding region, close to the AUG initiation codon.

2. Materials and methods

2.1. Antisense phosphorothioate (anti-S-) and phosphodiester (anti-O-) oligodeoxyribonucleotides, and transfection reagents

The antisense sequence, including the initiation region of the CXCR4 mRNA (MEDLINE, human mRNA for HM89), and its sense and scrambled sequences, were tested in the present study. The nucleotides within the initiation codon are underlined. S-ODNs, FITC-labeled anti-S-ODN-1 (5′-labeled), and anti-O-ODN-1 were purchased from KURABO Biomedical Co. (Japan). They were the antisense S-ODN-1 and the antisense O-ODN-1 [5′-GATCCCCTC-CATGGTAACCG-3′ (anti-S-ODN-1 and anti-O-ODN-1)], complementary to bases 69–88 of the human CXCR4 mRNA, the sense S-ODN-1 and the sense O-ODN-1 [5′-CGGTTACCATGGAGGG-GATC-3′ (sen-S-ODN-1 and sen-O-ODN-1)], corresponding to the sequence of bases 69–88 of the human CXCR4 mRNA, and the scrambled S-ODN-1 [5′-ATCGCCTAGCTACCTACGCG-3′ (scr-S-ODN-1)] with the same base composition as the antisense 1 target (69–88 bases). The antisense S-ODN-2 [5′-GTGTATATACTGA-TCCCCTC-3′ (anti-S-ODN-2)], complementary to bases 80–99 of the human CXCR4 mRNA, and the antisense S-ODN-3 [5′-TTA-

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TCTGAAGTGTATATACT-3′ (anti-S-ODN-3)], complementary to bases 89–108 of the human CXCR4 mRNA, were also synthesized.

The sequence of the FITC-labeled anti-S-ODN-1 was the same as that of the antisense.

Our originally constructed peptide transfectant (named RNP: reconstituted nucleosomal protein) was prepared with the nucleosomal histone subunits: H2A, H2B, H3, and H4. Histone subunits were purchased from Boehringer Mannheim (GmbH, Germany). The optimal proportions for ionic complex formation between the peptide and the ODNs were determined using a 20% denatured polyacrylamide gel electrophoresis (PAGE) retardation system. It was necessary to mix H2A (0.004 μg) and H2B (0.004 μg) in 20 mM phosphate buffer (pH 7.2) before the addition of any other subunit. Next, the antisense ODN (1 μg) was added to the solution. Then H3 (0.0047 μg) was added, followed by H4 (0.0034 μg). This mixture was added to the culture medium for the transfection of HeLa-CD4 cells with the S-ODNs by the use of the RNP.

2.2. Cells and viruses

The HeLa-CD4 cell line (human cervical carcinoma) expressing CXCR4 was grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (United Biotechnology), 50 units/ml penicillin, and 50 units/ml streptomycin at 37°C in a 5% CO_2-gassed incubator.

HIV-1 NL432 was produced by transfection of the proviral DNA clone, pNL432, into COS cells. The supernatants were clarified by filtration, and the levels of p24 antigen, the core antigen of HIV-1, were determined by ELISA. The amounts of viruses were expressed as the concentrations of p24 antigen. The virus stocks were analyzed for their p24 antigen levels and were stored at −80°C until use.

2.3. Anti-HIV-1 activity of anti-S-ODNs and anti-O-ODN-1

Several concentrations of anti-S-ODNs and anti-O-ODN-1, as an anti-HIV drug, were added to cultured HeLa-CD4 cells for 24 h in 24-well plates (1×10^5 cells/ml/well). After 24 h of culture, the HeLa-CD4 cells were washed twice with fresh medium, and then were infected with the HIV-1 NL432 virus (MOI: multiplicity of infection = 0.1) in 1 ml of medium at 37°C and in a 5% CO_2 atmosphere for 3 h. The cells were washed twice with fresh medium to remove the residual virions, gently resuspended in fresh medium with 10% FBS, and cultured for 24 h. Anti-HIV activity was determined by measuring the amount of p24 antigen in the supernatant, using an HIV-1 p24 ELISA.

2.4. Staining of treated HeLa-CD4 cells

To down-regulate CXCR4 expression, the naked and encapsulated anti-S-ODN-1 was incubated for 24 h with washed HeLa-CD4 cells (1×10^6 cells/2 ml/well) that had been inoculated 24 h previously and maintained as described above. After the anti-S-ODN-1 treatment, the cells were scraped, washed twice in cold PBS, and resuspended in PBS. After centrifugation for 2 min at 2000 rpm, the cells were stained with monoclonal antibodies specific for human CXCR4 (12G5 mouse IgG2a) (Pharmingen), and then were incubated with secondary FITC-labeled antibodies (rat anti-mouse IgG2a: FITC) (Serotec). As a control (data not shown), the cells were stained with monoclonal antibodies specific for CD4 (conjugated to phycoerythrin, PE) (Dako Japan Co., Ltd.). After staining, the cells were washed and resuspended in 0.5% HCHO/PBS.

2.5. Flow cytometry and analysis

CXCR4 expression in stained cells was analyzed by flow cytometry with a FACSCalibur (Becton Dickinson) flow cytometer and the Cell Quest software (Becton Dickinson). For each sample, 30 000 total events were analyzed, with sequential gating of HeLa-CD4 cells.

2.6. Intracellular localization of FITC-labeled anti-S-ODN-1 in HeLa-CD4 cells

HeLa-CD4 cells were incubated in RPMI 1640 at 37°C and in a 5% CO_2 atmosphere for 24 h. The cultures, in 6-well plates (1×10^6 cells/2 ml/well), were washed twice with PBS, and then 5 μM FITC-labeled anti-S-ODN-1 was added and the cultures were incubated for 4 h as above. The cells were washed twice with PBS, scraped off, resuspended in 0.5% HCHO/PBS and 0.5% glycerol/PBS, and observed by laser-assisted confocal microscopy (Molecular Dynamics, MultiProbe 2001).

3. Results and discussion

3.1. Sequence-specific inhibition of the CXCR4 chemokine receptor function by naked antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-1–3) in HeLa-CD4 cells

Antisense oligonucleotides have become widely used in research into the specific inhibition of gene expression. They are now being investigated as possible therapeutic agents. Such oligodeoxyribonucleotides may be designed to complement a region of a particular gene or messenger RNA. We selected an antisense oligodeoxyribonucleotide sequence, including the initiation codon. The translation initiation site was chosen as a target site in several other mRNAs. The HeLa-CD4 cells were used as the target cells, since they were infected with the X4 HIV-1 NL432.

It was reported previously that we evaluated the inhibitory effects on the functional expression of the CXCR4 receptor in HeLa-CD4 cells using the naked antisense phosphorothioate oligodeoxyribonucleotide (anti-S-ODN-1) containing the AUG initiation codon at its center [16]. The anti-S-ODN-1 was directly added to the cultured cells, and then the cells were incubated at 37°C. The anti-S-ODN-1 had an inhibitory effect on the HIV-1 gag p24 antigen at a highest concentration of 10 μM. Two different control oligodeoxyribonucleotides were prepared, the sen-S-ODN-1 and scr-S-ODN-1 sequences, with the same base composition as that of the anti-S-ODN-1 target. For control oligodeoxyribonucleotide sequences, sen-S-ODN-1 and scr-S-ODN-1, we could not detect any inhibitory effects on the HIV-1 gag p24 antigen at the highest concentration of 10 μM.

Next, we also synthesized the antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-2 and anti-S-ODN-3) complementary to the downstream coding region, close to the AUG initiation codon of the CXCR4 mRNA (see Section 2). However, they showed lower anti-HIV-1 activities than that of the anti-S-ODN-1 (Fig. 1). The anti-S-ODN-1 was the most potent among the antisense S-ODNs for the inhibition of the HIV-1 gag p24 antigen production. On the other hand, the sequence-specific down-regulation of surface CXCR4 expression on HeLa-CD4 cells with the anti-S-ODN-1 was detected by flow cytometry using the monoclonal

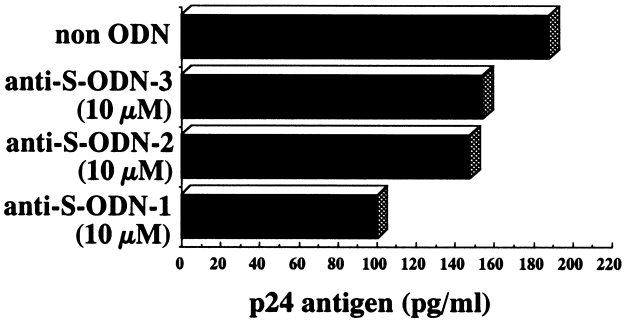

Fig. 1. Sequence-specific inhibition of HIV-1 infection in HeLa-CD4 cells by the naked antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-1–3). HeLa-CD4 cells were infected with HIV-1 NL432 in the presence or absence of different concentrations of the naked antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-1–3). Aliquots of the culture supernatant were assayed for HIV-1 gag p24 content by ELISA. The experiment reported is representative of a set of three different experiments.

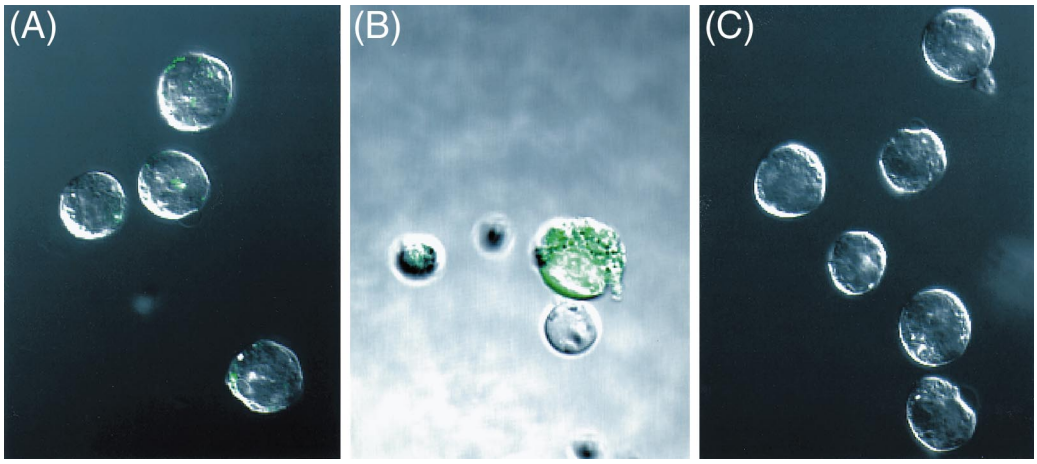

Fig. 2. Intracellular localization of FITC-labeled anti-S-ODN-1 delivered by a basic peptide transfection reagent, nucleosomal histone proteins (RNP) into HeLa-CD4 cells, observed by laser-assisted confocal microscopy. A: FITC-labeled anti-S-ODN-1 alone. B: FITC-labeled anti-S-ODN-1 encapsulated with RNP. C: HeLa-CD4 cells alone. The cells were washed twice with PBS, scraped off, resuspended in 0.5% HCHO/PBS and 0.5% glycerol/PBS, and observed by laser-assisted confocal microscopy.

anti-CXCR4 antibody (12G5 mouse IgG2a) [16]. The anti-S-ODN-1 had an inhibitory effect, causing more than 50% inhibition of surface CXCR4 expression at the highest concentration of 10 μM. On the other hand, with the control sequence, the sen-S-ODN-1, we could not detect any inhibitory effects on the surface CXCR4 expression in HeLa-CD4 cells. These results suggest that the down-regulation of CXCR4 expression by the anti-S-ODN-1 may block HIV-1 entry into human cells. Based on these results, the following antisense experiments were performed using the anti-S-ODN-1.

3.2. Confocal microscopic observation of the uptake of encapsulated and naked FITC anti-S-ODN-1 in HeLa-CD4 cells

Antisense ODNs have been used as antisense inhibitors of gene expression in various culture systems and are considered to be potential therapeutic agents against cancer and viral infectious diseases. In order to exert any of these effects, the anti-ODNs must enter the cytoplasmic and nuclear compartments of the cells. The problem in the use of antisense ODNs is that the cellular uptake of anti-ODNs is inefficient [28,29]. The use of various enhancers to increase the intracellular accumulation of the anti-S-ODNs has largely solved this problem, and has greatly facilitated their use as research tools in vitro. Many cellular uptake enhancers have been reported, including cationic lipids, liposomes, peptides, dendrimers, polycations, cholesterol conjugates, and electroporation. One of the most commonly used enhancers is a mixture of neutral and cationic lipids [30–35].

We analyzed the efficiency of the delivery of the encapsulated FITC-labeled anti-S-ODN-1 with a basic peptide transfection reagent, nucleosomal histone protein (RNP) [26,27], into HeLa-CD4 cells using laser-assisted confocal microscopy. The nucleosomal histone proteins, which are known as basic proteins, possess nuclear localization signals and helical domains. The RNP was prepared as indicated in Section 2. As shown in Fig. 2B, the FITC-labeled anti-S-ODN-1 with RNP revealing a major portion is localized within the nucleus and cytoplasms within HeLa-CD4 cells. In contrast to this observation, little fluorescent signals were observed either in the endosomes and cytoplasms of HeLa-CD4 cells treated with the naked FITC-labeled anti-S-ODN-1 (Fig. 2A). However, no cell surface-bound S-ODNs were detected. Thus, in the presence of the RNP transfection reagent, the anti-S-ODNs first enter the cytoplasm and then quickly accumulate in the nucleus. The localization of anti-S-ODN-1 with the nucleus has implications for the mechanism of action of this and other antisense phosphorothioate constructs. The uptake of antisense S-ODNs was greatly enhanced by RNP encapsulation. These results prove that the FITC-labeled anti-S-ODN-1 encapsulated with RNP penetrates the HeLa-CD4 cells, and then the anti-S-ODN can hybridize to the target sequence in the mRNA.

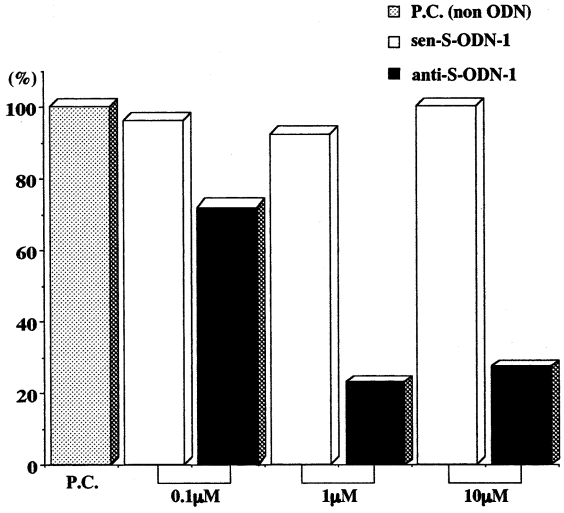

Fig. 3. Sequence-specific inhibition of the CXCR4 chemokine receptor function by the anti-S-ODN-1 encapsulated with RNP in HeLa-CD4 cells. HeLa-CD4 cells were incubated with 0.1, 1, and 10 μM of the phosphorothioate oligodeoxyribonucleotides (anti-S-ODN-1 and sen-S-ODN-1) encapsulated with RNP. The surface CXCR4 expression on HeLa-CD4 cells was detected by flow cytometry using the monoclonal anti-CXCR4 antibody (12G5 mouse IgG2a). The experiment reported is representative of a set of three different experiments.

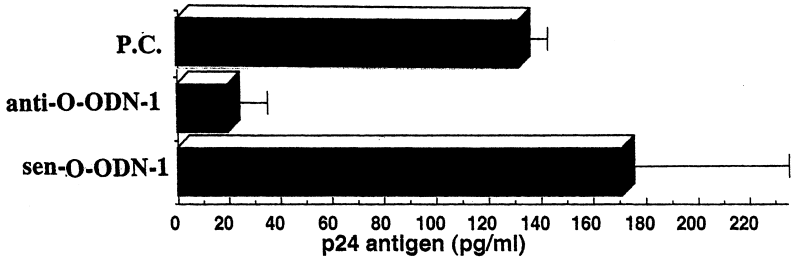

Fig. 4. Inhibitory effect of antisense phosphodiester oligodeoxyribonucleotide (anti-O-ODN-1) encapsulated with RNP in HeLa-CD4 cells. HeLa-CD4 cells were infected with HIV-1 NL432 in the presence of 1 μM concentrations of the encapsulated phosphodiester oligodeoxyribonucleotides (anti-O-ODN-1 and sen-O-ODN-1). Aliquots of the culture supernatant were assayed for HIV-1 gag p24 content by ELISA.

3.3. Sequence-specific inhibition of the CXCR4 chemokine receptor function by the anti-S-ODN-1 encapsulated with RNP in HeLa-CD4 cells

Synthetic antisense phosphorothioate oligodeoxyribonucleotides (anti-S-ODNs) have been widely used as tools for the specific inhibition of gene expression. They are considered to be a potential new generation of drugs. Antisense ODNs can inhibit various viral pathogens and regulate specific gene expression by inhibiting transcription or translation through their complementary interactions with targeted genetic segments. However, there are some problems with the use of ODNs, in that cells are not very permeable to ODNs, and the ODNs are not stable for in vivo applications. Thus, transport and intracellular delivery are important and fundamental considerations when developing an effective ODN-based therapy.

We evaluated the inhibition of the functional expression of the CXCR4 receptor gene using several concentrations of the anti-S-ODN-1 encapsulated with a basic peptide transfection reagent, nucleosomal histone proteins (RNP). The RNP was prepared as indicated in Section 2. The surface CXCR4 expression on HeLa-CD4 cells was detected by flow cytometry using the monoclonal anti-CXCR4 antibody (12G5 mouse IgG2a). The anti-S-ODN-1 encapsulated with RNP showed 80% inhibition of the surface CXCR4 expression on HeLa-CD4 cells at a 1 μM concentration, whereas at the highest concentration of 10 μM, it had an inhibitory effect similar to that of the 1 μM concentration (Fig. 3). However, at the lower concentration of 0.1 μM, the anti-S-ODN-1 encapsulated with RNP slightly suppressed the CXCR4 expression. In contrast, almost no inhibition was observed when the reaction was carried out using the control ODN, sen-S-ODN-1, instead of the anti-S-ODN-1, at concentrations of 0.1, 1, and 10 μM. For p24 assaying in HeLa-CD4 cells, the anti-S-ODN-1 encapsulated with RNP had a higher inhibitory effect (70%) at the 1 μM concentration [16]. However, naked anti-S-ODN-1 showed lower anti-HIV-1 activity (11%). For the control sequence, sen-S-ODN-1, we could not detect any inhibitory effects on the HIV-1 gag p24 antigen expression at a concentration of 1 μM. Of particular interest is the RNP encapsulated antisense phosphodiester oligodeoxyribonucleotide (anti-O-ODN-1), which was found to have an inhibitory effect on the HIV-1 gag p24 antigen expression of same order as those for the RNP encapsulated antisense phosphorothioate oligodeoxyribonucleotide (anti-S-ODN-1), probably due mainly to their relative nuclease resistance in culture medium (Fig. 4) [36,37]. For the control sequence, the sen-O-ODN-1, we could not detect any inhibitory effects on the HIV-1 gag p24 antigen expression at a concentration of 1 μM. These results suggest that the anti-ODNs encapsulated with RNP conferred sequence-specific inhibition. The use of an RNP transfection reagent confers a significant advantage over the use of the anti-ODNs alone for the delivery of the anti-ODNs into HeLa-CD4 cells. Furthermore, the antisense ODNs encapsulated with RNP might explain the enhanced antisense activity.

In conclusion, anti-S-ODN-1, containing an AUG initiation codon at the center of the oligodeoxyribonucleotide targeted to the CXCR4 mRNA, showed the greatest inhibitory effects among the S-ODNs on HIV-1 gag p24 antigen production and also on CXCR4 expression in HeLa-CD4 cells, consistent with the assumption that this region is important and accessible. The use of the cationic peptide, RNP, provides a simple and efficient method for the successful intracellular delivery of the oligodeoxyribonucleotides. The anti-S-ODNs encapsulated with RNP exhibited higher inhibitory activities than the naked anti-S-ODNs, and showed sequence-specific inhibition. The activities of unmodified oligodeoxyribonucleotides are effectively enhanced by using a cationic peptide, RNP. Consequently, the encapsulated anti-S-ODN-1 may be useful to block HIV-1 entry into human cells.

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