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Original Article

Down-Regulation of $CXCR_4$ Expression in MT4 Cells by a Recombinant Vector Expressing Antisense RNA to $CXCR_4$ and Its Potential Anti-HIV-1 Effect

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SUMMARY: CXC-chemokine receptor ($CXCR_4$) is one principle coreceptor for the entry of T cell line (T)-tropic HIV-1 virus into a cell. In order to find more efficacious therapeutic possibilities for people with an HIV-1 infection, we explored the inhibitory effects of antisense RNA on $CXCR_4$ expression in MT4 cells. First, we used RT-PCR to obtain DNA fragments from healthy adult peripheral blood mononuclear cells; these fragments targeted the initiation region of $CXCR_4$ mRNA translation. We then constructed a recombinant retroviral vector, pLXSN-X4a (containing antisense RNA to $CXCR_4$). After packaging by PA317 cells, the pseudovirion of the recombinant vector had formed and succeeded in transfecting MT4 cells (a kind of T-tropic HIV-1 susceptibility cell line). The PCR and RT-PCR results showed that the recombinant vector had integrated into the genome of MT4 cells and had been transcribed. The expression of $CXCR_4$ on the surface of MT4 cells transfected with antisense RNA was reduced by 30%, compared with those cells transfected with blank vector or untransfected cells. No change in the DNA synthesis rates or in cell proliferation was found in any of the transfected cells. After a challenge with HIV-1 SF33, the cells transfected with antisense RNA vector (pLXSN-X4a) produced reduced p24 levels compared with the cells transfected with blank vector (pLXSN) or untransfected cells. These results indicated that these $CXCR_4$-antisense expressing cells could resist T-tropic HIV-1 infection and could retain normal biological functions. These studies provide useful data for further experiments in this area.

INTRODUCTION

The application of highly active antiretroviral therapy (HAATR) has achieved long sustained suppression of viral replication in HIV-1-infected individuals (1). However, considering the high cost and the low compliance with long-term HAART therapy, as well as resistance to chemotherapy resulting from HIV-1 variants (2,3), it remains necessary to discover novel anti-HIV-1 agents with different mechanisms of action. Recently, studies have shown that chemokine receptors play a crucial role in HIV-1 infection, for HIV-1 infection occurs not only through the interaction of the virion with CD_4 molecules in target cells, but also by interaction with one of several chemokine receptors (i.e., coreceptors) (4-6). Although the list of possible coreceptors is continuously expanding, the major coreceptors are CCR_5 and CXC-chemokine receptor ($CXCR_4$), which facilitate the cell entry of the macrophage-tropic (R_5 virus, responsible for primary infection) and the T cell-tropic (X_4 virus, appearing later in the course of the disease) strains of HIV-1, respectively. Individuals with mutations in the CCR_5 gene (homozygosity for the ccr_5 Δ32 allele), a mutation with a 32 bp deletion in the encoding region of CCR_5, which completely abolishes the cell surface expression of CCR_5, demonstrate a high level of resistance against HIV-1 infection; this mutation is furthermore not associated with any other clinical conditions (7-10). T cell line (T)-tropic virus, predominantly found in the late course of HIV-1 infection, induces the formation of syncytia in CD_4^+ cell lines and replicates in these cells at a higher rate than M-tropic isolates (11). Occurrence of the T-tropic HIV-1 strain in individuals is associated with CD_4^+ cell decline and progression to AIDS. This viral strain was unable to enter into the CD_4^+ cells without $CXCR_4$ (macrophage cells). Based on these findings, we postulated that the down-regulation of coreceptor expression may be an effective means of blocking HIV-1 infection and subsequent progression to AIDS. In this study, we analyzed the down-regulation of $CXCR_4$ by antisense RNA, and we suggest its potential effect on HIV-1 replication.

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MATERIALS AND METHODS

Construction of a recombinant retroviral vector: The PCR primers were designed with Oligo 4.0 software, according to the sequence of $CXCR_4$ mRNA (Genbank Accession: NM_003467). The sequences of the primers were as follows: X4-1a: 5′-CCTGGATCCCTGGAGAACCAGCGGTTAC-3′, X4-2a: 5′-TCCGAATTCTGAAGGCCAGGATGAGGAC-3′.

The DNA fragment used to construct a vector expressing antisense RNA against $CXCR_4$ was PCR-amplified from the cDNA of healthy adult peripheral blood mononuclear cells (PBMCs); PCR was performed in a 25 μl reaction mixture containing 200 μM of each dNTP, 1.5U Tag DNA polymerase (Promega, Madison, Wis., USA), 0.8 μM of each primer, and 1.5 mM $MgCl_2$; initial denaturation (94°C for 5 min) was followed by 35 cycles of denaturation (94°C for 45 sec), annealing (69°C for 45 sec), extension (72°C for 70 sec), and an additional 10-min extension at 72°C. The fragments were

then cloned into the vector pLXSN (a type of retroviral vector derived from a plasmid, pBR322, with a neomycin selection marker) in the antisense orientation, and the resulting vector was designated as pLXSN-X4a (Fig. 1). The recombinant vector was then determined by DNA sequencing (Sangon, Shanghai, China).

Generation of a retroviral packaging cell line: The resultant retroviral vectors and their parental vector (pLXSN, as a control) were transfected into amphotropic packaging cells (PA317, derived from TK-NIH/3T3 cotransfected with packaging construct DNA – pPAM3 and herpes simplex virus thymidine – TK gene carried in pBR322) with lipofectAMINE (GIBCO-BRL, Gaithersburg, Md., USA). Seventy-two hours later, the transfected PA317 cells were selected with a G418 (800 μg/ml)-containing medium for 2 to 3 weeks. The G418-resistant colonies were subcloned and characterized by genomic PCR analysis to ensure the entire integration of the foreign genes into the chromosome of the packaging cells. The PCR primers corresponded to the sequences up- and downstream of the inserted site in the recombinant vector, respectively (JC1: 5′-CCGTCTCTCCCC CTTGAA-3′(1562-1579nt); JC2: 5′-GCGGGACTATGGTT GCTG-3′(1825-1842nt) (Fig. 2) (BioAsia, Shanghai, China). PCR was performed in the same reaction mixture as described above; initial denaturation (94°C for 5 min) was followed by 35 cycles of denaturation (94°C for 45 sec), annealing (65°C for 45 sec), extension (72°C for 70 sec), and an additional 10-min extension at 72°C. The PCR products were electrophoresed on 1.0% agrose gels, which were stained with ethium bromide and photographed using Bio-Rad Gel COD 100 (Bio-Rad Laboratories, Hercules, Calif., USA).

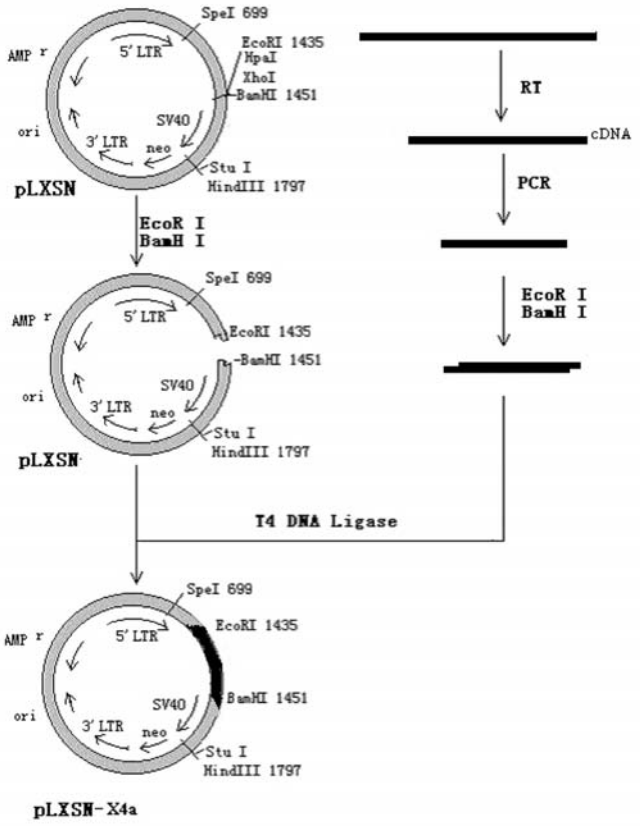

Fig. 1. Construction of a recombinant vector.
pLXSN: a retroviral vector with a neomycin selection marker; X4a was the DNA fragment (corresponding to $CXCR_4$ mRNA), which was RT-PCR-amplified from healthy adult peripheral blood mononuclear cells, and then was inserted inversely into pLXSN. The recombinant vector was designated as pLXSN-X4a.

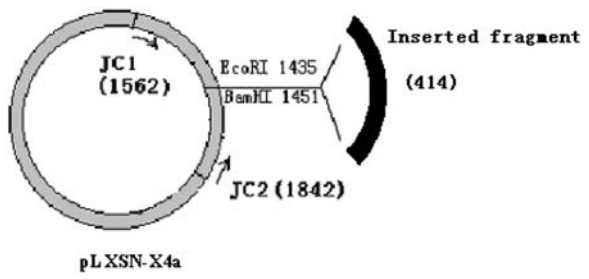

Fig. 2. Location of Primers JC1/JC2.
JC1/JC2: primers used to detect the existence of the foreign gene and its expression in transfected MT4 cells.

Detection of pseudovirion in a culture medium of PA317 cell: NIH/3T3 cells were seeded (1×10^4 cells/well) in 24-well cell-culture plates and were transfected with the supernatant of survival PA317 cells at three different dilutions (1:1, 1:10, and 1:100). Twenty-four hours later, the transfected NIH/3T3 cells were selected with a G418 (500 μg/ml)-containing DMEM. After 2 weeks of selection, single G418-resistant cells emerged and were counted under an inverted microscope (Olympus, Tokyo, Japan).

Generation of transfected MT4 cells: MT4 cells seeded (1×10^6 cells/well) into 6-well cell-culture plates were transfected by incubation with the supernatant of PA317 cells containing the pseudovirions of pLXSN-X4a and pLXSN in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, Mo., USA). Untransfected MT4 cells (used as a control) were incubated with the same quantity of fresh DMEM culture medium. Forty-eight hours later, the cells were selected with a G418 (400 μg/ml)-containing RPMI 1640 growth medium. The medium was replaced twice weekly. Three weeks later, all of the untransfected cells died, whereas the cells transfected with the pseudovirion survived. Then, G418-resistant cells were expanded in the culture medium (containing G418) for further experiments.

Analysis of the foreign gene and its expression in transfected MT4 cells: The total RNA from transfected MT4 cells (after 3 weeks of selection with G418) and from untransfected cells was extracted with Trizol reagent (GIBCO-BRL). The procedure was carried out as follows: 1×10^6 cells were lysed in 1 ml Trizol reagent, and then the homogenized samples were incubated for 5min at room temperature, followed by the addition of 0.2 ml of chloroform; the sample tubes were capped securely, and then were shaken vigorously by hand for 15 s. The samples were incubated at room temperature for 3 min, and centrifuged at 12,000 × g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube and then was mixed with 0.5 ml isopropyl alcohol to precipitate the RNA. The RNA pellet was washed once with 75% ethanol and dissolved in DEPC (diethylpyrocarbonate)-treated water. The RNA solution was then used in the next step. To remove possible contaminating DNA, a 4-μg RNA aliquot was digested with 4 U RQ1 RNase-Free DNase (Promega), in the presence of 12 U Rnasin and then was extracted by phenol-chloroform. A 3- μg treated sample of RNA was reverse- transcribed into cDNA using 40 U AMV Reverse Transcriptase in the presence of 1.6 μl primer (JC2, 20 μM), 50 U Rnasin, at 42°C for 60 min. The genomic DNA of the same cells was extracted with the Wizard genomic DNA purification kit (Promega) according to the manufacturer's

protocol. Then, the PCR of the cDNA and genomic DNA was performed in the same reaction mixtures and under the same conditions as those used for the PA317 cells. Each experiment included a negative control.

Measurement of cell proliferation and DNA synthesis rates of transfected MT4 cells: To measure cell proliferation, transfected and untransfected MT4 cells (1×10^4 cells/well) were seeded into 96-well cell-culture plates with 200 μl RPMI 1640 medium under the following conditions: 37°C, and 5% CO_2. Twenty-four hours later, 25 μl of MTT solution (1 mg/ml) was added into each well and the cells were cultured for an additional 4 h. Then, 100 μl of 10% sodium dodecyl sulfate (SDS) was added into each well in order to completely dissolve the formed blue particles. Absorbance of the solution in each well at 570 nm was read with a microplate reader (Bio-Rad). To measure the DNA synthesis rates, transfected and untransfected MT4 cells (1×10^5 cells/well) were seeded into a 96-well cell-culture plate with 200 μl RPMI 1640 medium (containing 10% FCS). [^{3}H] thymidine (1 μCi/well) was added into each well. After incubating the samples at 37°C for 24 h, they were harvested onto filter papers using a semiautomatic cell collector (DYQ-III, Shaoxing, China). Each piece of filter paper with the cells from one well was added to 1 ml of scintillation liquid in a micro tube, and the incorporation of isotopes into the cells was determined by a scintillation counter (Wallac Trilux 1450 microbeta liquid scintillation and luminescence counter, Perkin Elmer, Boston, Mass., USA).

Flow cytometric assay: Untransfected and transfected MT4 cell preparations were washed twice in washing solution (Ca^{2+}/Mg^{2+}-free PBS, containing 0.2% BSA). Then, 1×10^6 cells were resuspended in 30 μl of washing solution and incubated with human IgG (1 μg/1×10^5 cells) at room temperature for 15 min in order to block the Fc receptor. The cells were then stained with 10 μl PE-conjugated mouse monoclonal antibody against $CXCR_4$ (R&D Systems, Minneapolis, Minn., USA) or with 20 μl FITC/PE conjugated mouse monoclonal antibody against CD_3/CD_4 (Becton-Dickinson, Pharmingen, Calif., USA) and were incubated at 4°C for 45 min. As a negative control, FITC/PE conjugated irrelevant IgG was used. After staining, the cells were washed twice with the same washing solution and then were resuspended in 500 μl FIX buffer (Ca^{2+}/Mg^{2+}-free PBS, containing 1% formalin, 0.1% sodium azide, and 2.0% GS). Flow cytometry utilizing FACScan (Becton Dickinson) was performed on a minimum of 10,000 events per sample. The results were analyzed using specialized software supplied by the manufacturer.

Determination of HIV-1 infectivity: Transfected and untransfected (control) MT4 cells in RPMI 1640 medium with 10% FCS were infected with HIV-1 SF33 virus at 50 $TCID_{50}$. After a 2-h incubation at 37°C, the infected MT4 cells were washed once to remove the residual virions, and were gently resuspended in fresh culture medium and cultured. The culture supernatant was collected on the 6th day and anti-HIV-1 activity was determined by measuring the amount of p24 antigen in the supernatant using an HIV-1 p24 antigen ELISA kit (Organon Teknika, Durham, N. C., USA).

RESULTS

Construction of an expression vector: The recombinant retrovirial vector (pLXSN-X4a) was constructed by inserting the foreign gene fragment (corresponding to the initiation region of $CXCR_4$ mRNA translation) into the pLXSN plasmid (as depicted in Fig. 1). The foreign gene was controlled by the viral long terminal repeat (LTR) promoter (which can be activated by HIV-1 Tat protein). The constructed vector was verified by DNA sequencing.

Integration of foreign gene fragments into the genomic DNA of transfected PA317 cells and the formation of pseudovirions: The genomic DNA of transfected PA317 cells was PCR-analyzed and relevant bands of interest were amplified. The results indicated that the foreign gene fragment was integrated into the chromosome of the packaging cells (Fig. 3). After 2 weeks of selection with G418-containing DMEM, NIH/3T3 cells transfected with the supernatant of PA317 cells had survived, while the untransfected NIH/3T3 cells had died. This suggested that the supernatant of G418-resistant PA317 cells containing pseudovirions could infect NIH/3T3 cells.

Expression of a foreign gene in transfected MT4 cells and its effects on the expression of $CXCR_4$, CD_4, and CD_3: To identify the foreign gene fragment (X4a) in MT4 cells and its expression, we performed both genomic PCR and RT-PCR. Bands emerged in the products amplified from transfected MT4 cells, but not in those from control cells (Figs. 4A, 4B). These results suggested that the X4a fragment had

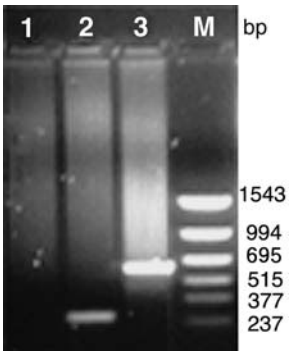

Fig. 3. Analyzing the foreign gene fragment in the transfected PA317 cells by PCR.
1, 2, 3: Genomic PCR products of untransfected PA317 cells (no band) and PA317 cells transfected with pLXSN (280 bp), pLXSN-X4a (678 bp), respectively; M: PCR Marker.

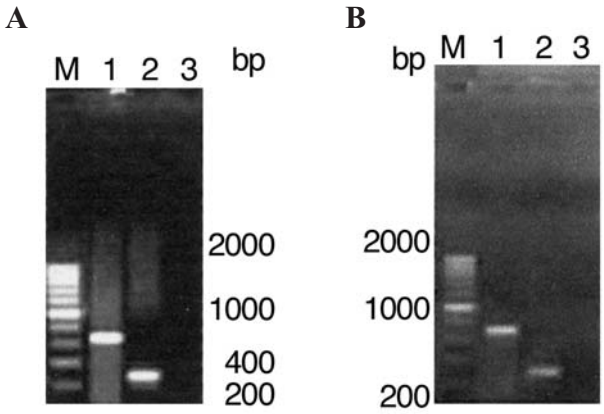

Fig. 4. Analyzing the foreign gene fragment in transfected MT4 cells and its expression by PCR and RT-PCR.
A, B: M: 200 bp DNA ladders. 3: PCR (in A) and RT-PCR (in B) products of untransfected MT4 cells, respectively (no band); 1, 2: Genomic PCR (in A) and RT-PCR (in B) products of MT4 cells transfected with pLXSN-X4a (678 bp) and pLXSN (280 bp), respectively.

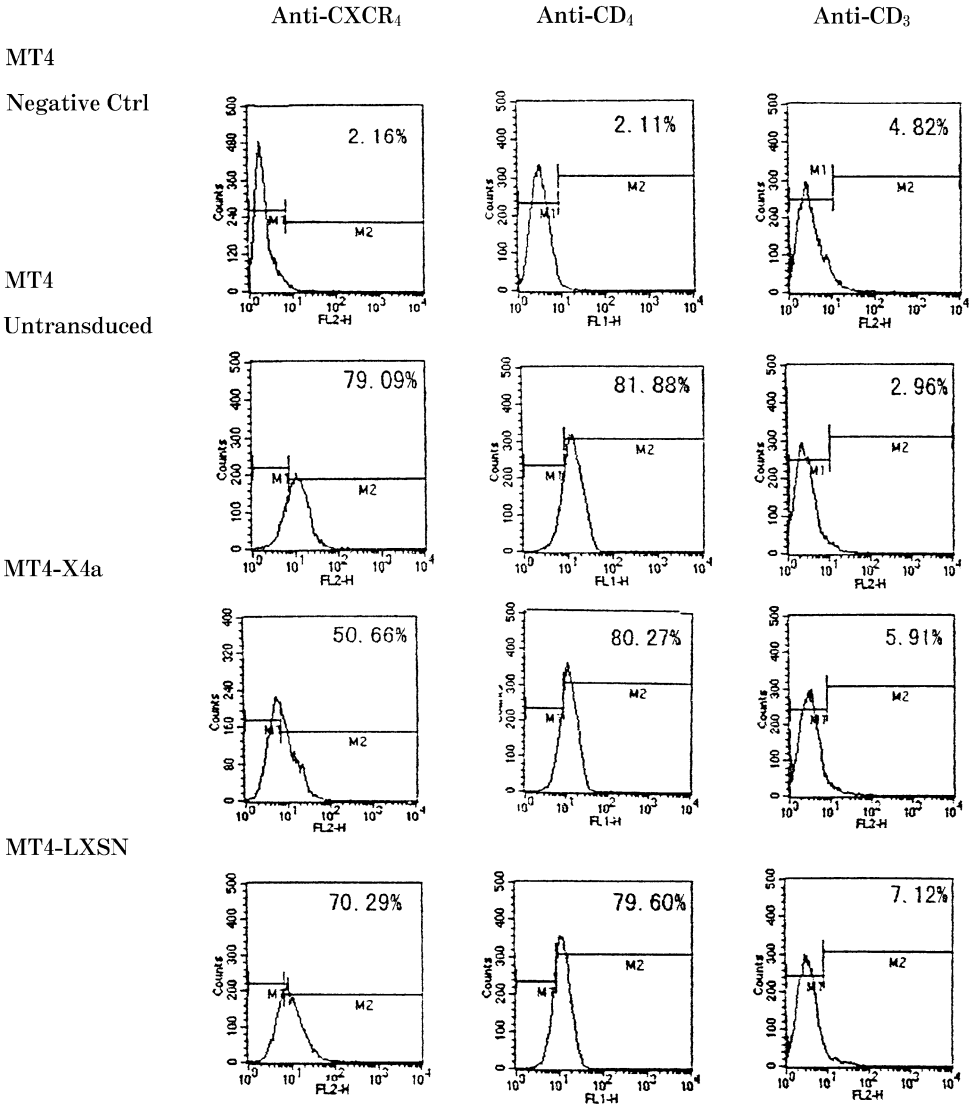

Fig. 5. Anti-sense RNA effects on the expression of $CD_{3/4}$ and $CXCR_4$ on MT4 cells.
Cells were stained with mouse monoclonal antibody against human $CD_{3/4}$ (conjugated with FITC/PE) and $CXCR_4$ (conjugated with PE), respectively; the cells were stained with irrelevant IgG conjugated with FITC/PE as a negative control. After staining, the cells were analyzed on a FACScan, and the percentage of positively stained cells is presented. MT4-X4a and MT4-LXSN are surviving MT4 cells selected by G418 after transfection with the pseudovirions of pLXSN-X4a and pLXSN, respectively.

been integrated into the transfected MT4 cells and had been transcribed.

Then, we carried out flow cytometry (FACScan) in order to measure the expression of $CXCR_4$ and CD_4/CD_3 on transfected cells (Fig. 5). There was comparable level of CD_4 expression on the MT4 cells transfected either with pLXSN-X4a or with pLXSN. There was no CD_3 antigen expression on a number of MT4 cells. In contrast, the expression of $CXCR_4$ on the surface of MT4 cells transfected with pLXSN-X4a was reduced by 30%, as compared with that on the surface of untransfected MT4 cells or on MT4 cells transfected with blank pLXSN.

Effect of the recombinant vector on MT4 cells: We assessed the biological features of the transfected MT4 cells in order to determine whether or not the inhibition of $CXCR_4$ by antisense RNA had an adverse effect on the MT4 cells. MT4 cells transfected with pLXSN-X4a, pLXSN and their parental cells (MT4 cells) were found to exhibit similar levels of cell proliferation, as determined by MTT (Fig. 6). [^{3}H] thymidine incorporation of these cells, reflecting the cellular DNA synthesis rate, was also comparable under the same culture conditions (Fig. 7). These results indicated that the recombinant vector, with or without antisense RNA to $CXCR_4$, did not have an apparent adverse effect on these MT4 cells.

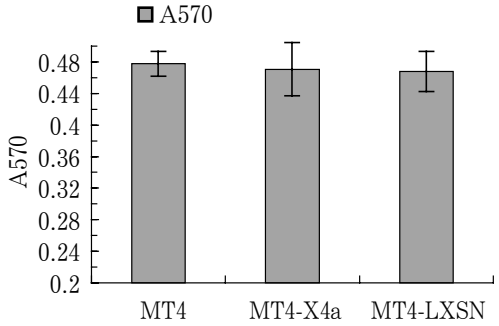

Fig. 6. Proliferation of MT4 cells transfected with different vectors.
MT4: untransfected cells used as a control, as measured by MTT.

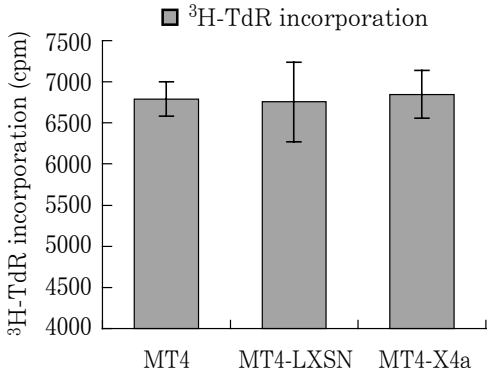

Fig. 7. DNA synthesis rates of MT4 cells transfected with different vectors.
Measured by [^{3}H] thimidine incorporation.

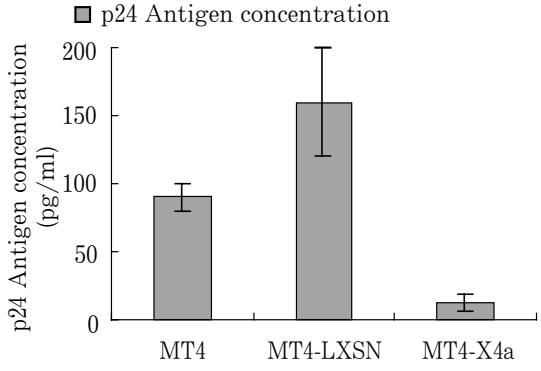

Fig. 8. p24 antigen levels produced by different MT4 cells infected with HIV-1 (SF33).
Different types of MT4 cells were infected with HIV-1 (SF33). On the 6th day of culture, levels of p24 antigen in the culture supernatant was assayed by ELISA.

Inhibition effect of antisense RNA on HIV-1 infectivity: In order to evaluate the effect of antisense RNA on HIV-1 infectivity, MT4 cells untransfected and transfected with different vectors were infected with T-tropic HIV-1 SF33. As shown in Fig. 8, low levels of p24 antigen were detected only in the culture medium of pLXSN-X4a -transfected MT4 cells, whereas high levels of p24 antigen were detected in the culture medium of pLXSN-transfected MT4 cells and blank MT4 cells (control). This indicated that the antisense RNA was able to protect MT4 cells from HIV-1 infection to a certain extent.

DISCUSSION

HIV infection continues to be a major global health concern, with millions of people infected worldwide. HIV-1 infection leads to the progressive depletion of CD_4^+ T lymphocytes, eventually causing the development of AIDS and ultimately leading to death by opportunistic infection. The recent widespread use of combination therapy can effectively reduce the viral load to undetectable levels in the peripheral blood, with a subsequent increase in CD_4^+ T cell numbers and improved clinical status. However, a number of problems such as the severe side effects associated with currently used drugs and the development of drug-resistant HIV (3,12,13) has emerged. Moreover, the existing drugs are only able to suppress viral replication, but cannot completely eliminate the virus in the body.

There are several gene therapy approaches against HIV infection, i.e., interfering directly with viral replication or eliminating infected cells, such as dominant-negative mutants of Gag, Env or Rev (14-16); the use of an RNA decoy (17,18), antisense RNA molecules, and ribozymes (19) has also been described. These approaches have yielded promising results. However, with frequent mutations, HIV-1 may be able to evade such strategies. In addition, these approaches only suppress viral replication in infected cells, but cannot block HIV entry into new target cells. The discovery that HIV-1 requires chemokine receptors in addition to CD_4 for entry and fusion into target cells has implied that down-regulation of coreceptor expression on targeting cells, or blockage of the interaction of HIV-1 with the coreceptor may provide possible approaches to anti-HIV therapy. One study has shown that SDF-1α (a natural ligand of $CXCR_4$) or Met-SDF-1α (SDF-1α modified with methionine) could block HIV-1 binding to $CXCR_4$ in vitro. However, these chemokines have a short half-life in vivo, and they therefore require frequent administration into infected individuals; thus, this approach could be ineffective and might even induce inflammatory reactions (20). In this study, we demonstrated that antisense RNA inhibited the surface expression of $CXCR_4$ on MT4 cells. Moreover, these $CXCR_4$-antisense expressing cells appeared to retain normal cell biological features, as indicated by normal proliferation and DNA synthesis rates and the normal expression of CD_4 on these cells. These findings, taken together, have suggested that the recombinant vector we used might be harmless to MT4 cells. Furthermore, $CXCR_4$ may not be essential to their existence (probably due to redundancy of their chemokine receptors). In addition, these $CXCR_4$-antisense expressing cells produced reduced p24 antigen, as compared with cells transfected with blank vector (pLXSN) and untransfected cells after infection with HIV-1. This result implied that $CXCR_4$-antisense RNA expressing cells can resist HIV-1 infection to a certain extent. Nevertheless, we noted that the inhibition of $CXCR_4$ expression of the antisense RNA was not fully satisfactory. This may account for why these $CXCR_4$-antisense RNA expressing cells exhibited only a limited anti-viral effect. We also observed that the levels of p24 antigen produced from MT4 cells transfected with blank vector were higher than those produced in MT4 cells alone, suggesting that pLXSN might enhance HIV-1 infectivity. MT4 cells are a human T cell line carrying human T cell lymphotropic virus-I (HTLV-I), which are easily infected with HIV-1. Although the exact mechanism is not yet known, it is likely that the interaction of the pLXSN gene with the HTLV-I gene might exert an effect on the expression of certain cellular elements except for $CXCR_4$, and subsequently affect HIV-1 proliferation. The present findings suggest that we need to perform additional studies of different target cells such as H9 or Jurkat cells in the future, and draw a comparison between cell types; this approach will help determine whether or not this strategy has any adverse effects on cells function in vivo (study underway). In addition, it will be necessary to enhance the expression of antisense RNA in target cells. Nevertheless, the present series yielded promising results, and provided a strong foundation for further study.

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