Cell lines and cell culture

All the cell lines, unless otherwise specifically mentioned, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum. Human glioma NP-2-$CD4^+$ cells transfected with a variety of chemokine receptors as indicated [27] were maintained in medium containing 500 μg/ml of G-418 (Promega, Wisconsin, USA) and 1 μg/ml of puromycin (Sigma). Human CD4-negative osteosarcoma (HOS) cells expressing either CXCR4 or CCR5 [28] were cultured in medium containing 1 μg/ml of puromycin. Human hepatoma cells Huh-7 and Hep-G2 [29] were obtained through the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Human primary cultured hepatocytes (p-hepatocytes, BD Bioscience, California, USA) were maintained on BD Matrigel with Hepato-STIM hepatocyte culture medium (BD Bioscience).

Reagents and antibodies

The CXCR4 antagonist AMD3100 [30], and the CCR5 antagonist TAK-779 [31] were provided by the NIH AIDS Research and Reference Reagent Programme and Takeda Chemical Industries, Ltd., Osaka, Japan, respectively. Recombinant human soluble CD4 (sCD4) was from ImmunoDiagnostics, Inc. (Woburn, Massachusetts, USA). Antialbumin-fluorescein isothiocyanate (FITC) antibody was from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anticytokeratin-18–phycoerythrin and antialpha fetoprotein (AFP)–FITC antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Anti-HIV-1-p24 (clone KC57)-FITC antibody was from Beckman Coulter. All other antibodies were from BD Pharmingen (San Diego, California, USA).

Pseudotyped virus infection assay

The HIV-1 Env-pseudotypes were generated as previously described [32]. Briefly, 293T cells (5×10^6 cells/10 cm-dish) were transfected with 5 μg of luciferase-expressing pNL4-3-Luc-R^-E^- [33] or green fluorescent protein (GFP)-expressing pNL4-3-GFP [34] plasmid in combination with 10 μg of one of the *env*-expressing plasmids, pSM-SDA-1, pSM-HXB2 (X4), pSM-ADA (R5), or pSM-89.6 (R5X4). The vesicular stomatitis virus-G pseudotypes were also prepared [35].

For infection assays of luciferase-pseudotypes (luc-p), 10 ng p24 of luc-p were added into each well of 24-well plates (5×10^4 cells/well). After 12 h infection, the cells were washed and incubated for an additional 36 h at 37 °C. The cells were then lysed using a Luciferase Assay kit (Promega) and the luciferase activity was examined by a luminometer (Lumat 9507, Germany). To determine the effects of various reagents related to the viral receptors, target cells were preexposed for 1 h with the indicated concentration of the antagonists, or the antibodies. For GFP-pseudotypes (GFP-p) infection, target cells were infected with 10 ng p24 of GFP-p virus for 48 h and fixed by 5% paraformaldehyde. Infectivities were visualized under a Zeiss LSM510 confocal microscopy and DIC images with a 512 × 512 resolution were acquired.

Chimeric viruses

All *env* recombinant chimeric viruses in this study were generated in the background of pNL43, an X4-tropic HIV-1 infectious clone [36]. Briefly, the fragment of pNL43 containing *Eco*RI (nt 5743–5748) and *Kpn*I (nt 6343–6348) was amplified by PCR with a F5671–R6472 primer pair (F5671, 5′-GGCTCCATAACTTAGGA CAAC, pNL43 nucleotide position 5671–5691; R6472, 5′-TACTTCTTGTGGGTTGGGGTC, pNL43 position 6452–6472), followed by insertion into the pSM–SDA-1 using *Eco*RI and *Kpn*I. The new *Eco*RI–*Xho*I fragment (3155 bp) covering the entire SDA-1 *env* gene was then replaced with the equivalent region of pNL43 to construct the Env-chimeric virus NL43_SDA-1. Similarly, Env-chimeras of ADA (NL43_ADA), 89.6 (NL43_89.6) or truncated *env* (NL43_env (-)) were created, respectively. All Env-chimeric viruses were prepared by transfecting 293T cells as described above. For infection assays, 100 ng p24 of the chimeric viruses or virus stock supernatants were added in each well of 24-well plates (5×10^4 cells/well). After 2 h adsorption, the cells were washed and incubated for 48 h. Viral replication was monitored by p24 antigen production.

Flow cytometry and apoptosis assay

We performed cell-surface staining for CD4, CXCR4 and CCR5 by flow cytometry. To determine the purification and differentiation of p-hepatocytes, we tested the specific markers using antialbumin–FITC, anti-AFP–FITC and anticytokeratin-18–phycoerythrin antibodies. Appropriate class matched antibodies were used in each experiment. To detect the proliferation and intracellular p24, p-hepatocytes were fixed and permeabilized using a Cytofix-Cytoperm kit (BD Bioscience). Subsequently, the cells were stained with anti-Ki-67–phycoerythrin and antip24–FITC antibodies. Apoptosis of the p-hepatocytes was determined using the Apoptosis Detection kit I (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, New Jersey, USA). All Data were acquired and analyzed using Cell Quest software (BD Bioscience).

Nucleotide sequence accession number

The GenBank accession number for the sequence determined in this study is AY902478 (SDA-1).

Results

Evaluation of SDA-1 viral quasispecies

In an attempt to isolate CD4-independent clinical HIV-1 strain(s), we performed virus isolation from a

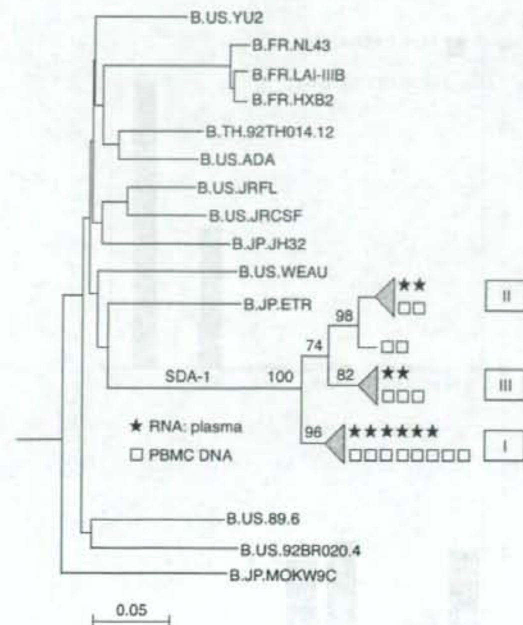

Fig. 1. Evolution of SDA-1 *env* quasispecies in plasma and PBMC. Phylogenetic analysis of newly characterized, SDA-1 gp120 *env* nucleotide sequences obtained from plasma ($n = 10$) and PBMC ($n = 15$) with representative sequences of HIV-1 subtype B. Numbers at branch nodes refer to the percentage of bootstrap values and symbols indicate individual clones.

therapy-naive HIV-1 and PJP patient with extremely low CD4 cell number, and successfully isolated the virus (peak of p24, 500 ng) from this patient and designated it SDA-1. To assess the quasispecies diversity present in *vivo*, we analyzed the SDA-1 *env* clones derived from plasma RNA and PBMC. As shown in Fig. 1, SDA-1 is grouped within the HIV-1 subtype B reference sequences. Within SDA-1's sequence cluster, three phylogenetic forms were identified. Supported by a significant bootstrap value (96%), form I was the predominant quasispecies, representing 70% of all sequences. Two minor quasispecies (forms II and III) had similar structures but differed in the position of the first breakpoint. The mean distances between major and minor quasispecies did not differ significantly from the sequence heterogeneity. Furthermore, the quasispecies diversities between plasma and PBMC were similar within each form, and were all below 5.0%.

Multicoreceptor usage and CD4-independent entry of SDA-1

To determine the receptor usage of SDA-1, we randomly selected five clones from the predominant quasispecies and generated Env-pseudotypes and Env-chimeric viruses as representatives. As a control, the Envs from a variety of HIV-1 subtypes with X4 (HXB2), R5 (ADA), and R5X4 (89.6) tropism were used. Utilizing luciferase-pseudotypes (luc-p), we first examined the coreceptor usage of SDA-1. We found that in the presence of CD4, all representative SDA-1 Env-pseudotypes were able to use efficiently both CXCR4 and CCR5, with additional moderate usage of CCR3 and CCR8 (Fig. 2a).

We next investigated whether SDA-1 Envs are capable of inducing CD4-independent infection. We found that SDA-1 Envs mediated entry into both HOS–CXCR4 and HOS–CCR5. However, the infectivities of SDA-1 for HOS–CXCR4 were approximately 2.5-fold higher than that for HOS–CCR5 (Fig. 2b). In stark contrast, none of the other types of luc-p viruses entered either of those cells. Furthermore, we evaluated the ability of SDA-1 Envs in mediating cell–cell fusion, a dye-transfer cell–cell fusion assay [37] was used with HOS–CXCR4 and HOS–CCR5 cells. Only in the cells expressing SDA-1 Envs (effector cells) did cell–cell fusion with CD4-negative, CXCR4- or CCR5-positive HOS cells (target cells) occur (data not shown).

In addition to the results with HOS–CXCR4 and CCR5, preexposure of HOS cells to Leu-3A, a CD4 monoclonal antibody (mAb) that recognizes the gp120 binding site on CD4 [38], failed to block SDA-1 infection. In contrast, pretreatment with antagonists for CXCR4 or CCR5 effectively inhibited infection (Table 1). Furthermore, the infectivities of SDA-1 on HOS–CXCR4 and HOS–CCR5 were enhanced by preexposure of the virus to sCD4 indicating that the binding of SDA-1 Env to CD4 induces further conformational changes in gp120 to fully expose the chemokine receptor binding domain. Collectively, SDA-1 Envs mediated the CD4-independent infection via both CXCR4 and CCR5.

Having clarified that SDA-1 is a CD4-independent isolate, we next investigated what types of $CD4^-$ cells are able to support SDA-1's entry. We focused first on human liver-derived cell lines, as the mechanisms of the liver damage in HIV-1-infected individuals are still unclear.

Two hepatoma cell lines, Huh-7 and Hep-G2, were used as targets. We first examined the expression of the receptors on the cell surface by flow cytometry and found that both CXCR4 and CCR5 were expressed on Huh-7 and Hep-G2 cells. In contrast, CD4 was not detected on either, which was confirmed by RT–PCR (data not shown). We then evaluated whether SDA-1 can enter into hepatoma cells with luc-p viruses. We found that only SDA-1 luc-p viruses efficiently infected Huh-7; however, its infectivity was marginal in Hep-G2 (Fig. 2c). Previous studies have shown that few HIV-1 variants can infect $CD8^+$ cells using CD8 as receptor [10,39]. Therefore, we further explored receptors used by

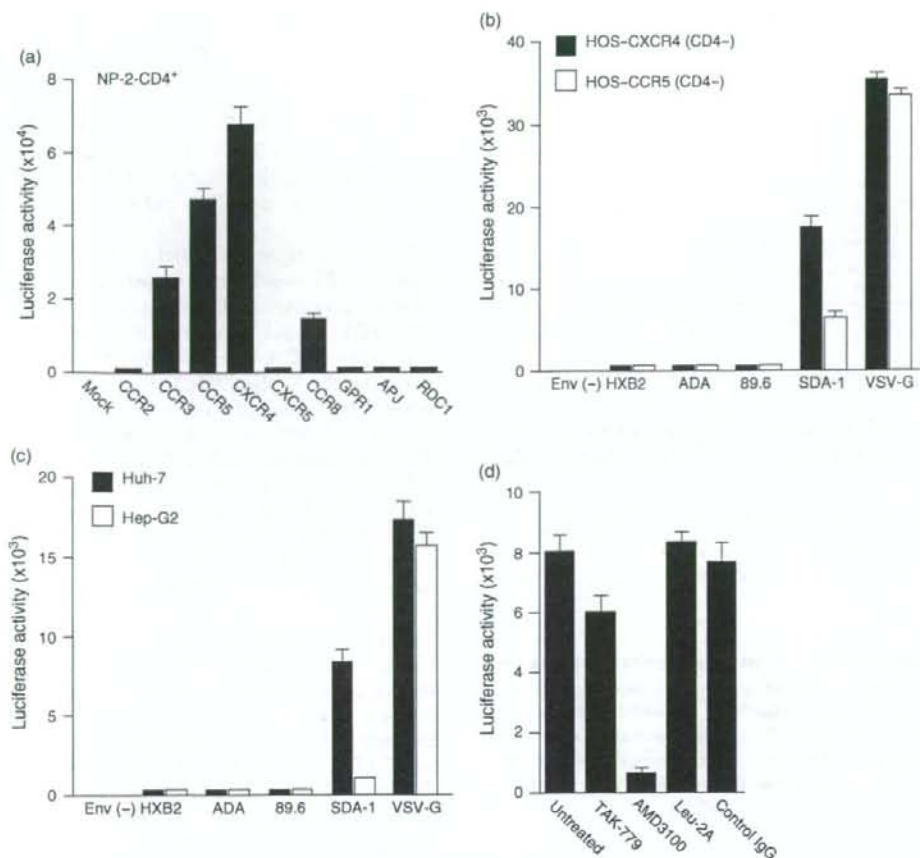

Fig. 2. Multicoreceptor usage and CD4-independent entry of SDA-1. (a) SDA-1 Envs mediate entry of $CD4^+$ cells using multiple coreceptors. NP-2-$CD4^+$ cells coexpressing one of the indicated chemokine receptors were exposed to SDA-1 luc-p viruses for 48 h and the luciferase activities were measured. (b) SDA-1 Envs mediate entry of $CD4^-$ cell lines through either CXCR4 or CCR5. The HOS cells ($CD4^-$) expressing either CXCR4 or CCR5 were exposed to the indicated HIV-1 luc-p viruses or VSV-G for 48 h, after which the infectivities were determined. (c) Entry of SDA-1 into $CD4^-$ human hepatoma cells. Huh-7 and Hep-G2 were exposed to the indicated HIV-1 luc-p viruses or VSV-G. Infectivities were determined at 48 h. (d) Effects of receptor-related antagonists or antibodies on the entry of SDA-1 entry into Huh-7 cells. Interaction of SDA-1 luc-p viruses with Huh-7 cells was tested in the absence or presence of AMD3100 (1.0 μM), TAK-779 (100 nM), anti-CD8 Leu-2A antibody (30 μg/ml) or class-matched control antibody (30 μg/ml). Results shown (a–d) are means of triplicate experiments. Bars, standard deviation. IgG, immunoglobulin G; VSV, vesicular stomatitis virus.

SDA-1 for entry into hepatoma cells. As shown in Fig. 2d, preexposure of Huh-7 to anti-CD8 Leu-2A mAb, as well as the CCR5 antagonist, TAK-779, failed to block SDA-1 infection of Huh-7, whereas anti-CXCR4 with AMD3100 effectively suppressed the infectivity. These results suggested that SDA-1 enters Huh-7 cells principally via CXCR4.

Replication of SDA-1 in human hepatoma cells

Although SDA-1 luc-p viruses infected some cells independently of CD4 cells, it was necessary to determine whether SDA-1 can replicate in those $CD4^-$ cells, particularly in hepatoma cells. For this purpose, we constructed NL43-based Env-chimeric viruses described above. We then examined whether the chimeric viruses were able to replicate in $CD4^-$ cells. As shown in Fig. 3a, the SDA-1 Env-chimeric viruses replicated efficiently in HOS–CXCR4 and HOS–CCR5 cells to similar levels. In contrast, none of the other Env-chimeric viruses infected either of those cells lines. Furthermore, we examined whether SDA-1 Env-chimeric viruses could replicate in hepatoma cells. As shown in Fig. 3b, high levels of NL43-SDA-1 replication were observed in Huh-7 cells. However, marginal replication was detected

Table 1. Inhibition of SDA-1 by blocking reagents in CD4⁻ cells.

	% Inhibition	
Reagent	HOS-CXCR4	HOS-CCR5
Medium	0	0
Control mAb (30 μg/ml)	0	0
Leu-3A (30 μg/ml)	10	12
Soluble CD4 (10 μg/ml)	225[a]	120[a]
AMD3100 (1.0 μM)	99	0
TAK-779 (100 nM)	0	97

CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; HOS, Human CD4-negative osteosarcoma; mAb, monoclonal antibody.
[a]Enhancement of entry.

in Hep-G2 cells. Although both Huh-7 and Hep-G2 cells are derived from human hepatoma, many potential host factors [40] could influence HIV replication, which for the most part remain unknown. Similarly, only Huh-7 cells, but not Hep-G2 cells, were susceptible to HCV [41,42]. These reasons may be related to the difference between Huh-7 and Hep-G2 regarding the level of replication by SDA-1.

SDA-1 replicates in both proliferating and static hepatocytes

To investigate further whether normal human hepatocytes could sustain entry and replication of SDA-1, p-hepatocytes were used for the following experiments. Among the three specific markers of human hepatocytes, both albumin and cytokeratin-18, but not alpha-fetoprotein were detected in the p-hepatocytes suggesting that the hepatocytes we used were well differentiated (data not shown). We also found that CXCR4 was expressed on the surface of p-hepatocytes. In contrast, neither CD4 nor CCR5 was detected on the p-hepatocyte surface or by real-time PCR (RT–PCR) (data not shown).

We next explored whether SDA-1 can enter p-hepatocytes by using GFP-p. As shown in Fig. 4a, only SDA-1 GFP-p viruses gave GFP-positive cells in p-hepatocytes, whereas other HIV-1 GFP-p viruses did not. The GFP-positive cells showed spindle-like shapes suggesting that the infection occurred in the p-hepatocytes but not in the contaminating lymphocytes. Furthermore, we studied whether SDA-1 can replicate in the p-hepatocytes. As shown in Fig. 4b, the p-hepatocytes were productively infected by the SDA-1 Env-chimeric viruses and SDA-1 virus stock itself but not by the other HIV-1 Env-chimeric viruses. Moreover, we found that AMD3100 inhibited the replication of SDA-1 in p-hepatocytes in a dose-dependent manner (Fig. 4c) indicating that the infection of p-hepatocytes by SDA-1 was mediated through CXCR4.

A previous study [19] reported that the HIV-1 gp120 *env* directly caused hepatocyte death by signaling through CXCR4 *in vitro*; however, most studies were performed using the hepatoma Huh-7 cells not hepatocytes, therefore, it may not really reflect the nature of liver damage. To explore the pathological effects of HIV-1 CD4-independent infection on hepatocytes, we exposed p-hepatocytes to the SDA-1 and analyzed cell viability. We found that the viability of the p-hepatocytes in cells cultured with or without SDA-1 Env-chimeric viruses was comparable (96%, *P* was not significant) indicating that HIV-1 CD4-independent infection rarely induces hepatocyte death via an apoptotic process (data not shown). To further examine whether the infection or replication of SDA-1 is limited only to a certain number of p-hepatocytes or whether the infectivity or replication is influenced by the cell cycle, we studied the intracellular expression by flow cytometry of p24 and Ki-67 [43], a marker strictly associated with cell proliferation, in the HIV-1-infected p-hepatocytes. As shown in Fig. 4d, we found that 32.49% of p-hepatocytes were infected by SDA-1. However, there was no significant difference in

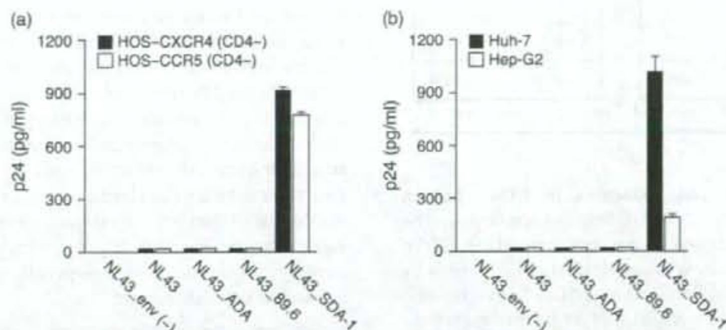

Fig. 3. CD4-independent infection of SDA-1 Env-chimeric viruses. The HOS cells (CD4⁻) expressing either CXCR4 or CCR5 (a) and two CD4⁻ human hepatoma cells (b) were incubated with the indicated HIV-1 Env-chimeric viruses. Virus replication was then monitored by p24 antigen production on day 3. Results shown (a, b) are means of triplicate experiments. Bars, standard deviation.

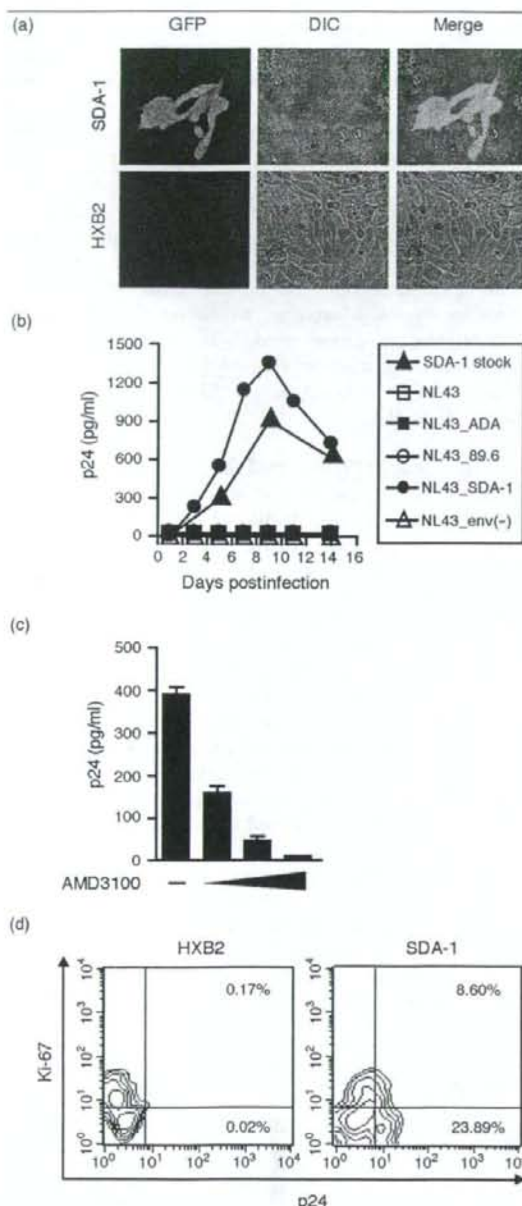

Fig. 4. SDA-1 enters and replicates in $CD4^-$ human p-hepatocytes. (a) Entry of SDA-1 into p-hepatocytes. The p-hepatocytes were exposed to the indicated HIV-1 GFP-p viruses for 48 h. Infectivity was determined as GFP^+ cells by confocal microscopy. (b) Replication of SDA-1 Env-chimeric viruses and SDA-1 virus stock in human p-hepatocytes. (c) SDA-1 infects p-hepatocytes through CXCR4. The inhibitory effects of AMD 3100 (0.1, 0.3 and 1.0 μM) on SDA-1 Env-chimeric viruses infection of p-hepatocytes were studied. Results shown are means of triplicate experiments. Bars, SD. (d) SDA-1 replicates in both proliferating and static

percentage of p24 expression between Ki-67^+ (31%) and Ki-67^- p-hepatocytes (33.1%), suggesting that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes.

Considering that SDA-1 can infect hepatocytes *in vitro*, it would have been interesting to determine whether the patient's liver was infected *in vivo*. However, consent for a liver biopsy was denied by the patient's family. There was no evidence of liver dysfunction. When virus was isolated from this patient; however, liver damage [an aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ≥ 1] was observed at the end of the clinical stage. Although the cause of liver injury was unclear, our present data suggest that CD4-independent HIV-1 infection may lead to hepatocellular damage.

Discussion

In this study, we characterized a quasispecies of a CD4-independent HIV-1 isolate, termed SDA-1, which was able to utilize either CXCR4 or CCR5 in the absence of CD4. Moreover, we demonstrated that SDA-1 efficiently entered and replicated in Huh-7 hepatoma cells and normal human hepatocytes, through CXCR4, without inducing apoptotic cell death.

Many SIV and HIV-2 isolates can infect cells without CD4, at least to some extent. However, CD4-independent HIV-1 viruses have been rarely isolated and, so far, only a few laboratory-adapted CD4-independent HIV-1 variants have been reported. It must be noted that CD4-independent HIV-1 variants, isolated *in vitro* by passage through cells lacking CD4, have been shown to be more sensitive to neutralizing antibodies than CD4-dependent viruses [44,45]. Therefore, we might hypothesize that the emergence of a quasispecies of HIV-1 with a reduced requirement for CD4 is likely to be at a low abundance relative to the more common $CD4^+$ strains. However, with disease progression, HIV-1 variants with reduced affinity for CD4 and with increased affinity for chemokine receptor could evolve and become more robust in the viral quasispecies, disseminate in a variety of $CD4^-$ tissues *in vivo* under conditions of both reduced immunological pressure and a dramatically reduced pool of target $CD4^+$ cells concomitant with high levels of virus replication. It will be important to search the viral quasispecies in other patients, especially in the later stages of HIV-1 disease for the existence of similar CD4-independent HIV-1 variants and expanded cellular tropism.

Fig. 4. (Continued)
human p-hepatocytes. Intracellular stainings of HIV-1-infected p-hepatocytes for p24 and Ki-67 were analyzed by flow cytometry. CXCR4, chemokine (C-X-C motif) receptor 4; GFP-p, GFP-pseudotypes.

Although the extent to which CD4$^-$ cells are infected *in vivo* is unclear, it has been widely thought to be low. Nonetheless, recent studies [11,12] utilizing the novel approach of laser capture microscopy have revealed HIV-1 sequences in isolated CD4$^-$ cells of kidney epithelium and neuronal cells, indicating that latent infection might occur in such cells or tissues *in vivo*. The mechanism of viral entry into CD4$^-$ cells remains unclear, but as we show here the evidence of emergence of CD4-independent strains *in vivo* must be kept in mind.

End-stage liver disease is now becoming a frequent cause of death in HIV-1-infected hospitalized patients. HCV and HBV coinfection with HIV-1 has been shown to enhance the progression of liver damage [16]. However, little attention has been given to the direct virological interaction between HIV and HCV/HBV in the liver, as HIV has been thought not to infect hepatocytes directly. Nonetheless, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection. In our study, we clearly demonstrated that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes through CXCR4. To our knowledge, this is the first report that HIV-1 can efficiently replicate in normal hepatocytes. Furthermore, we have shown that HIV-1 infection did not induce significant cytocidal effects in the hepatocytes. It is noteworthy that the liver is a continuously regenerating organ. Therefore, if HIV-1 enters and integrates its DNA into the host genome, liver cells containing HIV-1 DNA will be continuously generated by the division of the infected cells. Thus, the expression of HIV-1 proteins on the infected cell surface might result in chronic damage of the liver cells by inducing host immune responses. Direct virological interaction between HIV, HCV and HBV in the liver or enhanced production of HIV-1 by inflammatory cytokines produced by the HCV and HBV-activated immune cells might also exacerbate the liver injury. At present, however, we have no definite information concerning the extent to which patients' hepatocytes harbor HIV-1 and CD4-independent HIV-1 variants.

Finally, a particularly important area of vaccine research is to take advantage of gp120 structural information to guide the design of novel envelope immunogens. As has been reported, CD4-dependent viruses hide neutralizing epitopes and only CD4 binding to gp120 induces conformational changes in gp120 to fully expose epitopes for broadly neutralizing antibodies. The CD4-independent strain we isolated here seems particularly important, as it can efficiently replicate in CD4$^-$ hepatocytes. Therefore, the gp120 structural alterations, which might expose the coreceptor binding site without binding to CD4, may also open up other sites that could yield neutralizing antibodies. Nevertheless, evidence of a clinical CD4-independent R5X4 HIV-1 virus should have important implications concerning the range of mutability and tropism of HIV-1 and the pathogenesis of AIDS.

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P.X., H.L., Y.S. and T.H. designed the study. P.X., O.U., Y.S., M.Z., Y.A. and H.G. performed the experiments. P.X., O.U, Y.S., H.L. and T.H. analyzed the data. N.S. and H.H. contributed to the coreceptor expressing cell lines. P.X., H.L., Y.S., O.U., N.S., H.H. and T.H. contributed to writing the paper. T.H. contributed to grant application and financial support.

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Increased Synthesis of Anti-Tuberculous Glycolipid Immunoglobulin G (IgG) and IgA with Cavity Formation in Patients with Pulmonary Tuberculosis▽

Masako Mizusawa,[1]¶ Mizuoho Kawamura,[2] Mikio Takamori,[3] Tetsuya Kashiyama,[3] Akira Fujita,[3] Motoki Usuzawa,[1] Hiroki Saitoh,[1] Yugo Ashino,[1] Ikuya Yano,[4] and Toshio Hattori[1]*

Division of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8574,[1] Fuji Research Laboratories, Kyowa Medex, Co., Ltd., Shizuoka 411-0932,[2] Department of Respiratory Diseases, Tokyo Metropolitan Fuchu Hospital, Fuchu, Tokyo 183-8524,[3] and Japan BCG Central Laboratory, Kiyose, Tokyo 204-0022,[4] Japan

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Tuberculous glycolipid (TBGL) antigen is a cell wall component of *Mycobacterium tuberculosis* and has been used for the serodiagnosis of tuberculosis. We investigated correlations between the levels of anti-TBGL antibodies and a variety of laboratory markers that are potentially influenced by tuberculous infection. Comparisons between patients with cavitary lesions and those without cavitary lesions were also made in order to determine the mechanism underlying the immune response to TBGL. Blood samples were obtained from 91 patients with both clinically and microbiologically confirmed active pulmonary tuberculosis (60 male and 31 female; mean age, 59 ± 22 years old). Fifty-nine patients had cavitary lesions on chest X-rays. Positive correlations were found between anti-TBGL immunoglobulin G (IgG) and C-reactive protein (CRP) ($r = 0.361$; $P < 0.001$), between anti-TBGL IgA and soluble CD40 ligand (sCD40L) ($r = 0.404$; $P < 0.005$), between anti-TBGL IgG and anti-TBGL IgA ($r = 0.551$; $P < 0.0000005$), and between anti-TBGL IgM and serum IgM ($r = 0.603$; $P < 0.00000005$). The patients with cavitary lesions showed significantly higher levels of anti-TBGL IgG ($P < 0.005$), anti-TBGL IgA ($P < 0.05$), white blood cells ($P < 0.01$), neutrophils ($P < 0.005$), basophils ($P < 0.0005$), natural killer cells ($P < 0.05$), CRP ($P < 0.0005$), KL-6 (sialylated carbohydrate antigen KL-6) ($P < 0.0005$), IgA ($P < 0.05$), and sCD40L ($P < 0.01$). The observed positive correlations between the anti-TBGL antibody levels and inflammatory markers indicate the involvement of inflammatory cytokines and NKT cells in the immunopathogenesis of pulmonary tuberculosis.

There were an estimated 8.8 million new tuberculosis (TB) cases in 2005. TB incidence reached a peak worldwide, but the total number of new TB cases is still rising. The numbers of human immunodeficiency virus (HIV)-positive and multidrug-resistant TB patients diagnosed and treated are increasing (22). To develop new drugs and vaccines against TB, it is essential to study its immunopathogenesis. Lipoarabinomannnan (LAM), a complex glycolipid, is a major cell wall component of *Mycobacterium tuberculosis*. It has been researched extensively as an immunomodulator (4, 7, 9, 24, 26). LAM has also been used as a glycolipid antigen in the serodiagnostic method for TB. In addition to LAM, there are many glycolipids constituting the mycobacterial cell wall, such as trehalose 6,6-dimycolate (TDM). We used TDM, a glycolipid antigen purified from *Mycobacterium tuberculosis* H37Rv, in an enzyme-linked immunosorbent assay (ELISA) and reported that its sensitivity was 81% and its specificity was 96% (14). Subsequently, by combining TDM with more hydrophobic glycolipids, a new tuberculous glycolipid (TBGL) antigen was designed and a more sensitive serodiagnostic kit for TB, an anti-TBGL immunoglobulin G (IgG) test, was developed (11). Although TBGL has been used as a serodiagnostic antigen for TB and its clinical evaluations have been reported in several studies, how TBGL is involved in tuberculous pathogenesis has not been studied. Since TBGL is one of the cell wall components of *Mycobacterium tuberculosis*, like LAM, it may have some important roles in the immunopathogenesis of TB, as does LAM. In order to determine the mechanism underlying the immune response to TBGL, we measured plasma IgA, IgM, and IgG titers specific for TBGL and investigated correlations between those antibody titers and laboratory markers potentially influenced by TB infection in patients with active pulmonary TB. The measured markers were the numbers of white blood cells with differential counts, CD3-positive lymphocytes (T cells), CD20-positive lymphocytes (B cells), and CD56-positive lymphocytes (natural killer cells) and levels of serum IgG, serum IgA, serum IgM, serum albumin, serum creatinine, serum C-reactive protein (CRP), plasma soluble CD40 ligand (sCD40L), and plasma KL-6 (sialylated carbohydrate antigen KL-6). KL-6 is a mucinous high-molecular-weight glycoprotein expressed on type II pneumocytes, and it was reported to be elevated in the sera of patients with interstitial pneumonia (13). We used plasma samples, but the level of KL-6 is known to show no significant difference between serum and plasma. We measured KL-6 because TB patients with extensive radiographic changes were also reported to have higher KL-6 values (8).

* Corresponding author. Mailing address: Division of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi, Japan 980-8574. Phone: 81-227178220. Fax: 81-227178221. E-mail: hattori.t@rid.med.tohoku.ac.jp.

¶ Present address: Jichi Medical University Hospital, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan.

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CD40L is expressed on the surfaces of activated CD4+ T cells, basophils, and mast cells. The binding of CD40L to its receptor, CD40, on the surfaces of B cells stimulates B-cell proliferation, adhesion, and differentiation. A soluble isoform of CD40L has been shown to exist in the circulation, exhibiting full activity in B-cell proliferation and differentiation assays (16). It was reported that the treatment of wild-type CD40 mice with sCD40L fusion protein elicited a pulmonary inflammatory response that was not observed in identically treated CD40 knockout mice (21). Based on these reports, we measured sCD40L as a possible marker of pulmonary inflammation.

Furthermore, it was reported that the positive rate and the titers of anti-TBGL IgG were higher in pulmonary TB patients with cavitary lesions than in those without cavitary lesions (15). Considering this result, we subdivided the patients into two groups, those with cavitary lesions (cavity+ group) and those without cavitary lesions (cavity− group), and compared multiple laboratory markers to determine associations.

In addition, we categorized the patients into three groups based on chest X-ray findings, namely, minimal, moderately advanced, and far advanced, according to the classification of the National Tuberculosis and Respiratory Disease Association of the USA (NTA) (6), and compared all the measured laboratory markers, including anti-TBGL antibodies, among the three groups to determine if there were any parameters related to disease progression and severity.

MATERIALS AND METHODS

Subjects. We designed a cross-sectional study using 121 patients at Tokyo Metropolitan Fuchu Hospital between May 2004 and August 2005. These patients were clinically diagnosed as having active TB and admitted to the hospital for treatment. Medical histories were taken from the enrolled patients, and all of them underwent physical examination, chest X-rays, blood test and culture test for acid-fast bacilli, and/or TB-PCR test of sputum samples. Ninety-one subjects were selected (60 males and 31 females; mean age [± standard deviation], 59 ± 22 years old) for analysis according to the following criteria: (i) diagnosed as having pulmonary TB by positive culture or positive PCR for *Mycobacterium tuberculosis* in sputum, (ii) untreated or undergoing less than 2 weeks of TB treatment, (iii) negative for *Mycobacterium avium* complex infection, (iv) negative for HIV infection, (v) no malignancy, and (vi) no other active pulmonary diseases. The remaining 30 patients were excluded for the following reasons: 4 for both negative culture and a negative PCR test for *Mycobacterium tuberculosis* in sputum, 5 for more than 2 weeks of TB treatment, 2 for *Mycobacterium avium* complex infection, 4 for HIV infection, 3 for malignancy, 2 for interstitial pneumonia, and 10 for insufficient data collection. We enrolled patients with less than 2 weeks of treatment based on a report that anti-TBGL IgG did not decrease until 1 month after the commencement of chemotherapy (15). The study was approved by the Ethics Committee of Tokyo Metropolitan Fuchu Hospital. We obtained written informed consent from all the enrolled patients.

TBGL antibody. Anti-TBGL antibodies were measured using a Determiner TBGL antibody ELISA kit (Kyowa Medex, Tokyo, Japan), an in vitro ELISA for the quantitative measurement of anti-TBGL IgG antibody in serum or plasma. This assay employs glycolipid antigens purified from *Mycobacterium tuberculosis* H37Rv (TBGL antigen) coated on a 96-well plate. The details of the assay were described in our previous studies (2, 11), but briefly, plasma was diluted 41-fold and added to wells that bound TBGL antigen. The wells were washed, and horseradish peroxidase-conjugated rabbit anti-human IgG, IgA, and IgM, all of which are specific to each heavy chain (Dako Japan, Kyoto, Japan), were added, followed by 60 min of incubation at room temperature. The plates were washed three times with washing buffer, 100 μl of TMBZ (3,3′,5,5′-tetramethylbenzidine) solution was added to each well, and the plates were incubated for 15 min at room temperature. To stop the enzyme reaction, 100 μl of 1 M H_2SO_4 was added, and the absorbance at 450 nm was measured with an MTP-120 plate reader (Corona Electric Co., Tokyo, Japan). The antibody titer was expressed according to a cutoff index. We scored the sample as positive when the titer was above the cutoff index for anti-TBGL IgG of 2.0 U/ml, the cutoff point proposed by Kishimoto et al. for the screening of patients with TB based on the diagnostic efficiency by receiver operating characteristic curve analysis (12). The cutoff values for anti-TBGL IgA and IgM are not available.

Measured laboratory markers. We investigated the correlations between anti-TBGL antibodies and laboratory markers of TB infection, including immunocompetent cells. We measured the number of white blood cells with differential counts and the numbers of lymphocytes positive for CD3, CD20, and CD56 by FACSCalibur flow cytometry (Becton Dickinson and Company, NJ), using phycoerythrin-conjugated Leu-4 monoclonal antibody (MAb), fluorescein isothiocyanate-conjugated Leu-16 MAb, and phycoerythrin-conjugated Leu-19 MAb, respectively (Becton Dickinson and Company, NJ). Serum albumin and serum creatinine were measured because malnutrition and chronic renal failure are major risk factors for TB infection. We also measured IgA, IgG, IgM, and CRP by using serum and sCD40L and KL-6 by using plasma. The rationales for measuring sCD40L and KL-6 were stated in the introduction. sCD40L and KL-6 ELISA kits were purchased from Medsystems Diagnostics (Vienna, Austria) and from Sanko-Junyaku (Tokyo, Japan), respectively. The titers were measured according to the manufacturers' protocols.

Radiographic classification. We subdivided the patients into two groups, the cavity+ group and the cavity− group. We also categorized the patients into three groups based on chest X-ray findings, namely, minimal, moderately advanced, and far advanced, according to the classification of the NTA. Minimal lesions include those that are of slight to moderate density but do not contain demonstrable cavitation. They may involve a small part of one or both lungs, but the total extent, regardless of distribution, should not exceed the volume of lung on one side which is present above the second chondrosternal junction and the spine of the fourth or the body of the fifth thoracic vertebra. Moderately advanced lesions may be present in one or both lungs, but the total extent should not exceed the following limits: disseminated lesions of slight to moderate density may extend throughout the total volume of one lung or the equivalent in both lungs; dense and confluent lesions must be limited in extent to one-third the volume of one lung; and the total diameter of cavitation, if present, must be less than 4 cm. Far advanced lesions are more extensive than moderately advanced lesions (6).

Statistical analysis. Laboratory data were analyzed using Stat Flex, version 5 (Artec, Osaka, Japan), and Statcel 2 (OMS Publishing Inc., Saitama, Japan). Correlations between levels of each parameter were evaluated by Spearman's rank correlation coefficient. The significances of differences were evaluated by the Mann-Whitney test. P values of <0.05 were considered significant. Bonferroni adjustment was used for multiple comparisons.

TABLE 1. Health status of patients in the study

Parameter	Value for group		
	Total	Cavity+	Cavity−
No. of patients	91	59	32
Age (yrs [mean ± SD])	59 ± 22	56 ± 21	64 ± 22
Gender (no. of males/no. of females)	60/31	42/17	18/14
No. of patients with history of:			
TB	20	14	6
Gastrectomy	3	3	3
Diabetes mellitus	14	7	7
Chronic renal failure	5	2	3
Diabetes mellitus and chronic renal failure	1	0	1

RESULTS

Health status of patients and positive rate. The health status of the included patients is shown in Table 1. Among the 91 patients in this study, there were 20 patients with histories of TB, 3 patients with histories of gastrectomy, 14 patients with diabetes mellitus, 5 patients with chronic renal failure, and 1 patient with both diabetes mellitus and chronic renal failure. Fifty-nine patients had cavitary lesions on chest X-rays. The positive rate for the anti-TBGL IgG test, a commercialized

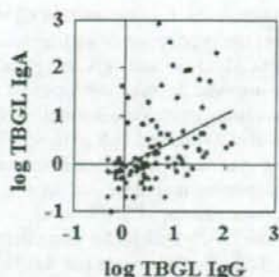

FIG. 1. Correlation between anti-TGBL IgG (TBGL IgG) and anti-TBGL IgA (TBGL IgA) ($r = 0.551$; $P < 0.0000005$).

diagnostic test for TB, was 79.7% (47/59 patients) for the cavity$^+$ group, 50% (16/32 patients) for the cavity$^-$ group, and 69.2% (63/91 patients) overall. No patient was on steroid therapy.

Correlations between anti-TBGL antibodies. We sought correlations between each of the anti-TBGL antibodies. Anti-TBGL IgG had a positive correlation with anti-TBGL IgA ($r = 0.551$; $P < 0.0000005$) (Fig. 1). No other correlations were shown between the anti-TBGL antibodies.

Correlations between anti-TBGL antibodies and influential laboratory markers. Anti-TBGL IgG had positive correlations with IgA ($r = 0.228$; $P < 0.05$), CRP ($r = 0.361$; $P < 0.001$), and KL-6 ($r = 0.275$; $P < 0.01$) and negative correlations with creatinine ($r = -0.249$; $P < 0.05$) and albumin ($r = -0.240$; $P < 0.05$). Anti-TBGL IgA had positive correlations with IgG ($r = 0.285$; $P < 0.01$), IgA ($r = 0.300$; $P < 0.005$), KL-6 ($r = 0.225$; $P < 0.05$), and sCD40L ($r = 0.404$; $P < 0.005$). Anti-TBGL IgM had positive correlations with IgM ($r = 0.603$; $P < 0.00000005$) and albumin ($r = 0.251$; $P < 0.05$).

TABLE 2. Measured parameters (mean ± SD) and comparison between cavity$^+$ group and cavity$^-$ group

Parameter	Value for group			P value[a]
	Total	Cavity$^+$	Cavity$^-$	
TBGL-IgG (U/ml)	13.2 ± 23.5	17.1 ± 27.6	6.0 ± 9.9	<0.005*
TBGL-IgA (U/ml)	22.6 ± 95.4	32.0 ± 117.2	5.3 ± 15.7	<0.05*
TBGL-IgM (U/ml)	6.0 ± 5.6	5.9 ± 5.8	6.2 ± 5.3	NS
IgG (mg/dl)	1,518 ± 471	1,523 ± 510	1,509 ± 395	NS
IgA (mg/dl)	416 ± 213	451 ± 236	348 ± 140	<0.05*
IgM (mg/dl)	106 ± 57	103 ± 58	111 ± 55	NS
White blood cells/μl	7,236 ± 2,706	7,830 ± 3,020	6,141 ± 1,513	<0.01*
Neutrophils/μl	5,567 ± 2,532	6,192 ± 2,798	4,415 ± 1,362	<0.005*
Monocytes/μl	397 ± 223	424 ± 243	347 ± 172	NS
Eosinophils/μl	115 ± 119	126 ± 135	94 ± 80	NS
Basophils/μl	24 ± 45	22 ± 46	29 ± 43	<0.0005*
Lymphocytes/μl	1,128 ± 740	1,061 ± 766	1,253 ± 685	NS
CD3$^+$ cells/μl	751 ± 509	715 ± 517	815 ± 496	NS
CD20$^+$ cells/μl	131 ± 115	114 ± 93	161 ± 143	NS
CD56$^+$ cells/μl	208 ± 197	197 ± 223	231 ± 140	<0.05*
CRP (mg/dl)	4.5 ± 5.2	5.7 ± 5.8	2.2 ± 2.9	<0.0005*
KL-6 (U/ml)	564 ± 459	662 ± 518	382 ± 241	<0.0005*
sCD40L (ng/ml)	1.8 ± 2.5	2.1 ± 3.0	1.2 ± 1.0	<0.01*
Creatinine (mg/ml)	1.0 ± 1.5	0.8 ± 0.6	1.5 ± 2.4	NS
Albumin (g/dl)	3.4 ± 0.8	3.3 ± 0.7	3.6 ± 0.8	NS

[a] Asterisks show significant differences between the cavity$^+$ group and the cavity$^-$ group. NS, no significant difference. The significances of differences were evaluated by the Mann-Whitney test. P values of <0.05 were considered significant.

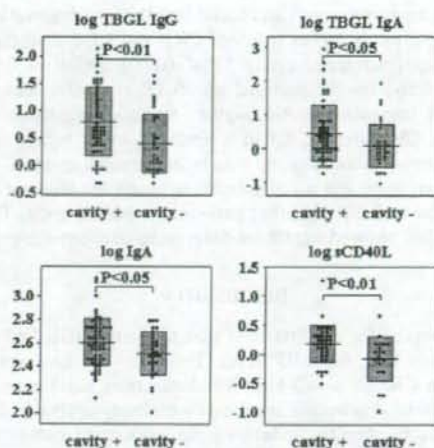

FIG. 2. Anti-TBGL IgG (TBGL IgG), anti-TBGL IgA (TBGL IgA), IgA, and sCD40L levels in cavity$^+$ and cavity$^-$ groups.

Comparison between patients with and without cavitary lesions. We compared all the measured laboratory markers between the patients with cavitary lesions (cavity$^+$ group) and those without cavitary lesions (cavity$^-$ group) in order to determine new differences apart from that of the anti-TBGL IgG level (15). As shown in Table 2, both anti-TBGL IgG and anti-TBGL IgA levels were significantly higher in the cavity$^+$ group ($P < 0.005$ and $P < 0.05$, respectively), but the anti-TBGL IgM titers showed no difference between the two groups. The numbers of white blood cells ($P < 0.001$), neutrophils ($P < 0.005$), basophils ($P < 0.0005$), and natural killer cells (CD56$^+$) ($P < 0.05$) were significantly higher in the cavity$^+$ group. The levels of CRP ($P < 0.0005$), KL-6 ($P < 0.0005$), IgA ($P < 0.05$), and sCD40L ($P < 0.01$) were also significantly higher in the cavity$^+$ group (Table 2; Fig. 2 and 3).

Radiographic changes and inflammatory markers. We compared the levels of the inflammatory markers CRP and KL-6

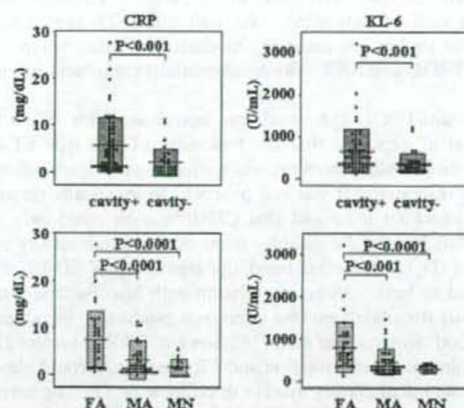

FIG. 3. CRP and KL-6 levels in cavity$^+$ and cavity$^-$ groups and NTA classification groups. FA, far advanced; MA, moderately advanced; MN, minimal.

among the three groups and found that the far advanced group had significantly higher levels of CRP and KL-6 than did the moderately advanced group ($P < 0.0001$ and $P < 0.0005$, respectively) or the minimal group ($P < 0.0001$ and $P < 0.00005$, respectively). Although no statistical significance appeared, CRP and KL-6 had a tendency to be higher in the moderately advanced group than in the minimal group (Fig. 3). However, there was no correlation between the levels of CRP and those of KL-6. No other parameters, including anti-TBGL antibodies, showed significant differences between the groups.

DISCUSSION

We report for the first time that the anti-TBGL IgG level correlates with the CRP level. This may not be surprising because CRP is a well-known inflammatory marker and inflammation is generally involved in antibody synthesis. However, the mechanism underlying the association between the anti-TBGL IgG level and CRP was not readily understandable. Trehalose 6,6′-dimycolate ("cord factor") is one of the principal antigens in TBGL, and cord factor has mycolic acid side chains. Mycolic acids are long-chain fatty acids that constitute the lipid-rich cell wall framework of mycobacteria, and their recognition is known to be mediated by CD1. Enomoto et al. discovered a CD1-restricted human T-cell line specific for glucose monomycolate, a glycosylated species of mycolic acids (5), and most CD1-restricted T cells are known to be natural killer T cells (NKT cells). Historical studies showed granuloma formation in the lungs of mice after intravenous administration of emulsified trehalose-6,6′-dimycolate ("cord factor") (23). The role of NKT cells in granuloma formation was also confirmed by the fact that granulomas were actually formed in wild-type mice injected with cell walls from *Mycobacterium tuberculosis* but not in $J\alpha 281^-$ mice, which lack NKT cells (1). On the other hand, Mempel et al. demonstrated that NKT cells migrate to and accumulate at inflammatory sites and behave like inflammatory cells independently of the CD1 molecules (17), which could lead to the production of inflammatory markers such as CRP. The possibility of NKT-cell involvement in anti-TB immunity was also suggested in a recent study describing that NKT cells are selectively lower in the peripheral blood mononuclear cells of individuals with pulmonary TB (19). More extensive studies are necessary to clarify the relationship between TBGL and NKT cells in tuberculous granuloma formation.

The anti-TBGL IgA level was correlated with sCD40L. Wiley et al. reported that the treatment of wild-type CD40 mice with sCD40L fusion protein elicited a pulmonary inflammatory response that was not observed in identically treated CD40 knockout mice and that CD40 ligation could play an important role in the establishment of the inflammatory response (21). On the other hand, the expression of CD40L was reported to have a direct correlation with *Mycobacterium tuberculosis*-stimulated gamma interferon production by peripheral blood mononuclear cells (18). Since sCD40L is involved in both pulmonary inflammation and TB infection, it could play a role as an inflammatory marker in pulmonary TB. The correlation between the anti-TBGL IgA level and sCD40L may also reflect the following immunopathogenesis of *Mycobacterium tuberculosis* infection. In the cavity$^+$ group, sCD40L and IgA were significantly elevated. It is known that CD40 engagement by CD40L induces the production of endogenous transforming growth factor beta (TGF-β) and IgA secretion (25) and that TGF-β may be involved in the development and/or consequences of tuberculous granuloma formation (3). Therefore, the higher levels of sCD40L and IgA in the cavity$^+$ group may reflect the intense granuloma formation in cavitary lesions, and these immune responses may have led to the correlation of anti-TBGL IgA level and the sCD40L level.

The level of anti-TBGL IgG had a correlation with that of anti-TBGL IgA. This correlation was not due to cross-reaction of the secondary antibodies because unwanted antibodies had been removed by solid-phase absorption. Julián et al. conducted a comparative study of IgG, IgM, and IgA antibody responses to four trehalose-containing glycolipids, including cord factor, purified from *Mycobacterium tuberculosis* in the sera from 92 TB patients. They concluded that IgG antibody was more sensitive, IgA antibody was more specific, and IgM reactivity was negligible for all the glycolipid antigens used (10). Since TBGL is a glycolipid antigen containing cord factor, anti-TBGL IgA may yield a higher specificity than does anti-TBGL IgG, and the detection of both anti-TBGL IgG and anti-TBGL IgA may improve the diagnostic value. A prospective controlled study on anti-TBGL IgA will be necessary to confirm this possibility.

There was a strong correlation between the levels of anti-TBGL IgM and serum IgM. However, we concluded that this did not reflect specific immunity in TB infection because IgM has a low affinity and cross-reactivity in addition to its pentameric structure (20).

Inoue et al. reported that the serum levels of KL-6 in 57 patients with active pulmonary TB rose significantly according to the increase in the extent of radiographic findings based on the classification of the NTA, but there was no significant difference between those with cavities and those without cavities (8). In our study, the far advanced group had significantly higher levels of KL-6 than did the moderately advanced group and the minimal group, and although the difference was not statistically significant, the moderately advanced group had higher levels of KL-6 than did the minimal group. In contrast to Inoue's data, KL-6 was significantly higher in the cavity$^+$ group. The same results were shown for the level of CRP (Fig. 3), but no correlation was seen between KL-6 and CRP. Based on these findings, the level of KL-6 or CRP may reflect a different component of disease progression and could be used to evaluate the severity of pulmonary TB.

Although we found interesting correlations between anti-TBGL antibody levels and inflammatory markers, suggesting the involvement of inflammatory cytokines and NKT cells, confirmatory experiments have not been done, which is a major limitation of this study. Demonstrating specific immune responses to glycolipid antigens by using T cells from TB patients and their characterization would help to elucidate the immunopathogenesis of pulmonary TB.

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Small RNA Molecules as Therapeutic Agents for Viral Infectious Diseases

[1]Jing Zhang and [2]Toshio Hattori
[1]Research and Development Center,
FUSO Pharmaceutical Industries, Ltd., Osaka, Japan
[2]Department of Infectious and Respiratory Diseases,
Tohoku University School of Medicine, Sendai, Japan

Abstract: The potential of using small RNA molecules as therapeutic agents has been extensively explored, antisense RNA, ribozyme, aptamer, decoy and more recently siRNA have been demonstrated to be highly efficient in inhibiting a number of pathogenic viruses including human immunodeficiency virus, hepatitis B and C virus, poliovirus and influenza virus. The specificity and potency of the sequence-specific agents such as antisense, ribozyme and siRNA in particular, imply that these strategies will prove to be promising therapeutics for treating viral infections, although the antiviral efficacy may be limited by emergence of escape variants. Distinct from the reagents targeting viral RNA, decoy and aptamer inhibit viral replication by binding and thus inactivating the viral component such as regulatory gene product and viral enzyme. This review provides an up-to-date overview of the progress and problems in small RNA-based antiviral approaches, with a focus on their therapeutic utility, delivery and unwanted side effects.

Key words: Antisense RNA, antiviral approaches, ribozyme, aptamer, decoy, siRNA, therapeutics utility

INTRODUCTION

Despite much effort towards preventing viral infectious disease, chronic infection with viruses such as Human Immunodeficiency Virus (HIV), hepatitis B and C viruses (HBV and HCV) have been increasing, remaining serious worldwide health problems. Additionally, the emerging of avian influenza virus that can infect humans and the outbreak of the Severe Acute Respiratory Syndrome (SARS) caused by SARS coronavirus imply the threat of a global virus pandemic. The approaches to combat viral infections include vaccine and drugs that are targeted to specific viral enzymes or other proteins. One unavoidable problem is selection of resistant mutants during long-term treatment and multiple targets are generally required to prevent the emergence of mutant variants.

The concept of using RNA molecules as therapeutic agents for viral infection has aroused increasing interest in the recent decade. Antisense strategies, which encompass antisense oligonucleotides, ribozymes and small interfering RNA (siRNA), involve small nucleic-acid-based molecules that inhibit viral replication in a sequence-specific manner. The antisense reagents can be designed to target any viral RNA provided its sequence is known, making them theoretically ideal antiviral therapeutics. However, because of the strict sequence-specific property intrinsic in these approaches, the possibility of emergence of escape mutants upon persistent treatment is concerned. Other RNA-based strategies for combating viral disease include apatmer and decoy, which inhibit viral replication by binding and consequently inactivating the viral component such as regulatory gene product and viral enzyme.

Corresponding Author: Jing Zhang, Research and Development Center, FUSO Pharmaceutical Industries, Ltd., Osaka, Japan Tel: 81-6-6969-3131 Fax: 81-6-6964-2706

Antisense RNAs

Antisense oligonucleotides are single-strand DNA or RNA oligomer of 18-25 nucleotides in length, designed to bind to its target RNA via Watson-Crick base pairing. Depending on the type of antisense oligonucleotides used, there are two different action modes involved in gene knockdown. Conventional oligonucleotides such as phosphodiesters and phosphorothioates, recruit cellular RNase H to the duplex to cleave the target RNA; later-generated oligonucleotides including the modified oligonucleotides derivatives such as morpholinos, locked nucleic acids and peptide nucleic acids, do not active RNase H but inhibit translation by steric hindrance instead. In addition to their application in analysis of gene function, intensive studies have been conducted to explore the potential of antisense oligonucleotides as therapeutic modalities for diseases caused by the expression of deleterious genes, especially in the field of cancer and viral infections.

Viruses with the RNA genomes are particularly well suited to be targeted by antisense oligonucleotides, because both the mRNA and genomic RNA can be targeted, destruction of viral RNA could eliminate not only viral protein synthesis but also viral replication. On the other hand, in the case of DNA viruses or retroviruses, only mRNA can be targeted by antisense nucleotide, making virus clearance theoretically impossible and persistent treatment necessary. The first attempt employing antisense nucleotides as the viral therapeutic was published in 1978, which reported an inhibitory effect of oligodeoxynucleotides on replication of Rous-Sarcoma virus (Zamecnik and Stephenson, 1978). Since then, a lot of studies have demonstrated that antisense oligonucleotides are effective in fighting many pathogenic viruses. Theoretically, antisense oligonucleotides can be designed to target any region of viral RNAs, however, in view of the genetic diversity among viral isolates, it is necessary to target viral RNA sequences that are conserved and normally invariant among different strains. Actually, the 5'-untransalted region (UTR) is one of the most highly conserved regions in the HCV genome and has most frequently been targeted with antisense oligonucleotide. The 5'-UTR region, however, constitutes the internal ribosome entry site (IRES) capable of initiating cap-independent translation of the viral protein. Highly ordered RNA structures and multiple sites participating RNA-protein interaction within 5'-UTR have been documented to be crucial for translation and/or replication, both of which make the RNA inaccessible by oligonucleotides, extensive efforts were made to identify effective target sites.

In addition to mediating degradation of the target RNA, antisense oligonucleotides have been designed to inhibit essential processes of viral life cycle by steric blockade. For example, chimeric 2-*O*-methyl/LNA oligoribonucleotides against trans-activation response element (TAR) of HIV, which interacts with Tat trans-activator protein and cellular factors to stimulate transcriptional elongation, were reported to block HIV transcription by steric hindrance of the tat-TAR interaction (Arzumanov *et al*., 2001). Additionally, it was reported that antisense RNA targeting the splice donor-packaging signal of HIV inhibited viral replication via inhibition of both viral protein synthesis and virion RNA packaging (Chadwick and Lever, 2000).

Antisense oligonucleotides are usually delivered with cationic lipid reagents that are positively charged and capable of neutralizing the negative charge of the oligomleotides. One of the major challenges of antisense oligonucleotide approach is the stabilization of oligomers, since single-stranded nucleic acid molecules are unstable and are degraded in blood stream with a few hours. A number of chemically modified nucleotides have been employed to enhance nuclease resistance. Phosphorothioates are the major representatives of first generation of modified oligonucleotide. Phosphorothioates are advantageous over unmodified oligonucleotides in resistant to nucleolytic degradation, but they also have undesirable feature such as decreased binding affinity to target RNA and propensity to bind to various protein, which may result in toxic side effects (Levin, 1999). Second generation oligonucleotides represented by 2'-*O*-methyl and 2'-*O*-methyoxy-ethyl RNA were developed to solve these problems, they are less toxic and have enhanced affinity with target RNA,

but the unsatisfied feature is that they cannot activate RNase H to cleave the target RNA. More recently, novel chemically modified nucleotides, so-called third generation oligonucleotides, have been developed. Most of them exhibit improved properties such as enhanced stability, higher target affinity and lower toxicity. Further, gapmers with a stretch of unmodified or phosphorothioate DNA monomers in the center of the oligonucleotide are widely used to overcome the shortcoming of inability to activate RNase H.

Although nearly 20 antisense oligonucleotides have progressed to the stage of clinical trials, only one drug was approved by the Food and Drug Administration, which is used to treat cytomegalovirus-induced eye infection in AIDS patients. Most clinical trials were interrupted because of the unsatisfactory effectiveness.

Ribozymes

Ribozymes, catalytic RNAs that are capable of cleaving target RNA, are another important category of sequence-specific gene-silencing molecules. Since the discovery of the first group I nitron ribozymes in the early 1980s, a variety of ribozymes, including hammerhead and hairpin ribozymes, have been developed. Ribozymes have a catalytic domain that is flanked by sequences complementary to the target RNA. The hammerhead ribozyme was first isolated from viroid RNAs, it can be transformed from a *cis*-cleaving molecule into a target-specific *trans*-cleaving enzyme by dissecting the catalytic and substrate strands of the ribozyme. Hammerhead and hairpin ribozymes are the most intensively studied ribozymes. Similar to those for antisense nucleotides, the problems that should be cleared in developing therapeutic ribozymes are: 1) accessible target sites have to be selected; 2) the oligoribonucleotides have to be stabilized against nucleolytic degradation and 3) the ribozymes have to be delivered to target cells.

Hammerhead ribozyme consists of two substrate binding arm and a catalytic core cleaving any NUH triplets (where N can be any ribonucleotide and H can be any ribonucleotide except guanosine) with AUC and GUC triplets being processed most efficiently. Hairpin ribozymes usually cleave after BNGUC (where B can be any nucleotide except adenosine). Because of secondary and tertiary structures of the target RNAs, not all sequences that are theoretically cleavable by ribozymes can be served as the target sites for efficient cleavage. Computer predictions of the secondary structure of the target RNA and systemic experiments with a number of antisense oligonucleotides or ribozymes have been made to identify accessible target sites. Another approach to facilitate the access and subsequent cleavage of the ribozyme was reported by Taira and coworkers, who developed novel ribozymes with the ability to access any target site regardless of the secondary structure by combining with the unwinding activity of the endogenous RNA helicase eIF4AI (Kawasaki and Taira, 2002). In addition to the secondary structure, cellular compartmentalization of target RNAs is also thought to influence their susceptibility to ribozyme cleavage. Enhanced cleavage efficacy was reported by co-localization of the viral RNA and ribozyme tethered with the retroviral packaging signal (Sullenger and Cech, 1993).

As the delivery of ribozymes, chemically synthesized ribozymes can be exogenously introduced into the target cells using the reagent as described for antisense oligonucleotides. However, stabilization of ribozymes is more difficult than that of antisense oligonucleotides, since introduction of modified nucleotides most likely causes conformational changes that attenuate its catalytic activity. Indeed, a number of attempts exploiting uniform structural modifications to enhance nuclease resistance of ribozymes were demonstrated to be infeasible due to the reduced catalytic activity (Paolella *et al.*, 1992; Pieken *et al.*, 1991; Shimayama *et al.*,1993). A systemic study of a variety of modified hammerhead ribozymes led to the identification of a consensus ribozyme motif with enhanced nuclease resistance while maintaining the catalytic activity by keeping the 5 purine ribonucleotides in the catalytic core unmodified (Beigelman *et al.*, 1995). In addition to directly introducing the synthesized one, ribozyme can also be endogenously expressed from plasmids inside the target cells, which can

elicit constant and long-lived ribozyme expression. Because RNA polymerase III (pol III) promoter is highly productive and capable of generating complex RNA structures with high integrity, pol III promoters such as the tRNA promoters are widely used to direct the expression of both hammerhead and hairpin ribozymes (Medina and Joshi, 1999; Yamada *et al.*, 1994).

Ribozymes have widely been used to inhibit virus replication. Combined with retrovirus system, hammerhead and hairpin ribozymes direct target various HIV-1 regions were demonstrated to be effective in inhibiting viral replication (Zhou *et al.*, 1994; Ojwang *et al.*, 1992). Another ribozyme-based anti-HIV approach was accomplished by cleavage of the chemokine receptor CCR5 or CXCR-4 and thus perturbing their coreceptor function (Goila and Banerjea, 1998). Further, the protective effect of ribozymes against HIV-1 infection has also been demonstrated *in vivo*. Using the SCID-hu mouse *in vivo* human thymopoiesis model, CD34$^+$ hematopoietic progenitor cells transduced by retrovirus expressing anti-tat-rev and -env ribozymes and Rev aptamers were showed significantly resistant to HIV-1 infection upon challenge (Bai *et al.*, 2002).

In addition to HIV-1, the potential to use ribozymes as tools to control HCV infection has also been studied. Extensive knowledge of IRES structure and high conservation among HCV genotypes have rendered the IRES element attractive as the target for ribozymes. Chemically synthesized hammerhead ribozymes targeting the conserved sites of HCV IRES significantly inhibited (>90%) the replication of HCV/poliovirus chimera (Macejak *et al.*, 2000). Adenoviral vectors have been considered to be an attractive candidate to deliver anti-HCV ribozymes because of the hepatotropic property, it was reported that adenovirus-delivered anti-HCV ribozymes were effective at eliminating HCV RNA in infected primary human hepatocytes (Lieber *et al.*, 1996). Simultaneous expression of multiple ribozymes targeting different conserved HCV RNA region from a single vector was attempted to circumvent the emergence of resistant viral mutants (Welch *et al.*, 1998).

Encouraged by successful inhibition of viral replication in cell culture and *in vivo*, several ribozymes have subsequently been tested in clinical trials. The first clinical trials was conducted with retroviral-delivered hammerhead and hairpin ribozymes against HIV-1 RNA and another one used chemically synthesized hammerhead ribozyme targeting HCV (Hepatazyme), but unfortunately, both of which had to be quitted because of the poor therapeutic efficacy (Michienzi *et al.*, 2003; Peracchi, 2004).

RNA Interference

In the past few years, research in the antisense field was revolutionized by discovery of RNA interference (RNAi). RNAi is an evolutionarily conserved phenomenon of posttranscriptional gene silencing that has been described in plants, invertebrates and vertebrates. When double-stranded RNAs (dsRNAs) are introduced into these organisms, they are cleaved into small interfering RNAs (siRNAs) of 21-23 nt by the endonuclease Dicer, followed by incorporation of siRNA into a RNA-Induced Silencing Complex (RISC), which unwinds the duplex and uses the antisense strand as a guide to seek and degrade homologous RNA. In mammals, however, introduction of long dsRNA (>30 bp) induces systemic, nonspecific inhibition of translation due to activation of the interferon response. A breakthrough was achieved by the finding that specific gene silencing in mammalian cells can be mediated by siRNAs of 21 nt, which can bypass dsRNA-induced nonspecific interferon response (Elbashir *et al.*, 2001). This finding triggered numerous studies using siRNA in mammalian cells. RNAi is the most potent antisense reagent discovered thus far, it was reported that siRNA-mediated gene silencing is about 100-1000 fold more efficient than that by antisense oligonucleotides (Bertrand *et al.*, 2002).

Similar to ribozymes, siRNA can be either introduced as synthetic short dsRNA molecules or intracellularly transcribed from plasmids. siRNA is of relatively high stability and efficient siRNA delivery and silencing can be achieved by use of the cationic lipid reagent, but the silencing effect

mediated by exogenously introduced siRNA is short-lived. When longer lasting gene silencing is desired, plasmids or viral vectors are employed to deliver siRNA expression cassette. The pol III promoters of small nuclear RNA U6 and the H1 RNA component of RNase P have been widely used to direct the siRNA expression. Double-stranded RNA molecules can be expressed separately as sense and antisense RNA using two promoters or transcribed as short hairpin RNAs (shRNAs) which are then processed to give siRNAs. Inducible knockdown of gene expression was achieved by incorporated the doxycycline-responsive element into pol III promoter (Van de Wetering *et al*., 2003) or by coupled with Cre-loxP recombination system (Kasim *et al*., 2004). Additionally, it was reported that transport of shRNAs from the nucleus to the cytoplasm is likely to be an event involving Exportin-5, a karyopherin participating in the nuclear export of pre-microRNA, thus efficient nuclear export could be obtained by artificial modification to render the loop sequences of shRNA analogous to that of pre-microRNA (Yi *et al*., 2003).

The first study using siRNA as a antiviral reagent was reported by Bitko and Barik (2001), who demonstrated an inhibitory effect of synthetic siRNAs direct target viral polymerase and fusion protein F on respiratory syncytial virus. Afterwards, many studies have described RNAi-mediated inhibition of a large variety of viruses. One common approach in siRNA-based antivial strategy is to directly target key RNA sequences within viral genome. Inhibition of HIV-1 has been achieved by siRNAs directed against tat and rev (Coburn and Cullen, 2002; Lee *et al*., 2002), reverse transcriptase (Surabhi and Gaynor, 2002), trans-acting response region (TAR), 3'-UTR and vif (Jacque *et al*., 2002). Similarly, replication of HCV replicon RNA was suppressed by siRNAs targeting the capsid and nonstructural protein (NS) 4B (Randall *et al*., 2003), NS3 and NS5B (Wilson *et al*., 2003; Kapadia *et al*., 2003), 5'-UTR (Yokota *et al*., 2003). Besides these two viruses, siRNA-based antiviral strategy has also been successfully applied to other pathogenic viruses, including poliovirus, dengue virus, influenza virus, SARS coronavirus and many others. In addition to direct targeting viral sequence, another approach is to target host proteins considered to be crucial for the life cycle of viruses. It was reported that siRNA targeted to HIV-1 receptor CD4 (Novina *et al*., 2002), or co-receptor CCR5 (Qin *et al*., 2003) block the entry and replication of HIV-1. Also, evidence obtained from our group and those from others showed that knock down of cellular co-factors polypyrimidine tract binding protein, La antigen and human VAMP-associated protein of 33 kDa inhibits HCV RNA replication (Zhang *et al*., 2004; Domitrovich *et al*., 2005; Gao *et al*., 2004). Cellular genes are less prone to mutation and antiviral approach by knock down host factors is therefore less likely to allow viral escape of silencing. However, the unintended side effects of knocking down cellular gene are concerned and must be addressed thoroughly prior to therapeutic application.

By demonstrating that a great variety of viruses can be successfully targeted by siRNAs, it is conceivable that these powerful antisense molecules can be used to target any preexisted or newly emerging human pathogenic virus. However, siRNA-mediated antiviral technology faces several important challenges that must be circumvented. Like other sequence-specific antisense reagents, one outstanding drawback of the approach using siRNA direct against viral genome is that emergence of siRNA-resistant variants. In fact, it was shown that a single point mutation in the siRNA target region conferred escape in poliovirus (Gitlin *et al*., 2002) and more recently HIV-1 was shown to elude siRNA targeting by the evolution of an alternative structure in RNA genome (Westerhout *et al*., 2005). Additionally, increasing evidence showed that viruses counteract RNAi effect by encoding viral proteins that act as the suppressor of RNAi pathway (Bennasser *et al*., 2005; Li *et al*., 2002). Several strategies may be useful to prevent the escape of mutant variants, for example, 1) simultaneous expression of multiple anti-viral siRNAs targeting different conserved viral sequences, 2) combination with siRNA against cellular co-factors indispensable for viral replication. Indeed, Schubert *et al*. (2005) reported that the silencing effect of the vector doubly expressing two different siRNAs was maintained even after artificially introducing a point mutation that disabled the respective mono-expression vector.

Although siRNAs are considered to be of high specificity for their targets, unwanted off-target effects may occur by siRNA recognition of other mRNAs with partially complementary sequence. Moreover, siRNAs can act like microRNAs to inhibit translation if there is a consecutive base pairing between siRNA and mRNA. Therefore, to minimize potential off-target effects, it is important to carefully comparing the candidate siRNA sequences with mRNAs in the human genome to avoid long stretches of homology.

Another potential toxicity of siRNA is that endogenous RNAi pathway, which is important in regulating the expression of developmentally essential genes, may be competitively inhibited by exogenously introduced siRNA, because the RNAi machinery such as Dicer and RISC may be limit in amount (Bitko *et al*., 2005). This is especially in the cases that high siRNA dose is administrated or multiple siRNAs are simultaneously expressed to avoid escape viral mutants.

As mentioned above, outstanding progress has been made since RNAi was shown to work in mammalian cells three years ago. The first phase I clinical trials targeting the VEGF angiogenic pathway in age-related macular degeneration have begun. Although the issues such as delivery and unwanted side effects are still the problems to be further cleared to turn RNAi from an effective functional genomics tool into a potent antiviral therapy, the prospects for overcoming these are good as we improve our understanding of the RNAi mechanism.

RNA Aptamers and RNA Decoys

RNA aptamers are short RNA ligands with binding and inhibitory activity to small molecule or protein targets. The screening and identification of such molecules with unique binding properties from very large random RNA libraries are generally accomplished by a technology termed systemic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold, 1990). The SELEX process starts with a large library of randomized RNA sequences containing 10^{14}-10^{15} different RNA species. The library is incubated with the target protein of interest and the RNAs that bind the protein are separated, amplified, cloned and sequenced. The high affinity and specificity of aptamers make them attractive therapeutic agents and increasing evidence indicated that the use of SELEX technique could generate aptamers to many disease relevant targets. The therapeutic utility of aptamers has been studied in a variety of human maladies including cancer, cardiovascular disease and infectious diseases.

In the case of using aptamers for antiviral purpose, the proteins essential for viral replication such as regulatory protein and viral enzyme are good target. Indeed, it was reported that RNA aptamers selected by SELEX against HIV-1 Tat (Yamamoto *et al*., 2000) or reverse transcriptase (Joshi and Prasad, 2002) inhibited HIV-1 replication by up to 99%. Similarly, RNA aptamers specific for HCV NS3 (Hwang *et al*., 2004; Nishikawa *et al*., 2003) or RNA-dependent RNA polymerase (Biroccio *et al*., 2002; Vo *et al*., 2003) were shown to block the replication of HCV replicon by inhibiting the viral enzymatic activities. In addition to vial proteins, antiviral aptamers were also selected to target key viral RNA sequences. Nishikawa and coworkers reported an inhibitory effect on IRES-directed translation by RNA aptamers binding to HCV IRES sequences (Kikuchi *et al*., 2005).

Another approach using small RNAs to target pathogenic protein is RNA decoys. RNA decoys are small RNA molecules analogous to *cis*-acting element, which can compete with the corresponding endogenous sequences for *trans*-factors binding, thereby attenuating the authentic *cis-trans* interaction. Since the concept of using decoys as therapeutic agents emerged 15 years ago, increasing evidence has shown that the decoy approach may be useful in the treatment of a variety of human disease. The first study performed to determine if an RNA decoy could be used to inhibit the activity of a pathogen protein was published by Sullenger *et al*. (1990) who demonstrated cells over expressing TAR decoy are highly resistant to viral replication. Later, same group reported that over expression of RRE-derived decoys inhibited HIV-1 replication by preventing the binding of Rev protein to the vial RNA (Lee *et al*., 1992). Also, it was demonstrated that RNA decoys mimicking the Stem-Loop (SL)

structures of HCV 5'-UTR inhibited IRES-dependent translation (Ray and Das, 2004). Additionally, considering that the SL structures in the NS5B coding region were demonstrated to function as *cis*-replicating elements (CREs) and replication of HCV is likely initiated by interaction between replicase complex and SL structures containing CREs, we explored the possibility of using RNA species corresponding such SL structures as antiviral decoys and provided the evidence showing that pol III-directed over expression of SL RNA inhibited HCV replication by sequestering the replication complex and preventing its binding to the physiological target in the viral RNA (Zhang *et al*., 2005).

Distinct from sequence-specific antisense reagents, the efficacy of antiviral approach by aptamers and decoys may be less affected by the extensive variability encountered among viral isolates. Aptamer resistance may be less of a problem because RNA-protein interactions are not easily disrupted by mutations in the protein. Indeed, the effective cross-clade inhibition of HIV-1 by gp120 aptamers (Khati *et al*., 2003) and to a lesser extent by reverse transcriptase (Joshi and Prasad, 2002) was documented. Additionally, because the interaction between *cis*-acting element and *trans*-acting factor is usually essential for viral replication and mutation in the *trans*-acting factor that blocks its binding to RNA decoy also blocks its binding to the authentic target in viral RNA. To circumvent RNA decoy-mediated inhibition, double mutation, one in *trans*-acting protein and another compensatory one in the *cis* element is simultaneously required, thus making the chance of emergence of escape mutants lower.

To provide the resistance to nuclease degradation in biological fluids, aptamers are routinely selected with amino-or fluoro-modifications at the 2' position of pyrimidines which are prone to nuclease attack. Further stabilization of selected RNA aptamers includes *O*-methyl-substitutions in purine nucleotides, which requires chemically synthesis of modified RNA molecules. An alternative to chemical modification is the application of L-nucleic acids during and after *in vitro* selection. Modified aptamers can be delivered with the same reagents used for antisense oligonucleotides, the half-life of aptamers in the plasma is increased by coupling with high molecular linkers such as PEG. Similar to ribozymes and siRNAs, both aptamers and decoys can also be intracellularly expressed from plasmid-or vector-delivered expression cassette. Intracellularly expressed aptamers (intramers) and decoys could elicit long-term and stable effect, which is particularly essential for antiviral purpose.

Despite of the high binding specificity and affinity, the efficiency of aptamer-or decoy-mediated inhibition may be not efficient enough in some cases. This can be improved by combination with other antisense modalities. Because aptamers and decoys aim at inhibition of protein function, whereas ribozyme and siRNA destroy target protein-encoding mRNA or viral RNA; so the additive or synergistic antiviral effect can be expected by combining these two conceptually different reagents.

Another issue that limits the therapeutic use of aptamers is relatively high cost for aptamer manufacturing and delivery of aptamers and decoys faces the same problems as with other antisense reagents. Further progress in aptamer and decoy therapeutic field will depend on the breakthrough of such problems.

CONCLUSION

Small RNA-based antiviral approach represents a useful alternative to small molecular compounds for combating viral infection. Antisense oligonucleotides and ribozymes have been used for many years to inhibit viral replication. The therapeutic efficiency of antisense oligonucleotides and ribozymes was far from satisfied. The more recently developed RNAi strategy, however, is obviously advantageous over the older generation antisense reagents due to the high efficiency in knockdown gene expression. RNAi field is moving at an unprecedented speed and the first clinical trials using RNAi have already commenced. The problem for emergence of siRNA resistant variants can be dissolved either by simultaneously targeting different conserved region or combination with other small RNA-based modalities such as aptamer and decoy, which bind and subsequently inactivate target protein. With the

advances in developing efficient delivery systems, one could expect that RNAi and other small RNA-based approaches can become realized as effective therapies to treat viral infections in the near future.

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