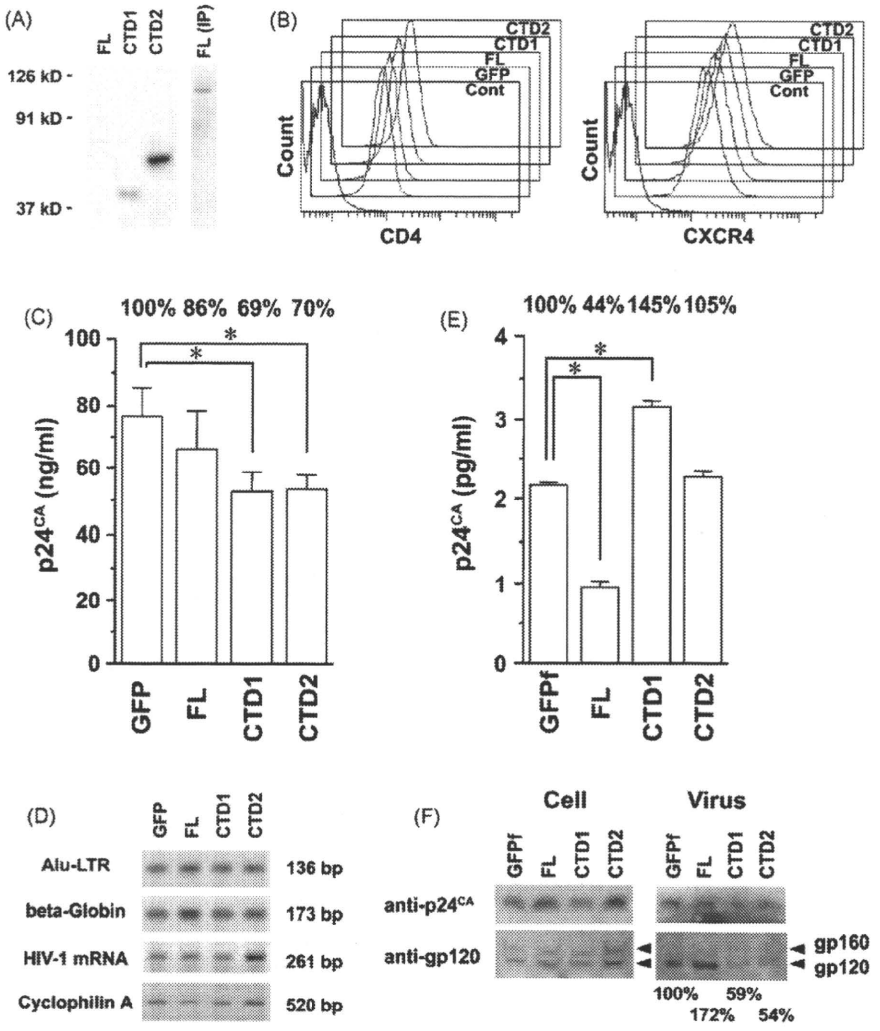

Fig. 2. Functional characterization of the SEC14L1a derivatives. (A) Detection of stable expression of FL, CTD1, and CTD2 in MT-4 cells by Western blotting using anti-FLAG antibody. FL was detected by the immunoprecipitation (IP) assay using agarose beads conjugated with anti-FLAG antibody. The flow cytometric analysis of the cell surface expression of HIV-1 receptors CD4 and CXCR4 in MT-4 cells stably expressing GFP, FL, CTD1, and CTD2. (C) Constitutive expression of CTD1 and CTD2 limited the replication of HIV-1 in MT-4 cells. The concentration of viral $p24^{CA}$ antigen in the culture supernatant was measured at 4 d post-infection. The results represent the average of seven independent experiments ± the standard error of the mean. The reduction of viral $p24^{CA}$ concentration relative to GFP was shown on the top. Asterisks indicate the statistical significance compared to GFP ($P < 0.05$ by two-tailed Student's *t*-test). (D) The PCR-based assay to examine the effect of SEC14L1a derivatives on the early phase of viral life cycle (top two panels) and the transcription from LTR promoter (bottom two panels). The HIV-1 entry efficiency was examined by Alu-LTR PCR. Beta globin was used as an internal control. The HIV-1 transcription efficiency was examined by RT-PCR targeting spliced viral mRNA. Cyclophilin A was used as a control. The expected length of each PCR amplicon was indicated. (E) The effect of SEC14L1a derivatives on the HIV-1 production. The 293T cells grown in a well of a 6-well plate were transfected with 200 ng of HIV-1 proviral DNA and 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The culture supernatant was recovered at 2 d post-transfection and the $p24^{CA}$ concentration was measured. The representative data from five independent experiments was shown. The results indicate the average ± the standard deviation. The relative $p24^{CA}$ concentration compared to GFPf was shown on the top. Asterisks indicate the statistical significance compared to GFPf ($P < 0.001$ by two-tailed Student's *t*-test). The *Env* incorporation onto the virus-like particles (VLP) produced by 293T cells expressing SEC14L1a derivatives. The 293T cells grown in a well of a 6-well plate were transfected with 1 μg of *gag-pol* (pCMVR8.91) and *Env* (pIIIex) expression vectors along with 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The cell lysates (Cell) and VLP fractions (Virus) were subjected to Western blot analysis detecting gp120 and $p24^{CA}$ harvested at 2 d post-transfection. The *Env* incorporation levels normalized to $p24^{CA}$ relative to GFPf were shown at the bottom.

G-pseudotyped HIV-1 vector, and the cellular genomic DNA was recovered at 4 d post-infection. The amount of Alu-LTR PCR products from FL-, CTD1-, or CTD2-expressing MT-4 cells was almost equal to that from GFP-expressing cells, suggesting that the early phase of the viral life cycle is not inhibited by any of the SEC14L1a derivatives (Fig. 2D). To examine the viral production phase, we examined the LTR-driven viral gene transcription by RT-PCR. Cellular RNA was extracted from the same MT-4 cells infected with VSV-G-pseudotyped HIV-1 vector, and RT-PCR was conducted to amplify LTR promoter-driven spliced HIV-1 mRNA. The amount of viral RNA expressed in FL-, CTD1-, or CTD2-expressing cells was not lower than that in GFP-expressing cells when the levels of the internal control was taken into account (Fig. 2D). Given that the similar number of viral genome was integrated as indicated by the Alu-LTR PCR, these data suggest that viral transcription is not inhibited by any of the SEC14L1a derivatives, and that the action point of CTD1 and CTD2 should be at post-transcriptional levels of the viral production phase.

Next, the FL, CTD1, or CTD2 expression vector was co-transfected with HIV-1 proviral DNA into 293T cells, and viral production was quantified by $p24^{CA}$ ELISA. The FLAG-tagged GFP (GFPf) was used as a control hereafter. We found that the FL expression significantly reduced the production of HIV-1 (44.2%, $P < 0.001$, two-tailed Student's *t*-test) compared to the GFPf control (Fig. 2E). In contrast, the CTD1 enhanced the production of HIV-1 (145.9%, $P < 0.001$, two-tailed Student's *t*-test; Fig. 2E). However, CTD2 did not measurably affect the HIV-1 production (105.1%, not statistically significant; Fig. 2E). As the ELISA assay examines the effect

of CTDs on *Gag* functions, we next tested the functional interaction between CTDs and *Env*. The *Env* incorporation onto the virion was examined by tripartite-transfection of expression vectors for *Env*, *gag-pol*, and SEC14L1a derivatives into 293T cells, and the VLP was collected by centrifugation. The immunoblotting against gp120 was performed on the cell lysate and the VLP fraction. The cellular *Env* and *Gag* expressions were not detectably affected by any of the SEC14L1a derivatives (Fig. 2F, left panel). The *Env* incorporation onto the VLP was slightly enhanced by FL (157%; Fig. 2F, right panel). In contrast, the VLP produced from CTD1- or CTD2-expressing cells incorporated substantially fewer *Env* than those from GFPf-expressing cells (59% or 54%, respectively; Fig. 2F, right panel). These data were reproducible in independently performed experiments. The densitometric analysis of Western blot image showed that the average ± the standard error of the mean of Env incorporation onto the virion was 129.7 ± 39.9%, 54.8 ± 24.7%, and 25.5 ± 10.3% for FL, CTD1, and CTD2 compared to GFPf, respectively (3–4 independent experiments). The *Env*-mediated cell-to-cell fusion assay indicated that SEC14L1a derivatives did not limit the cell surface targeting and function of *Env* (data not shown). In addition, the *Gag* processing in virion was unaffected by any of the SEC14L1a derivatives (data not shown). Collectively, these data suggest that the HIV-1 replication is inhibited by CTD1 and CTD2 due to the inefficient *Env* incorporation onto the virion. To test this possibility, we infected fresh MT-4 cells with the equal amount of HIV-1 propagated in CTD1- or CTD2-expressing MT-4 cells (1–2 ng $p24^{CA}$), and the viral replication was monitored at 3–4 days post-infection by measuring the $p24^{CA}$ concentration. The infectivity of HIV-1 propagated in CTD1- or CTD2-expressing cells was attenuated to 83.1 ± 17.9% or 82.4 ± 5.5% relative to the virus recovered from GFP-expressing cells, respectively (the average ± the standard error of the mean of 3 independent experiments). Altogether, these data suggest that the inhibition of HIV-1 replication by CTD1 and CTD2 is attributed to the attenuation of viral infectivity by lowering the *Env* incorporation onto the virion.

4. Discussion

In the present study, we provide the first evidence that the C-terminal fragment of SEC14L1a functions as an inhibitor of HIV-1 replication. The advantage of this system is that, since MT-4 cells are stably transduced with a cDNA library, the anti-HIV-1 function of a candidate gene is not due to a perturbed cell physiology. This system has been successful in identifying CD14, CD63, and Brd4-CTD as regulators of HIV-1 replication [1,3,4], and more candidates are being analyzed. Among the candidates, SEC14L1a CTD appeared to be one of the relatively modest inhibitors of HIV-1 replication. However, of note, the SEC14L1a derivatives have not been identified in other genetic screening systems. These facts point that our T cell-based system is sensitive in detecting the modest anti-HIV-1 activity of a gene, and is a unique tool in the pursuit of HIV-1 regulatory factors to complete the HIV-1-host interactome.

SEC14L1a may affect the Golgi-mediated vesicular trafficking since SEC14L1a lowers the cell surface levels of cholinergic transporters [23]. However, we do not have any data to suggest that SEC14L1a and its derivatives affect the cell surface targeting of membrane proteins including CD4, CXCR4 and *Env*. These data suggest that SEC14L1a's effect on cholinergic receptor expression is specific, and that the CTD's ability to inhibit HIV-1 replication is independent from SEC14L1a's regulatory functions on vesicular trafficking. The action point of CTD1 and CTD2 was shown to be the late phase of the viral life cycle. Given that CTD1 and CTD2 did not inhibit the biogenesis and the cell surface targeting of *Gag* and *Env*, the major mechanism of CTD1 and CTD2 to inhibit HIV-1 replication was to reduce the infectivity of HIV-1 by limiting the *Env* incorporation onto the virion. Consistent with this idea, the viral infectivity of virions produced in CTDs-expressing cells was attenuated. Then, how do CTDs block the *Env* incorporation onto the virion? We detected a weak interaction between *Gag* and CTD1 or CTD2 by immuno-coprecipitation analysis. Thus, we speculate that the interaction between *Env* and *Gag* at the plasma membrane is interfered by *Gag*-CTDs interaction, resulting in the reduction of *Env* incorporation onto the virion.

The CTD1 was an inhibitor of HIV-1 replication. While the CTD1 negatively affected the *Env* incorporation onto the virion, it positively affected the HIV-1 production. These observations may be seemingly controversial. However, the SEC14L1a derivatives' effect on HIV-1 replication is a summation of their effects of on each step of the viral life cycle. Therefore, it is conceivable that CTD1 can serve as a negative regulator of HIV-1 replication as well as a positive and negative factor on distinct steps of the viral life cycle. These seemingly controversial findings may be in part due to the cells in which the biological functions of SEC14L1a derivatives were examined. The effect of SEC14L1a derivatives on HIV-1 replication was investigated in MT-4 cells, whereas those on the HIV-1 production and *Env* incorporation onto the virion were examined in 293T cells. Although the basic biological features are largely shared among different cell types, it is possible that the SEC14L1a derivatives may function slightly differently in MT-4 cells from 293T cells given that the intracellular distribution of SEC14L1a derivatives in MT-4 cells was not identical to that in 293T cells (Fig. 1E and 1F).

Elucidating the molecular mechanism underlying CTDs' activity not only provides a hint to understand how the HIV-1 virion actively uptakes *Env* through the *Gag-Env* interaction, but also leads to the development of a novel anti-retroviral drug that lowers the infectivity of the virus by preventing *Env* incorporation onto the virion. This is the strength of our T cell-based assay since CTDs inhibit HIV-1 replication specifically. In the previous study, we proposed that a small portion of Brd4 may serve as a therapeutic molecular target for HIV-1 infection, since the constitutive expression of Brd4-CTD limited HIV-1 replication specifically [3], akin to the SEC14L1a CTDs. However, it remains to be examined whether the SEC14L1a and Brd4 derivatives inhibit HIV-1 replication in primary HIV-1 target cells.

The genome-wide screening has potential caveats, including a cDNA bias and a cell line bias. A cDNA library is not a perfect representation of mRNA expressed in the cells from which the library is constructed. For example, the longer the mRNA, the less efficiently the full-length cDNA is synthesized. In fact, we isolated Brd4-CTD from the PBL cDNA library as a potent inhibitor of HIV-1 replication [3]. However, although Brd4 (approximately 5000 nt mRNA in length) is expressed in MT-4 cells, we were unable to recover Brd4-CTD from the MT-4 cDNA library [3]. This clearly demonstrates the cDNA bias in the genetic screening. A cDNA library derived from non-T cells does not contain genes specifically expressed in T cells. Thus, we have to explore many more cDNA libraries to completely cover the genetic diversity of human cells. The cDNA libraries isolated from long-term non-progressors of HIV-1-seropositive individuals or from elite controllers might be of particular interest, considering that a dominant innate HIV-1 resistance gene, such as CCR5 delta 32, may partly account for the slow progression of AIDS. Similarly, use of a particular cell line and/or virus strain may bias the results. MT-4 cells are positive for HTLV-1, and are able to support robust HIV-1 replication. MT-4 cells do not express CCR5, and are unable to support R5-tropic HIV-1 strains. What if other T cell lines and R5-tropic viral strains are used? What if we assay the same cDNA library in TZM-bl cells? We plan to address these issues in the future studies.

In conclusion, genome-wide genetic screening is a powerful tool for identifying the regulatory factors of HIV-1 replication and innate HIV-1 resistance factors that limit HIV-1 infection and AIDS progression. The HIV-1-host interactome should also reveal poten-

tial therapeutic molecular targets that may be used to develop novel anti-AIDS drugs to tackle the emerging drug resistant viruses. However, the fact that different experimental systems often yield non-overlapping candidates suggests that we have to explore more experimental systems to fully understand the HIV-1-host interactome. Our T cell-based system provides an alternative tool for identifying novel HIV-1 regulatory factors, and should help us understand the HIV-1-host interaction in more detail.

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Conflict of interest statement

The authors state that they have no conflict of interest.

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Inhibition of HIV replication by a CD4-reactive Fab of an IgM clone isolated from a healthy HIV-seronegative individual

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HIV replication is restricted by some anti-CD4 mouse mAb *in vitro* and *in vivo*. However, a human monoclonal anti-CD4 Ab has not been isolated. We screened EBV-transformed peripheral B cells from 12 adult donors for CD4-reactive Ab production followed by functional reconstitution of Fab genes. Three independent IgM Fab clones reactive specifically to CD4 were isolated from a healthy HIV-seronegative adult (~0.0013% of the peripheral B cells). The germ line combinations for the V_H and V_L genes were V_H3-33/$_L$6, V_H3-33/$_L$12, and V_H4-4/$_L$12, respectively, accompanied by somatic hypermutations. Genetic analysis revealed a preference for V-gene usage to develop CD4-reactive Ab. Notably, one of the CD4-reactive clones, HO538-213, with an 1×10^{-8} M dissociation constant (Kd) to recombinant human CD4, limited the replication of R5-tropic and X4-tropic HIV-1 strains at 1–2.5 μg/mL in primary mononuclear cells. This is the first clonal genetic analysis of human monoclonal CD4-reactive Ab. A mAb against CD4 isolated from a healthy individual could be useful in the intervention of HIV/AIDS.

Key words: Autoimmunity · CD4-reactive Ab · IgM · Inhibition of HIV replication

Supporting Information available online

Introduction

CD4 is a T-cell marker that serves as a principal receptor for HIV. CD4-reactive Ab are detected in HIV-infected individuals (~13%) [1, 2] and HIV-exposed seronegative individuals (34%) [3]. In addition, some healthy individuals are positive for anti-CD4 Ab (~0.6%) [4]. Replication of multiple HIV clades is blocked by mouse mAb against CD4 *in vitro* and *in vivo* [5–12]. Thus, it is possible that anti-CD4 Ab play a role in protecting individuals from HIV infection and delaying AIDS disease progression. Similar arguments have been made for Ab against CCR5, a coreceptor for HIV [3, 10, 13]. Furthermore, some clinical studies suggest that CD4-reactive Ab, including a humanized mAb, has therapeutic potential against HIV infection and AIDS progression [5, 8, 10, 12]. However, the development and pathophysiological roles of self-recognizing Ab in healthy individuals are still largely unknown, and a human mAb against CD4 has not yet been isolated.

To gain insights into the genesis of auto-reactive Ab and to characterize the nature of CD4-reactive auto-Ab, we conducted experiments to isolate human monoclonal anti-CD4 Ab from PBMC of 12 HIV-seronegative adult donors. We succeeded in isolating

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three independent IgM clones recognizing CD4 from a healthy donor. Analysis of the V-region sequences of CD4-reactive Ab revealed a preference for V gene usage to give rise to CD4-reactive Ab. This is the first report describing CD4-reactive human mAb.

Results and discussion

Isolation of CD4-reactive IgM clones from a healthy individual

PBMC were collected from 12 HIV-seronegative adult volunteers, including two healthy and ten with autoimmune disorders, and B-lymphoblastoid cell lines (B-LCL) were established by infecting the cells with EBV (for experimental procedure, see Supporting Information Fig. 1). B-LCL were propagated in oligoclonal pools. In 790 cultures from one healthy donor, we identified two cultures positive for recombinant human CD4 (rhCD4) reactivity, HO538 and HO702, using ELISA (Fig. 1A). This donor may have a unique Ab repertoire, as auto-reactive B-LCL cultures were identified significantly more frequently in this donor than in the others (Fig. 1A). The rhCD4 reactivity was specific, as no binding was observed to 72 other viral, bacterial, and auto-Ag screened in parallel (Supporting Information Fig. 2). We amplified the Ig genes encoding the Fab regions by RT-PCR and cloned them into the bacterial expression vector pFabI-His2 that produces Fab fragments of an inserted set of V_H and V_L genes. We expected that some clones should reconstitute the CD4-reactive Fab present in the original B-LCL cultures. After screening by ELISA, one CD4-reactive Fab clone, HO538-213, was isolated from the HO538 culture, and two independent clones, HO702-001 and HO702-016, were isolated from the HO702 culture. These Fab clones originated from IgM, as determined by the sequence analysis. The estimated efficiency of peripheral B cells producing CD4-reactive Ab was ~0.0013% (three clones/2.4×10^5 estimated screened B cells × 100 (%), given that the B cells compose 10% of PBMC and that EBV immortalization is 30% efficient on average) [14]. According to the ELISA data, the Fab concentrations that yielded 50% maximal binding were ~8 μg/mL for HO538-213, and ~1 μg/mL for HO702-001 and HO702-016 (Fig. 1B). Consistent with these data, the BIACORE assay revealed that the dissociation constant (Kd) of HO538-213, HO702-001, and HO702-016 to rhCD4 was 6.5×10^{-8}, 7.7×10^{-9}, and 2.7×10^{-9} M, respectively (Fig. 1C), which is relatively weak compared with average Ab–Ag interactions (*e.g.* the Kd of mouse mAb Leu-3a to rhCD4 is 2.2×10^{-10} M).

Genetic analysis of CD4-reactive IgM clones

The Fab sequences were analyzed by the Kabat database (http://www.ncbi.nlm.nih.gov/igblast/) in GenBank, as previously described [15, 16]. The Ig gene family of each gene and the most homologous germline are indicated (Fig. 2A). All the three clones were of the IgM class and had a κ-chain for V_L. Comparison of the

A

Donor	Number of tested cultures	CD4	Nuclear antigen	Blood type i-glycolipid
Healthy 1	790	2 (0.3%)*	21 (2.6%)*	28 (3.5%) *
Healthy 2	160	0	0	0
Autoimmune (10 individuals)	435	0	3 (0.7%)	0

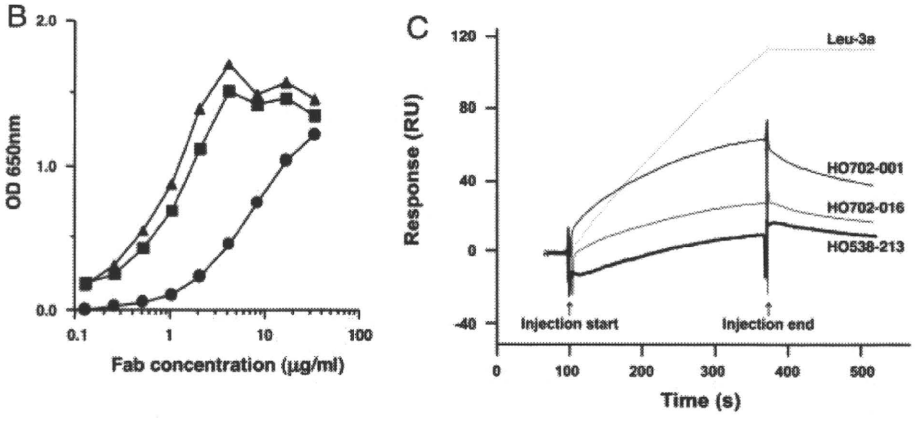

Figure 1. Isolation and characterization of healthy human-derived CD4-reactive Ab. (A) Summary of the frequency of B-LCL cultures that reacted with representative auto-Ag. The number of cultures positive for rhCD4 reactivity, HeLa cell nuclear staining, and blood type i-glycolipid are shown. *$p<0.05$, compared with other donor groups, Fisher's exact test. (B) CD4-binding kinetics of CD4-reactive IgM Fab. Serial dilutions of HO538-213 (circles), HO702-001 (triangles), and HO702-016 (squares) were incubated in microtiter plates pre-coated with rhCD4. (C) Surface plasmon resonance analysis of CD4-reactive IgM FAb HO702-001 (black), HO702-016 (dark gray), HO538-213 (bold), and mAb Leu-3a (gray) binding to immobilized rhCD4. The concentration of Ab was 0.3 μg/mL, flow rate 20 μL/min, and reaction time 270 s. RU, resonance units.

heavy chain with the germlines revealed that the μ-chains of HO538-213 and HO702-001 were 95 and 97% homologous to germ line V_H3-33, respectively, while HO702-016 was 96% homologous to germline V_H4-4 [17]. For the light chains, the κ-chain Vkappa3 of HO538-213 was 97% homologous to germline L6 [6, 18, 19], and κ-chain Vkappa1 of both HO702-001 and HO702-016 was 97% homologous to the germline L12 [6, 18, 19]. These data suggest that there is a preferential use of V_H and V_L genes to develop CD4-reactive Ab, considering the number of V_H and V_L genes present before the Ig gene rearrangement. According to the sequence analysis, the V_H amino acid sequences of HO538-213 and HO702-001 carried distinct mutations, although both were derived from the same germline V_H3-33. The mutations were more frequent in the CDR regions (Fig. 2B and C, Supporting Information Fig. 3), which is characteristic of somatic hypermutation (SHM) associated with affinity maturation. Unlike most SHM, however, mutations involving G/C were not dominant.

A

Clone	Ig class	VH family	Closest germline	% identity	VL family	Closest germline	% identity
HO538-213	IgM	VH 3	VH3-33	95%	Vk3	L6	97%
HO702-001	IgM	VH 3	VH3-33	97%	Vk-1	L12	97%
HO702-016	IgM	VH 4	VH4-4	96%	Vk-1	L12	97%

B

Clone			Number of mutations associated with: A	G	C	T	ins[a]	del[a]	GC involvement (%)[b]	Total	aa changes[c]	(%)
HO702-001	heavy chain	FR	3	2	1	0	0	0	66.6	6	2	33.3
		CDR	0	0	0	0	0	0	0.0	0	0	0.0
	light chain	FR	0	0	0	2	0	0	0.0	2	1	50.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	4	2	1	2	11	0	33.3	20	3	15.0
HO702-016	heavy chain	FR	1	0	0	0	0	0	0.0	1	0	0.0
		CDR	0	2	1	0	11	0	100.0	6	0	0.0
	light chain	FR	0	0	0	2	0	0	0.0	2	1	50.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	2	2	1	2	17	0	42.9	24	1	4.2
HO538-213	heavy chain	FR	2	1	1	4	0	0	25.0	8	3	37.5
		CDR	2	2	5	1	16	0	70.0	26	5	26.0
	light chain	FR	1	2	1	0	0	0	75.0	4	3	75.0
		CDR	3	1	0	0	2	0	25.0	6	3	50.0
		Total	8	6	7	6	17	0	66.0	43	13	30.2

C

Light chain

	FR1	CDR1	FR2	CDR2
HO538-213	EIVMTQSPATLSLSPGERATLSC	RASQSVNNYLA	WYQQRPGQAPSLLIY	DASNRAT
HO702-001/016	D-QL----S---A-V-D-V-IT-	------ISSH--	-----K--K--K----	K---SLES

	FR3	CDR3	FR4
HO538-213	GIPARFSGSGSGTDFTLTISSLEPEDFAVYYC	QQ.RG.NWPHT	FGQGTKLEIKRT
HO702-001/016	-V-S--------E---------Q-D---I----	--YNSYSRTY-	----------

Heavy chain

	FR1	CDR1	FR2	CDR2
HO538-213	QVQLVQSGGGVVQPGRSLRLSCA.ASG.FTFS	SYGMH	WVRQAPGKGLEWVA	VVCYDGRTTYYADSVKG
HO702-001	-----QEW----------	-----	----------	-IN---SNK----------
HO702-016	----QE--P-L-K-SQT-S-T-TVSG-SISSG	--YWS	-I--PA------EG	.RI-YSGS-N-NP-L-S

	FR3	CDR3	FR4
HO538-213	RFTISRDISENTLYLQMNSLRAEDTAVYYCAK	DLNKGYCNNGGCYG.VDC	WGQGTLVTVSS
HO702-001	---------N-K----------	-..RD.-SG-S--SWF-P	----------
HO702-016	-V--SV-T-K-QFS-KLS-VT-A---------R	T..PTL.GR-L.F...-Y	----------

Figure 2. Genetic analysis of CD4-reactive Ab. (A) Summary of the Ig class, V-gene family, closest germ line, and percentage identity of the closest germ line of CD4-reactive Ab. (B) The mutation profiles of the CD4-reactive IgM Fab fragments. (C) The deduced protein sequences of the V_H and V_L genes of the CD4-reactive Fab fragments are aligned. FR, framework region. Dashes and dots indicate identical residues and deletions, respectively. See Supporting Information Fig. 2 for further detail.

Inhibition of HIV replication by a Fab fragment of a CD4-reactive IgM

We next examined the potential impact of these CD4-reactive Fab Ab on HIV replication. Viral replication was monitored in PBMC by measuring $p24^{CA}$ viral Ag levels in the culture supernatant. Among the three IgM Fab clones, HO538-213 suppressed R5-tropic virus HIV-1_{JR-FL} replication by 3.5±1.5-fold at 1–2.5 μg/mL (average±SD from four independent experiments, Fig. 3A). There was a modest but consistent suppression of X4-tropic virus HIV-1_{HXB2} replication (1.4±0.2-fold, average±SD from three independent experiments). BIACORE and ELISA revealed that HO538-213 did not compete with the anti-CD4 mAb Leu-3a [20, 21] for CD4 binding. Leu-3a restricts HIV-1 replication by physically blocking the *Env*-CD4 interaction (data not shown), suggesting that the epitope recognized by HO538-213 is distinct from the *Env*-interacting domain of CD4 [7, 22, 23]. The monoclonal anti-CD4 Ab OKT4a does not block the *Env*-CD4 interaction, but restricts HIV-1 infection, although decreasing CD4 lateral diffusion on the cell surface [24–26]. We hypothesized that HO538-213 may have a similar mechanism of action. CD4 localizes to lipid rafts, and CD4-crosslinking activates signal transduction involving tyrosine kinases [27–29]. Thus, we treated MOLT-4 cells with HO538-213, and the lipid raft fraction was isolated by a membrane floatation assay as verified by the raft markers glycosphingomyelin 1 and sphingomyelin (Fig. 3B, left panel). Tyrosine kinase activitiy was examined by immunoblotting the lipid raft fractions using a PY20 anti-phosphotyrosine mAb (Fig. 3B, right panel, arrowhead). We detected a significant amount of tyrosine phosphorylation in the lipid raft fraction after HO538-213 treatment, indicating that HO538-213 can assemble cell surface CD4. This is consistent with our hypothesis that HO538-213 inhibits HIV-1 infection by decreasing the lateral movement of cell surface CD4.

Does CD4-reactive IgM function as a natural HIV resistance factor?

We then further characterized the donor from which the CD4-reactive Ab was isolated. The donor serum did not show a strong reactivity to rhCD4 at 1:10 dilution, where the non-specific effect was no longer detected. We analyzed the HIV-inhibition titer of the donor plasma. In a TZM-bl cell assay, the plasma did not block HIV replication at 1:50 dilution (data not shown). These data suggest that the CD4-reactive IgM circulates at very low titers in the donor and may not be sufficient to block HIV infection *in vitro*. However, it is possible that the CD4-reactive IgM may be able to limit HIV-1 propagation under *in vivo* conditions.

We next investigated the immunological status of the donor. IgG and IgM levels were within the normal range, and the plasma was negative for rheumatoid factor, anti-DNA, and anti-ribonucleoprotein Ab. However, the donor serum reacted to nuclear Ag at a titer of 1:160 (1:40 or less is considered normal), and the staining patterns were nucleolar (1:160) and speckled (1:80). Consistent with these data, the frequency of auto-reactive

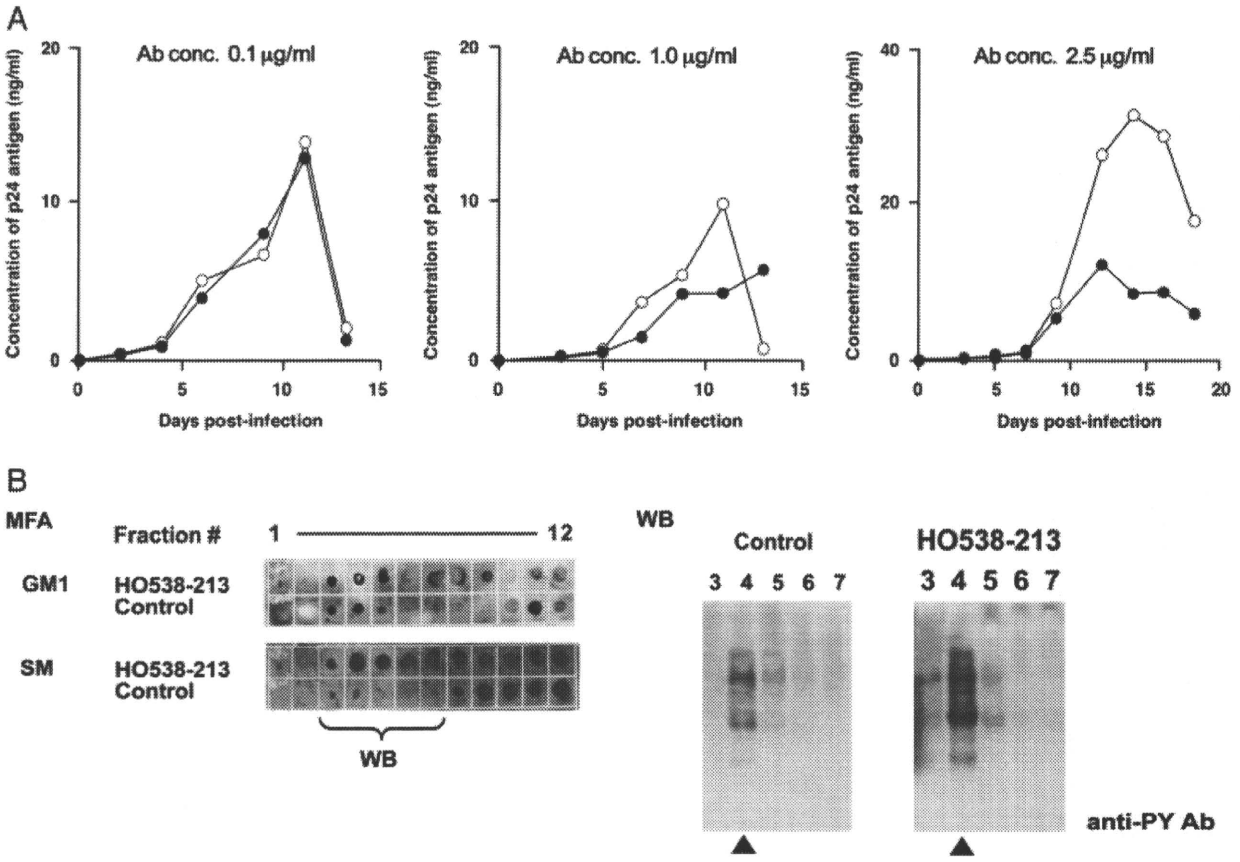

Figure 3. The effect of CD4-reactive Fab clone HO538-213 on HIV-1 replication. (A) The Fab clone HO538-213 (filled circles) was tested for its ability to inhibit HIV-1_{JR-FL} replication at a concentration of 0.1 (left), 1.0 (middle), and 2.5 (right) μg/mL. The CD4 non-reactive Fab clone 13-3 (open circles) was used as a negative control. Representative data from four independent experiments are shown. (B) Activation of the tyrosine kinase signaling cascade by HO538-213 in MOLT-4 cells. The detergent-resistant membrane fraction (arrowhead) was isolated by a membrane floatation assay (MFA) from MOLT-4 cells treated with HO538-213, and phosphotyrosine levels were examined by immunoblotting. GM1, glycosphingomyclin 1; SM, sphingomyelin.

Ab-producing cells from the same donor, namely against nuclear Ag and blood group i-glycolipid, was significantly higher than the other donors (Fig. 1A). In addition, we isolated anti-TNF-α IgG and IgM clones from this donor [16]. Although clinical manifestations of autoimmune disorders were lacking, it is likely that the donor may have an immunological background that generates auto-reactive Ab and tolerates them. Moreover, the donor has been healthy for 29 years, at the time the CD4-reactive Ab was first isolated, suggesting that such CD4-reactive Ab may not disturb host immunity.

Considering that the IgM-producing B cells we isolated went through positive/negative selection, their original target should not be CD4. It is thus likely that the IgM genes accumulated SHM that resulted in cross-reactivity to CD4 in the periphery after B-cell maturation. To better understand the unique immunological features of individuals with CD4-reactive Ab and their auto-reactive Ab repertoire, more human monoclonal self-reactive Ab are needed to analyze both their V-region sequences and cross-reactivities. Our experimental approach might be useful for addressing these issues. Unfortunately, however, we were unable to characterize the CD4-reactive Ab-producing cells, as the oligoclonal cultures of B-LCL were terminated after RNA extraction for our Ig gene cloning strategy. We speculate that B-1 cells could be the source of the CD4-reactive Ab, because B-1 cells produce IgM that often cross-reacts with auto-Ag.

Our genetic data indicated that only a fraction of the CD4-reactive Ab could have some HIV-inhibitory function. It is an open question whether such CD4-reactive HIV-inhibitory Ab may be present in the other healthy individuals, as well as in HIV-seropositive long-term non-progressors.

HIV-inhibitory CD4-reactive Ab are effective against multiple HIV clades, as CD4 is the major HIV receptor for all the viral clades [11]. A clinical trial is being conducted to examine the therapeutic efficacy of a humanized CD4-reactive mAb in patients with HIV infection [8, 12]. Although CD4-reactive Ab can be detected in healthy individuals, safety is always a concern when using self-recognizing Ab as therapeutic drugs. Given that HO538-213 was isolated from a healthy individual and that it recognized a different epitope than Leu-3a, HO538-213 might effectively inhibit HIV without disturbing $CD4^+$ T-cell functions. As noted above, the donor from which the three CD4-reactive IgM Fab were isolated has been healthy for more than 29 years since PBMC collection, suggesting that these Ab may not seriously inhibit $CD4^+$ T-cell functions *in vivo* and thus may be useful in treating HIV infection and other disorders [4].

Concluding remarks

This report provides the first clonal genetic analyses of human monoclonal anti-CD4 Ab. IgM is considered to function in "natural humoral immunity", as it has a relatively low affinity for pathogens and confers natural resistance to infectious agents. However, the pathogen-specific immunity function of IgM has not been demonstrated at a clonal level. Our data suggest that CD4-reactive IgM is present in healthy individuals and can contribute to natural resistance to HIV infection and AIDS progression. This is the first clear demonstration of a natural humoral immunity function of IgM against HIV.

Materials and methods

Functional cloning of heavy and light chain Ab genes

The establishment of Ab-producing cells, cloning of Ig genes encoding V regions, ELISA, and the purification of Fab fragments from *Escherichia coli* have been described previously [16]. The experimental procedure is schematically shown in the Supporting Information Fig. 1. In brief, PBMC from 12 donors, including two healthy individuals and ten individuals with autoimmune disorders, were infected with the B95-8 strain of EBV, and 1×10^4 cells were propagated in 96-well plates. The supernatant was analyzed by ELISA using rhCD4 derived from a baculovirus system (50 ng/well; INTRACELL) as an Ag. Other Ag tested, including viral, bacterial, and auto-Ag, are listed in the Supporting Information Fig. 2. Total cellular RNA was isolated from oligoclonal cell populations positive for anti-CD4 Ab production (RNeasy mini kit, Qiagen). cDNAs were synthesized and amplified by PCR with specific primers for human Ig μ-, γ-, λ-, and κ-chains. Only the μ- and κ-chains were amplified from HO538 and HO702 cultures and cloned into the pFab1-His2 vector, generating bacterial Fab-expression libraries [30]. The pFab libraries were screened for the production of CD4-reactive Fab by ELISA. The Fab fragments were purified using an anti-Fab Ab affinity column. The eluted Fab was dialyzed against PBS and concentrated by centrifugation (VIVASPIN concentrator, Vivascience AG). The purity of the Fab Ab was greater than 95% as determined by SDS-PAGE analysis (data not shown).

Surface plasmon resonance biosensor analysis

Surface plasmon resonance analyses were performed using BIACORE 3000 (GE Healthcare). The hrCD4 was immobilized onto CM5 sensor chips using standard amine-coupling chemistry. The purified Fab was diluted in a running buffer (10 mM HEPES, 0.15 M NaCL, 3 mM EDTA, surfactant P 20, pH 7.4) to 0.3–20 μg/mL and injected at a rate of 20–30 μL/min. The Fab was allowed to associate and dissociate for 120–270 s.

Cells

B-LCL and 293 T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) supplemented with 10% fetal bovine serum (Japan Bioserum), penicillin, and streptomycin (Invitrogen). The primary mononuclear cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, 5 μg/mL plasmocin (InvivoGen), 10 mM HEPES, 5 μg/mL anti-CD3 mAb (OKT3, Janssen Pharmaceutical), 70 U/mL recombinant human IL-2 (Shionogi Pharmaceutical), GlutaMax-I (Invitrogen), insulin–transferrin–selenium-A (Invitrogen), and 10 mM HEPES (Invitrogen). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Other experimental procedures

Procedures for monitoring HIV-1 replication [31] and membrane floatation assays [32] were described previously. Standard auto-Ab was tested by the clinical laboratory testing service SRL (Tokyo, Japan).

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Abbreviations: **B-LCL:** B-lymphoblastoid cell lines · **rhCD4:** recombinant human CD4 · **SHM:** somatic hypermutation

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Identification of the P-TEFb complex-interacting domain of Brd4 as an inhibitor of HIV-1 replication by functional cDNA library screening in MT-4 cells

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Abstract We conducted a phenotypic cDNA screening using a T cell line-based assay to identify human genes that render cells resistant to human immunodeficiency virus type 1 (HIV-1). We isolated potential HIV-1 resistance genes, including the carboxy terminal domain (CTD) of bromodomain-containing protein 4 (Brd4). Expression of GFP-Brd4-CTD was tolerated in MT-4 and Jurkat cells in which HIV-1 replication was markedly inhibited. We provide direct experimental data demonstrating that Brd4-CTD serves as a specific inhibitor of HIV-1 replication in T cells. Our method is a powerful tool for the identification of host factors that regulate HIV-1 replication in T cells.

Keywords: HIV-1 replication; Host factor; cDNA library; Brd4; P-TEFb complex; Tat-dependent LTR transcription

1. Introduction

The identification of specific molecular interactions required for efficient HIV-1 replication should provide clues towards improved understanding of the mechanisms of viral pathogenesis, as well as of host defence against HIV-1. In addition, this may help design highly specific inhibitors against HIV-1. Genome-wide screening for HIV-1 replication regulatory factors has been attempted by using various experimental approaches. Most of them were based on adherent epithelial cells, because these cells exhibit higher transduction efficiencies (by transfection or by viral vector transfer) when compared with T cell lines [1,2]; however, cells of epithelial origin are not relevant hosts for HIV-1 in vivo. Furthermore, viral vectors pseudotyped with vesicular stomatitis virus-G (VSV-G) are often used for screening purposes, instead of wild-type HIV-1. These vectors enter cells via the VSV-G-restricted route, which is different from the HIV-1 envelope-mediated entry pathway. These factors constitute potential caveats of these assays.

To overcome these potential problems, we carried out a phenotypic screen to identify human cDNAs that confer resistance to HIV-1 replication, without affecting cell proliferation. The assay was performed in a human T cell line, a physiologically relevant host, stably transduced with a human cDNA library. We isolated several potential HIV-1 resistance genes successfully, many of which were not known as HIV-1 regulatory factors. In this work, we studied Brd4 in detail to demonstrate the applicability of our phenotype screening. Our study of Brd4-CTD suggests the presence of a potential anti-HIV-1 drug target in the host transcription regulator cyclin T1 (CCNT1).

2. Materials and methods

2.1. Cells and transfection

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Tokyo, Japan), and then incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

2.2. Plasmid construction

The Brd4-CTD was amplified from 293T RNA by reverse transcriptase PCR (RT-PCR) using the primers 5′-AGATCTCTCATCCGACCACCCCCTCCTCC-3′ and 5′-TCAGGATCCCGAAAAGATTTTCTTCAAATATTG-3′. The BglII–BamHI fragment of the PCR product was cloned into the corresponding restriction sites of the pEGFP-C2 (Clontech, Palo Alto, CA). The XhoI–MfeI fragment from the resulting plasmid was cloned into the corresponding restriction sites of the pCMMP KRAB vector, creating the pGFP-Brd4-CTD. The cDNA encoding firefly luciferase (Luc^+) was amplified by PCR from the pGL3-Basic (Promega, Madison, WI) using the primers 5′-ACCGGTCTCGAGGGCCACCATGGAAGACGCCAAAAACATAAAGAAAGG-3′ and 5′-GAATTCGGATCCTTACACGGCGATCTTTCCGCCCTTCTTGGCC-3′. The PCR product was digested with AgeI and BamHI, and cloned into the corresponding sites of the pCMMP GFP vector, generating the pCMMP Luciferase. The BamHI–XhoI fragment of pLenti6/V5-GW/lacZ (Invitrogen) was removed, and Luc^+ was inserted with BglII and SalI sites artificially attached at its extremities, creating the pLenti Luciferase. Other plasmids used in this study were described previously [3,4].

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2.3. Selecting human cDNAs that confer resistance to HIV-1

The lentiviral vector carrying an hPBL cDNA library was described previously [5]. MT-4 cells (1×10^6) transduced with the cDNA library were infected with HIV-1_{HXB2} propagated in MT-2 cells, by resuspending MT-4 cells in a viral preparation containing 70–1250 ng/ml p24 viral capsid antigen in 20 ml of culture medium for 30 min at room temperature with continuous mixing. Anti-CD4 magnetic beads ($0.5–1.0 \times 10^7$; Dynal, Oslo, Norway) were added to the cell suspension to prevent cell-to-cell contact, and the cells (1×10^3 cells per well in 200 μl of culture medium) were plated in flat-bottomed 96-well plates. At 3–4 weeks post-infection, cells from four wells positive for cell outgrowth were pooled and genomic DNA was extracted. The cDNA inserts were PCR-amplified and sequenced using primers described previously [5].

2.4. Generation of viruses and infection

Viruses were produced as described previously [3,4]. Human T cell lines (MT-4 and Jurkat cells; 1×10^5 cells) were incubated with 500–1000 μl of MLV preparations in the presence of 8 μg/ml polybrene for 1 h at 4 °C with continuous agitation. For HIV-1 infection, 1×10^5 cells were incubated with an HIV-1-containing culture supernatant (*ca.* 5–5000 pg p24), for 30 min at room temperature. HIV-1 replication was monitored as described previously [3,4].

2.5. Western blotting

Western blotting was performed according to techniques described previously [4]. The following antibodies were used: anti-CCNT1 (ab2098, Abcam, MA), anti-Brd4 (ab46199, Abcam), anti-GFP (MAB3580, Chemicon International, Temecula, CA or 632381, Clontech), anti-p24 (183-H12-5C, NIH AIDS Research and Reference Reagent Program), anti-HEXIM1 (ab25388, Abcam), anti-Bip/GRP78 (clone 40, BD Transduction Laboratories), and EnVision+ system (Dako, Glostrup, Denmark).

2.6. Reporter assay

The 293T cells grown in 48-well plates were co-transfected with 20 ng of pLTR-Luc or pCMMP Luciferase, together with pGFP-Brd4-CTD. The total amount of transfected DNA was adjusted by pCMMP GFP. To measure the effect of Tat, cells were co-transfected with 100 ng of pSVtat in addition to the above-mentioned plasmids. Cells were replated in 96-well plates in triplicate at 2–4 h post-transfection. Luciferase activity was measured 48 h after transfection using the Dual-Glo assay kit (Promega).

2.7. RT-PCR

RT-PCR was performed as described previously [4]. For amplification of HIV-1 mRNA, forward (5′-CTCGACGCAGGACTCGGCTTGC-3′) and reverse (5′-AGTTCCACTCCTGCCCAAGTATCC-3′) primers were used. The mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the primers 5′-GTGGAAGGACTCATGACCACAGTC-3′ and 5′- CATGTGGGCCATGAGGTCCACCAC-3′.

2.8. Quantitative real-time PCR

The real-time PCR reaction was performed as described previously [4]. Amplifications were performed using the QuantiTect SYBR Green RT-PCR/PCR Kit (QIAGEN). To estimate the amount of integrated HIV-1 DNA, Alu-LTR PCR was performed as described previously [6] using the following primers: first PCR reaction, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-TGCTGGGATTACAGGCGTGAG-3′; and second PCR reaction, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-CTGCTAGAGATTTTCCACACTGAC-3′.

3. Results

To isolate cDNA clones that confer resistance to HIV-1 without negatively affecting cell proliferation, we performed phenotype screening using MT-4 cells stably transduced with a lentiviral vector carrying a cDNA library from human peripheral blood lymphocytes (hPBL). The complexity of the lentiviral cDNA library was on the order of 10^6. The lentiviral vector encoded a GFP expression cassette. Approximately 70% of the MT-4 cells became GFP-positive after infection of the lentiviral vector, suggesting that a portion of the cells were infected with multiple lentiviral vectors. The GFP-positive cells were collected using a FACS sorter and subsequently exposed to replication-competent HIV-1. The surviving cell clones were propagated and their transduced cDNAs were examined. The average length of hPBL cDNA in the lentiviral vector was ~0.7 kbp, which is shorter than the average human cellular mRNA length (~2 kbp). A gene was considered an innate

Table 1
Summary of cDNAs recovered in an HIV-1-resistant phenotype screening in MT-4 cells.

Gene category	# of independent clones	Frequency (%)	Frequently isolated genes[a] (# of independent clones)
Metabolism	16	24.6	Haemoglobin (7) Pyridoxal kinase, PDXK (3)
Transcription	7	10.8	Bromodomain containing 4, Brd4 (3) Zinc finger protein 26, ZNF26 (3)
Ribosomal proteins	7	10.8	Ribosomal protein L14, RPL14 (3)
Signal transduction	7	10.8	Zinc finger protein 36, ZFP36L2 (2) transducin beta-like 1X-linked, TBL1X (1)[c] Chromosome 22 open reading frame 5, C22orf5 (1)[c]
Trafficking	6	9.2	Chromosome 9 open reading frame 86, C9orf86 (1)[b] Chromosome 1 open reading frame 142, C1orf142 (1)[b] Nedd4-binding partner 3, N4BP3 (1)
Immunology	2	3.1	MHC class II, DR alpha (1)
Oncogenesis	2	3.1	AXIN1 up-regulated, AXUD1 (1)[c]
Glycosylation	2	3.1	Hyaluronan and proteoglycan link protein 3, HAPLN3 (1)
Differentiation	2	3.1	Jumonji AT rich interactive domain 2, JARID2 (1)
Cytoskeleton	2	3.1	Beta actin (1)
Cell cycle control	1	1.5	CWF19-like 1, CWF19L1 (1)
Apoptosis	1	1.5	Chromosome 2 open reading frame 28, C2orf28 (1)
DNA repair	1	1.5	Non-SMC element 1 homolog, NSMCE1 (1)[b]
Non-ORF coding	9	13.8	–
Total	65	100.0	–

[a]All the clones isolated more than three times are listed. A representative clone is shown for categories with a few candidates.
[b]These genes exhibited regulatory functions on HIV-1 production.
[c]These genes exhibited no effect on HIV-1 production.

negative factor for HIV-1 replication if the full-length open reading frame (ORF) was recovered. Alternatively, if a portion of a gene was recovered, the full-length gene was considered a potential positive factor for HIV-1 replication. We recovered 65 independent cDNA clones (43 genes, Table 1). A number of cDNAs encoded abundant cellular transcripts, including haemoglobin. In addition, cDNAs encompassing non-ORFs were isolated. The isolation of these cDNAs was likely due to the infection of a single cell with multiple cDNA-transducing lentiviral vectors, one of which encoded an HIV-1 resistance gene. If we disregard these cases, 26 genes were potential HIV-1 regulatory gene candidates, of which seven were examined for a potential HIV-1 regulatory functions as shown in Fig. 2. Four genes exhibited HIV-1 regulatory phenotypes (4/7 genes, 57.1%; Table 1). In addition to Brd4-CTD, C9orf86 and NSMCE1 scored as positive factors for HIV-1 replication, and C1orf142 was scored as a negative factor. This suggested that our screening was successful in selecting for HIV-1 regulatory genes. While each candidate gene will be studied in detail in future studies, here we focused on Brd4.

Brd4 was chosen for three reasons: (1) three independent Brd4 cDNAs were recovered; (2) Brd4 binds to the CCNT1/T2-bearing P-TEFb complex [7,8]; and (3) 13 independent Brd4 cDNA clones (13/42 clones, 31.0%) were isolated from a similar experiment in which the cDNA library from an *Oryctolagus cuniculus* kidney derived cell line was used. All the three Brd4 cDNA clones encoded Brd4-CTD: two encoded amino acids (aa) 1260–1362 and the third encoded aa 1209–1362 (Fig. 1A). The first two clones were translated using the Met-encoding codon at Brd4 nucleotide position 3778–3780, and the third was translated from the aberrant start codon in the primer upstream of the Brd4 ORF 3628 nt. To our surprise, aa fragment 1209–1264 of Brd4 was recently reported as an interactor of CCNT1 that inhibits Tat-dependent LTR-driven transcription [9]; however, the specific effect of this region on HIV-1 replication in human T cells was not fully investigated.

We hypothesized that the repression of HIV-1 replication in MT-4 cells may be due to the selective inhibition of viral gene transcription by Brd4-CTD. To test this, we cloned Brd4-CTD spanning aa 1209-1364 into a retroviral plasmid and fused GFP to its carboxy-terminus (GFP-Brd4-CTD, Fig. 1B). Confocal microscopy revealed that GFP-Brd4-CTD was localized mainly in the cytoplasm of MT-4 cells, with some signal found in the nucleus (Fig. 1C). A transient transfection assay revealed that the expression of GFP-Brd4-CTD modestly enhanced the luciferase expression driven by both the LTR and CMV promoters (Fig. 1D). In the presence of Tat, LTR

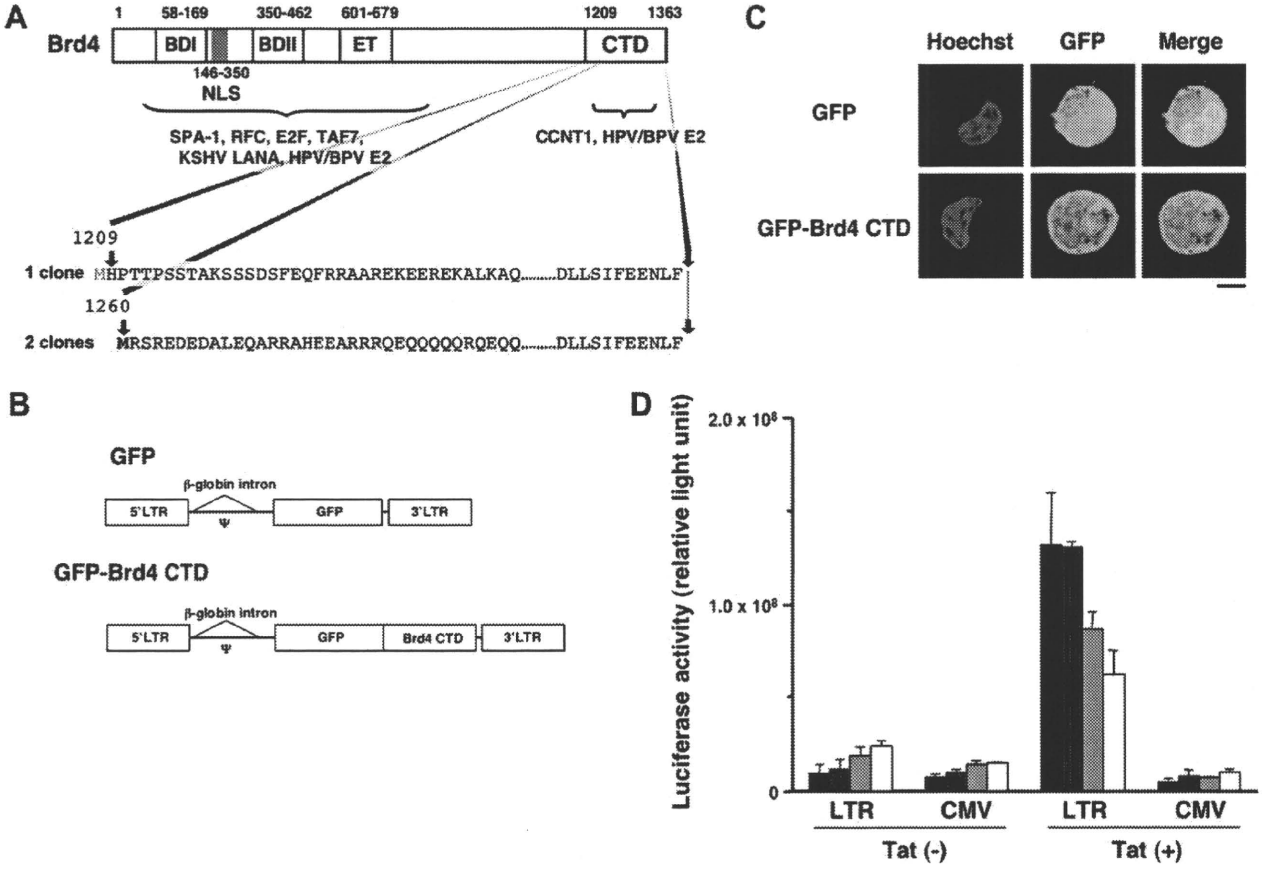

Fig. 1. Specific inhibition of Tat-dependent LTR transcription by GFP-Brd4-CTD. (A) Functional properties of the Brd4 domains and isolated Brd4 cDNAs. (B) Construction of MLV vector-based mammalian expression plasmids encoding GFP or GFP-Brd4-CTD. (C) Confocal microscopy images of MT-4 cells stably expressing GFP or GFP-Brd4-CTD. Green and blue represent GFP and the Hoechst 33258-stained nuclei, respectively. Magnification, 630×; scale bar, 5 μm. (D) Effect of GFP-Brd4-CTD on LTR and CMV promoter activities in the absence or presence of the Tat expression plasmid. Cells transfected with 0, 16, 80, and 400 ng of pGFP-Brd4-CTD correspond to black, dark gray, light gray, and white bars, respectively. Representative data from five independent experiments are shown.

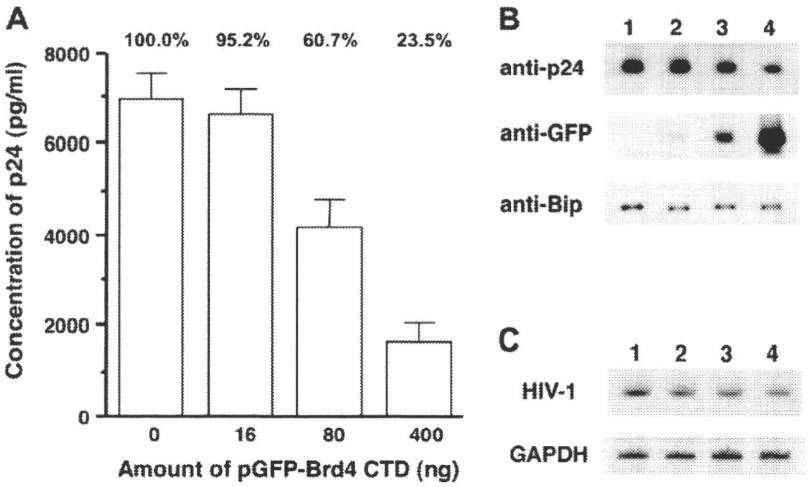

Fig. 2. Inhibition of HIV-1 production by GFP-Brd4-CTD. (A) Viral production was quantified by ELISA detecting p24 viral antigen. The relative decrease in viral levels are indicated on the top of the graph. (B) The viral protein levels in transfected cells were analyzed by Western blotting using the antibodies indicated. (C) The viral spliced transcript and GAPDH mRNA were amplified by RT-PCR. Lanes 1–4 in B and C correspond to the amount of pGFP-Brd4-CTD (0, 16, 80, and 400 ng, respectively). Quantification of these data is summarized in Table 2.

activity was markedly enhanced. When the GFP-Brd4-CTD expression vector was co-transfected, the Tat-dependent enhancement of LTR promoter-driven luciferase expression decreased. A similar trend was not observed for the CMV promoter. These data suggest that the Brd4-CTD specifically limits Tat-dependent LTR transcription.

We also investigated the effect of GFP-Brd4-CTD expression on HIV-1 production by using a proviral DNA mimicking the late phase of the viral life cycle. Consistent with the results described above, transfection of HIV-1 proviral DNA together with an increasing amount of the GFP-Brd4-CTD expression vector led to a decrease of viral yield, as well as of the levels of viral protein and mRNA in the transfected cells (Fig. 2). The viral RNA levels dropped in parallel with the protein levels, as demonstrated by real-time RT-PCR analysis (Fig. 2C and Table 2). These data suggest that GFP-Brd4-CTD inhibits HIV-1 production by blocking viral transcription.

To confirm the blockage of HIV-1 replication by Brd4-CTD, GFP-Brd4-CTD was transduced into MT-4 and Jurkat cells using an MLV-based vector (Fig. 3A). Green fluorescence indicated that the efficiency of MLV-mediated gene transduction in MT-4 cells was >90%, with a lower transduction efficiency observed in Jurkat cells, as estimated by FACS analysis. The GFP-positive Jurkat cells were collected using a FACS sorter. The expression of GFP and GFP-Brd4-CTD was verified by Western blot analysis (Fig. 3B). The expression levels of transcription-related gene products were not detectably affected by the constitutive expression of GFP-Brd4-CTD (Fig. 3B). In addition, there was no detectable difference in the levels of cell-surface HIV-1 receptors (CD4 and CXCR4), cell morphology, and cell proliferation rates between GFP- and GFP-Brd4-CTD-expressing cells (Fig. 1C and Supplementary data). We found that HIV-1 replicated less efficiently in GFP-Brd4-CTD-expressing cells than in GFP-expressing cells, in both cell lines tested, which confirms the HIV-1-resistant phenotype of MT-4 cells (Fig. 3C). The efficiency of viral genome integration into GFP-Brd4-CTD-expressing cells was indistinguishable from that of GFP-expressing cells (103.2 ± 24.1%) as examined by Alu-LTR PCR, suggesting that the early phase of the viral life cycle was not blocked by GFP-Brd4-CTD.

4. Discussion

Our phenotype screening method proved to be a powerful tool because a human T cell line was subjected to HIV-1 resistance screening by stable and non-transient introduction of a human cDNA library, and because wild-type HIV-1 was used; thus, the effect of candidate gene expression on cell proliferation was less of a concern in this system when compared with transient assay systems. In addition, HIV-1 inhibitory genes were isolated at a frequency of ~15% (4/26 genes), 75% of which were novel. We therefore believe that our system is remarkable in selecting genes that confer HIV-1 resistance in T cells. By applying this assay to other cDNA libraries, we

Table 2
Effect of GFP-Brd4-CTD on viral production examined by quantitative real time RT-PCR and ELISA.

pGFP-Brd4-CTD (ng)	HIV-1 mRNA (copy)[a]	GAPDH mRNA (copy)[a]	Ratio (HIV-1/GAPDH)	Normalized (%)[b]	p24 ELISA (%)[c]
0	274250	261750	1.048	100.0	100.0
16	221600	228850	0.968	92.4	95.2
80	138050	311450	0.443	42.3	60.7
400	120850	347750	0.348	33.2	23.5

[a]Copy per 100 ng total cellular RNA.
[b]Relative reduction of HIV-1 mRNA considering pGFP-Brd4-CTD 0 ng as 100%.
[c]See Fig. 2.

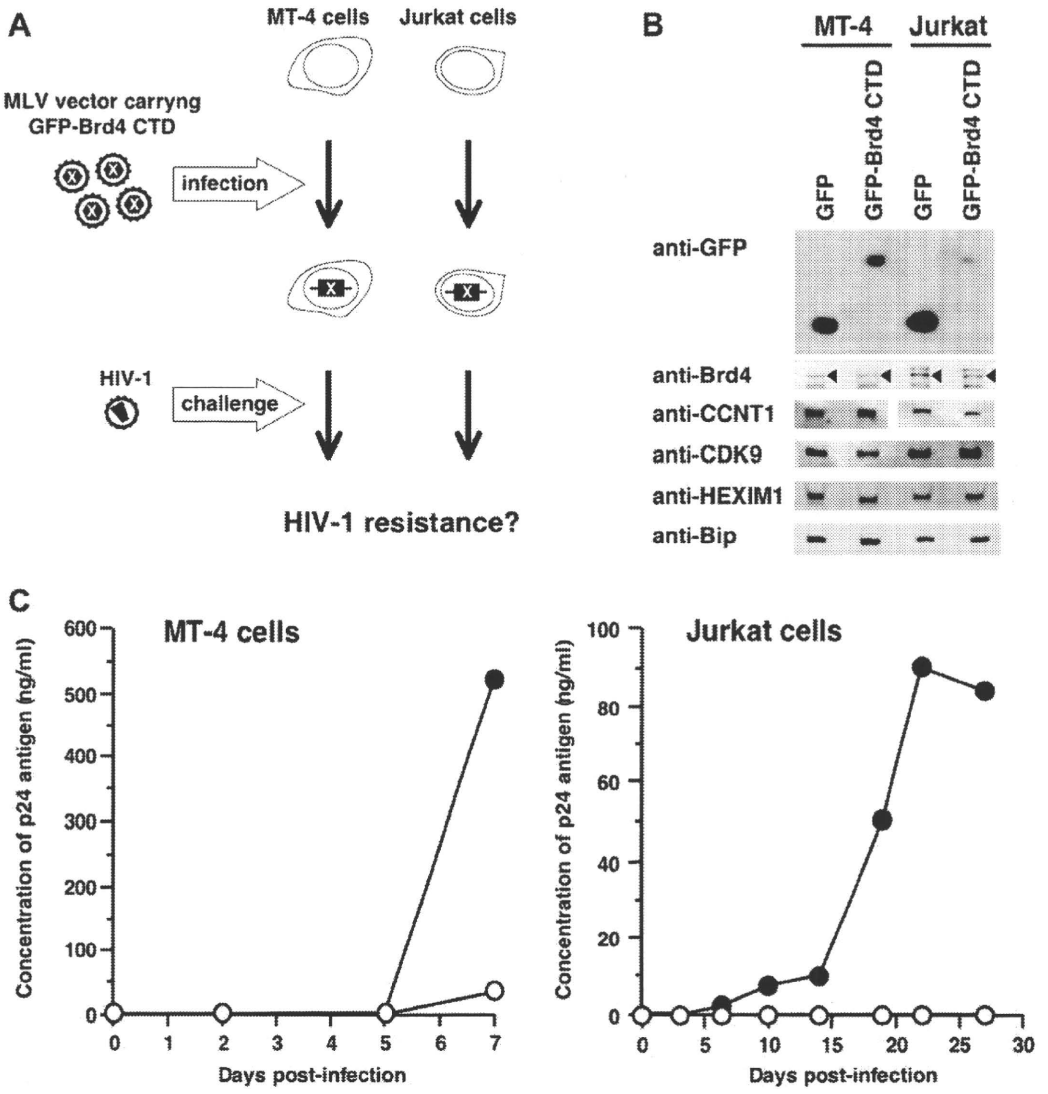

Fig. 3. Constitutive expression of GFP-Brd4-CTD limited the replication of HIV-1. (A) Experimental design. MT-4 and Jurkat cells were transduced with an MLV vector expressing GFP-Brd4-CTD. Cells were challenged by HIV-1 and the efficiency of viral replication was examined. (B) Western blot analysis of the expression levels of GFP, GFP-Brd4-CTD, Brd4 (arrowhead), CCNT1, CDK9, HEXIM1, and BiP in established MT-4 and Jurkat cells. (C) HIV-1 replication kinetics in MT-4 and Jurkat cells constitutively expressing GFP (black circles) or GFP-Brd4-CTD (white circles). Representative data from two independent experiments are shown.

may be able to isolate novel cellular factors that regulate HIV-1 replication.

The assessment of the selective impact of altered candidate gene expression or function on HIV-1 replication (without the alteration of cell proliferation) is critical to the identification of cellular molecular targets for novel anti-retroviral drugs. We demonstrated that Brd4-CTD was a specific silencer of HIV-1 replication, and verified that it effectively blocked HIV-1 replication in multiple human T cell lines without affecting cell proliferation. Our data indicate that primate lentiviral replication is more heavily dependent on the CCNT1-containing P-TEFb complex than cellular gene transcription, which is consistent with previous findings [4,10–11]. This implies that HIV-1 replication can be controlled by selectively restricting the CCNT1-containing P-TEFb complex. Our transcription assay indicated that the Brd4-CTD is not an inhibitor of the P-TEFb complex, but is rather a functional Tat inhibitor. Previous biochemical studies have suggested that Brd4-CTD and Tat bind to CCNT1 in a reciprocally exclusive fashion [7,9]. Given that the binding regions of these two proteins do not overlap, Brd4-CTD may be an allosteric inhibitor of the Tat-CCNT1 interaction. Taken together, our results indicate that the Brd4-interacting region of CCNT1 is a potential molecular target for the development of a novel HIV-1 inhibitor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.10.047.

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Prevention of hepatic ischemia–reperfusion injury by pre-administration of catalase-expressing adenovirus vectors

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ABSTRACT

Liver ischemia/reperfusion (I/R) injury, which is mainly caused by the generation of reactive oxygen species (ROS) during the reperfusion, remains an important clinical problem associated with liver transplantation and major liver surgery. Therefore, ROS should be detoxified to prevent hepatic I/R-induced injury. Delivery of antioxidant genes into liver is considered to be promising for prevention of hepatic I/R injury; however, therapeutic effects of antioxidant gene transfer to the liver have not been fully examined. The aim of this study was to examine whether adenovirus (Ad) vector-mediated catalase gene transfer in the liver is an effective approach for scavenging ROS and preventing hepatic I/R injury. Intravenous administration of Ad vectors expressing catalase, which is an antioxidant enzyme scavenging H_2O_2, resulted in a significant increase in catalase activity in the liver. Pre-injection of catalase-expressing Ad vectors dramatically prevented I/R-induced elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and hepatic necrosis. The livers were also protected in another liver injury model, CCl_4-induced liver injury, by catalase-expressing Ad vectors. Furthermore, the survival rates of mice subjected to both partial hepatectomy and I/R treatment were improved by pre-injection of catalase-expressing Ad vectors. On the other hand, control Ad vectors expressing β-galactosidase did not show any significant preventive effects in the liver on the models of I/R-induced or CCl_4-induced hepatic injury described above. These results indicate that hepatic delivery of the catalase gene by Ad vectors is a promising approach for the prevention of oxidative stress-induced liver injury.

1. Introduction

Hepatic ischemia/reperfusion (I/R) injury occurs in a variety of clinical settings, such as in liver transplantation, hepatic failure after shock, and liver surgery, and results in severe damages that substantially contribute to the morbidity and mortality of such cases [1–3]. Hepatic I/R injury is caused by reactive oxygen species (ROS), including superoxide anion, hydrogen oxide, and hydroxyl radical, which are generated by reperfusion of the ischemic tissue. ROS induce lipid peroxidation and damages to proteins and nucleic acids, leading to parenchymal cell dysfunction and necrosis, increased vascular permeability, and inflammatory cell infiltration [4]. Therefore, ROS should be detoxified to prevent hepatic I/R injury.

In previous animal studies, antioxidative enzyme catalase and superoxide dismutase (SOD) were systemically administered to neutralize ROS and prevent I/R-induced hepatic injury. Although catalase and SOD are endogenously expressed in the cells, the expression levels of these enzymes are insufficient to prevent I/R injury. Administration of antioxidant enzymes exhibited therapeutic effects on ROS-induced diseases, including I/R-induced hepatic injury, in several studies [5–8]; however, these enzymes are known to be rapidly eliminated from the circulation following systemic administration, which limits their therapeutic potential, [9,10] although chemical modification of antioxidant enzymes has been carried out to enhance their plasma half-lives and tissue accessibility [5,6,9]. In addition, systemically administered antioxidant enzymes might be degraded in the endosomes/lysosomes because they are internalized into the cells via the endocytosis pathway.

The delivery of therapeutic genes encoding antioxidant enzymes into the liver is considered to be a promising strategy to overcome these problems. Previous studies have demonstrated that ROS-mediated injury was efficiently prevented by over-expression of antioxidant

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enzymes in various tissues, including the artery, pancreatic islets, and brain [11–13]. A variety of types of gene delivery vehicles have been employed for delivery of antioxidative genes so far, and replication-incompetent adenovirus (Ad) vectors have several advantages over other vehicles to deliver antioxidant genes to the liver. First, Ad vectors have high tropism to livers. A more than 10^3-fold higher transgene expression is found in the liver, compared with other organs, following systemic administration [14–16]. Second, non-dividing cells are efficiently transduced with Ad vectors. Hepatocytes do not actively divide under normal conditions. Non-viral gene delivery vehicles have been used for prevention of hepatic I/R injury in previous studies [17,18]; however, non-viral gene delivery vehicles mediate inefficient transfection in non-dividing cells. Third, the Ad vector genome is not integrated into the host genome, indicating that transduction with Ad vectors is unlikely to induce insertional mutagenesis in hepatocytes. Fourth, Ad vector-mediated gene expression in liver persists for 1–2 weeks, [19,20] in contrast, rapid reduction in plasmid DNA-mediated transgene expression in organs is found after injection of non-viral gene delivery vehicles [21,22]. In spite of these advantages of Ad vectors, the ability of Ad vectors expressing antioxidant enzymes to prevent hepatic I/R injury has not been fully examined probably because Ad vectors are generally considered more toxic than non-viral gene delivery vehicles; however, our group demonstrated that intravenous administration of Ad vectors induces less amounts of inflammatory cytokines than cationic lipid/plasmid DNA complexes [23]. In addition, fiber-modified Ad vectors carrying a stretch of lysine residues in the C-terminus of a fiber knob have been demonstrated to poorly activate innate immune responses after systemic injection, compared with conventional Ad vectors [24]. These results suggest that Ad vectors, including fiber-modified Ad vectors, would be suitable for prevention of I/R injury by delivering antioxidant genes to livers.

Among antioxidant enzymes, SOD is often used for detoxifying ROS in previous studies [8,17,25,26]. SOD catabolizes superoxide anion to H_2O_2; however, H_2O_2 is converted to hydroxyl radicals, which are extremely reactive and more toxic than other ROS. H_2O_2 should be removed to effectively reduce I/R injury. Another antioxidant enzyme, catalase, prevents the generation of hydroxyl radicals by catabolizing H_2O_2 to H_2O and O_2, suggesting that catalase is promising for prevention of I/R injury. However, there are few studies reporting therapeutic effects of catalase gene delivery on I/R injury [17,27].

In the present study, catalase-expressing Ad vectors were intravenously pre-administered to prevent I/R-induced hepatic injury. Pre-injection of catalase-expressing Ad vectors successfully prevented not only I/R-induced hepatic injury but also CCl_4-induced liver damages. Furthermore, mice receiving pre-injection of catalase-expressing Ad vectors showed improved survival rates after partial hepatectomy followed by hepatic I/R.

2. Materials and methods

2.1. Cells

A549 (a human lung adenocarcinoma epithelial cell line), HepG2 (a human hepatocellular liver carcinoma cell line), and 293 (a human embryonic kidney cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum under 5% CO_2 at 37 °C.

2.2. Ad vectors

Ad vectors were constructed by means of an improved *in vitro* ligation method [28–30]. Briefly, the LacZ gene, which is derived from pCMVβ (Marker Gene, Inc., Eugene, OR) and the catalase gene, which is derived from pZEOSV2-CAT (a kind gift from Dr. J. Andres Melendez, Albany Medical College, Albany, NY) [31,32] were inserted into pHMCA5, [33] creating pHMCA5-LacZ and pHMCA5-CAT, respectively. pHMCA5-LacZ and pHMCA5-CAT were then digested with I-CeuI and PI-SceI, and ligated with I-CeuI/PI-SecI-digested pAdHM4 [28], resulting in pAdHM4-LacZ and pAdHM4-CAT, respectively. To generate the viruses, PacI-digested Ad vector plasmids were transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The viruses were prepared by the standard method, then purified with $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −80 °C. The determinations of infectious titers and virus particle (VP) titers were accomplished using 293 cells and an Adeno-X rapid titer kit (Clontech, Mountain View, CA) and the method of Maizel et al. [34], respectively. Catalase-, or β-galactosidase-expressing fiber-modified Ad vectors carrying a stretch of lysine residues (K7 (KKKKKKK) peptide) in the C-terminus of a fiber knob, AdK7-CAT and AdK7-LacZ, respectively, were similarly prepared using pAdHM41K7 [35]. The ratios of the biological-to-particle titer were 1:20, 1:31, 1:45, and 1:39 for Ad-LacZ, AdK7-LacZ, Ad-CAT, and AdK7-CAT, respectively.

2.3. Western blot analysis for catalase expression

A549 cells were transduced with Ad vectors at 3000 VP/cell for 2 h. Forty-eight hours later, cells were harvested and lysed with lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease inhibitor cocktail (Sigma Chemical., St. Louis, MO). Equal quantities of protein (5 μg), as determined by a protein assay (Bio-Rad, Hercules, CA), were subjected to sodium dodecyl sulfate/12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking nonspecific binding, the membrane was incubated with anti-catalase antibody (diluted 1/8000; Calbiochem, San Diego, CA) at room temperature for 3 h, followed by reaction with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted 1/3000; Cell Signaling Technology, Beverly, MA) at room temperature for 1 h. The band was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ), and the signals were read using an LAS-3000 imaging system (Fujifilm, Tokyo, Japan). For detection of the internal control, a polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (diluted 1/5000; Trevigen, Gaithersburg, MD) and an HRP-conjugated anti-rabbit IgG were used.

2.4. In vitro protective effect of catalase-expressing Ad vectors on ROS-induced cell damage

HepG2 cells (5000 cells/well) were seeded onto a 96-well plate. On the following day, the cells were transduced with Ad-LacZ, AdK7-LacZ, Ad-CAT, or AdK7-CAT at 300 or 3000 VP/cell for 2 h. After a 48-h incubation, the medium was exchanged for normal medium containing 30 mM menadione (Sigma Chemical), which is a ROS inducer. On the following day, the cell viability was determined by Alamar blue staining (BioSource, San Diego, CA).

2.5. Catalase activities in the liver after intravenous administration of Ad vectors

Ad vectors (Ad-LacZ, AdK7-LacZ, Ad-CAT, and AdK7-CAT) were intravenously administered into C57BL/6 mice (7–8-week-old females; Nippon SLC, Shizuoka, Japan) at a dose of 1×10^{10} VP/mice. Forty-eight hours later, the livers were isolated and homogenized with 50 mM potassium phosphate buffer containing 1 mM EDTA. The supernatants were recovered after centrifugation of the homogenates, and catalase activity in the supernatants was measured using a CalBiochem Catalase Assay Kit (Calbiochem).

2.6. Hepatic ischemia/reperfusion experiment

Mice were intravenously administered PBS (control) or Ad vectors via the tail vein at a dose of 10^{10} VP/mice. A partial hepatic ischemia/reperfusion experiment was performed as previously described [36,37]. Briefly, 2 days post-administration of Ad vectors, mice were anesthetized with a peritoneal injection of pentobarbital sodium (50 mg/kg). An incision was made in the abdomen, and all structures in the portal triad (hepatic artery, portal vein, bile duct) were occluded with a vascular clamp for 1 h to induce hepatic ischemia. Then, blood was allowed to flow through the liver again by removal of the clamp (reperfusion). After an appropriate period of reperfusion (0, 1, 6, 24 h), blood was collected via retro-orbital bleeding, and serum was obtained by centrifugation. The aspartate aminotransferase (ALT) and alanine aminotransferase (AST) activities in serum, as indicators of liver injury during reperfusion, were assayed using a transaminase-CII test (Wako, Osaka, Japan). In separate experiments, histology in the liver sections was evaluated 24 h after reperfusion. The livers were recovered and fixed by immersion in 10% buffered formalin, embedded in paraffin and processed for histology. Tissue damage was assessed in hematoxylin and eosin-stained sections. A sham surgery was performed under anesthesia but without occluding the vessels.

2.7. CCl_4-induced liver injury experiment

Ad vectors were intravenously administered into mice as described above. Forty-eight hours after Ad vector injection, CCl_4 dissolved in olive oil was intraperitoneally administrated to the mice at a dose of 1 ml/kg body weight to induce acute liver failure. Twenty-four hours after CCl_4 administration, blood was collected via retro-orbital bleeding, and the levels of ALT and AST in the serum were determined as described above.

2.8. Partial hepatectomy

Ad vectors were intravenously administered into mice as described above. Forty-eight hours after Ad vector injection, mice were anesthetized and subjected to two-thirds hepatectomy as described previously [38,39]. Subsequently, liver I/R was conducted by occlusion of the blood vessel to block the blood flow into the remnant liver for 8 min followed by reperfusion as described above. After the surgery, the mice were maintained under conventional conditions to monitor survival rates.

2.9. Statistical analysis

Results were expressed as the means ± S.D. Statistically significant differences between groups were determined by the two-way analysis of variance, followed by Student's *t*-test. The levels of statistical significance were set at $p<0.05$ and $p<0.01$.

3. Results

3.1. Ad vector-mediated catalase expression in vitro

First, to examine *in vitro* catalase expression levels following Ad vector infection, Western blotting analysis was performed. We observed an apparent increase in the catalase expression after transduction with Ad-CAT or AdK7-CAT in A549 cells (Fig. 1). In addition, AdK7-CAT mediated higher catalase expression than Ad-CAT, probably due to the higher transduction activity of AdK7 vectors than conventional Ad vectors [35]. The control Ad vectors, Ad-LacZ and AdK7-LacZ, did not increase catalase expression, indicating that transduction with Ad vectors alone does not induce any change in the antioxidant systems.

Next, to examine whether Ad vector-mediated over-expression of catalase prevents ROS-induced cellular toxicity, the cells were incubated with 30 mM menadione following transduction with Ad vectors, and the cell viabilities were determined. It is well known that menadione produces superoxide radicals in cells, leading to oxidative stress-induced cell death [40,41]. As shown in Fig. 2, the cell viability was significantly reduced to less than 50% in the presence of 30 mM menadione. In contrast, transduction with catalase-expressing Ad vectors dramatically improved the cell viabilities. Transduction with Ad-CAT and AdK7-CAT at 300 VP/cell resulted in cell viabilities of 70.8% and 79.1% of the cell, respectively. These results indicate that Ad vector-mediated over-expression of catalase is beneficial in preventing oxidative stress-induced cell death by efficiently deleting ROS.

3.2. Catalase activity in the liver following catalase-expressing Ad vector injection

Next, to measure catalase activities in the liver following intravenous administration of Ad vectors, the livers were recovered 48 h after Ad vector injection, and catalase activities in the liver were determined. The catalase activities were 2.4-fold and 4.3-fold increased following administration of Ad-CAT and AdK7-CAT, respectively (Fig. 3). By contrast, we found no elevation in the catalase activity by LacZ-expressing Ad vectors. These results indicate that catalase activity in the liver is significantly elevated by intravenous administration of catalase-expressing Ad vectors.

3.3. Prevention of hepatic I/R injury by pre-administration of catalase-expressing Ad vectors

To evaluate the ability of catalase-expressing Ad vectors to prevent hepatic I/R injury, serum ALT and AST levels were measured after 1 h of hepatic ischemia followed by reperfusion. Both serum ALT and AST levels were highly elevated at 1 h and 6 h after the reperfusion of hepatic flows in the mice pre-injected with PBS, indicating that hepatic injury was induced by I/R (Fig. 4). At 1 h after reperfusion, the ALT and AST levels increased from 59.3 to 184.0 and from 421.2 to 1174.7 IU/L, respectively. However, pretreatment with Ad-CAT or AdK7-CAT significantly reduced the serum ALT and AST levels at 6 h after reperfusion. The ALT and AST levels in mice pre-injected with AdK7-CAT were 3.9- and 4.4-fold lower than those in mice pre-injected with PBS. Reductions in the ALT and AST levels were also observed at 1 h after reperfusion, although these changes were not statistically significant. The control Ad vectors, Ad-LacZ and AdK7-LacZ, exhibited no suppressive effects on the I/R-induced elevation of serum ALT and AST levels.

Furthermore, to histologically evaluate the preventive effects of catalase-expressing Ad vectors, liver sections were prepared 24 h after reperfusion. An extensive necrotic area was observed in the mice pretreated with PBS or AdK7-LacZ (Fig. 5B, C). In contrast, transduction with Ad-CAT or AdK7-CAT resulted in a dramatic decrease in the necrotic area induced by hepatic I/R (Fig. 5D, E). In particular,

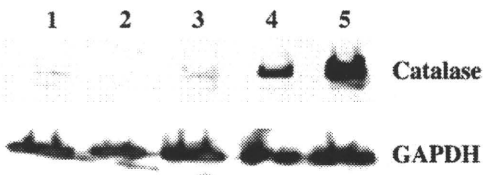

Fig. 1. Catalase expression following Ad vector transduction. A549 cells were transduced with Ad-LacZ, Ad-CAT, or AdK7-CAT at 3000 VP/cell for 2 h. Protein samples were collected after a 48-h incubation and analyzed by Western blotting. Lane 1, mock; lane 2, Ad-LacZ; lane 3, AdK7-LacZ; lane 4, Ad-CAT; lane 5, AdK7-CAT. The results are representative of two

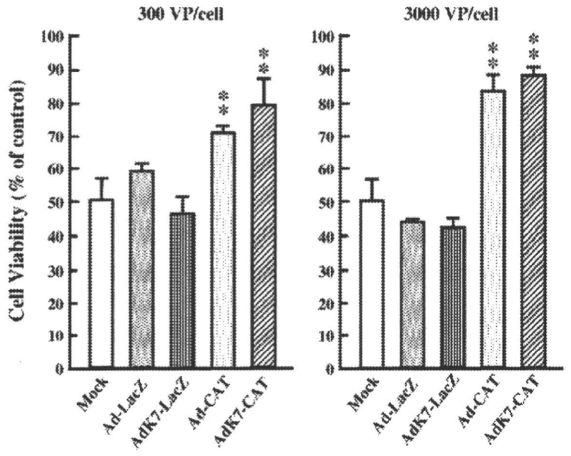

Fig. 2. Protective effects of catalase-expressing Ad vectors against menadione-induced cell death. HepG2 cells were transduced with Ad vectors at 300 or 3000 VP/cell for 2 h. After a 48-h incubation, menadione was added to the medium at a final concentration of 30 mM, and the cells were cultured for an additional 24 h. The cellular viabilities were then determined by Alamar blue staining. The cellular viabilities were normalized to the viability of Ad vector-infected HepG2 cells in the absence of menadione. The data are expressed as the means ± S.D. ($n=4$). **Significantly different from the mock-infected group at $p<0.01$.

pre-injection of AdK7-CAT almost completely prevented necrosis in the liver, although there were several small necrotic areas in the liver pretreated with Ad-CAT, probably due to the higher transduction efficiency and less liver toxicity profile of AdK7 vectors in the liver compared with conventional Ad vectors [24]. A TUNEL assay indicated that Ad-CAT and AdK7-CAT prevented the DNA fragmentation caused by hepatic I/R in hepatocytes (data not shown). These results indicate that the I/R-induced histological damages were also significantly attenuated by pretreatment with catalase-expressing Ad vectors.

3.4. Preventive effect of catalase-expressing Ad vectors on CCl_4-induced liver injury

To explore whether Ad vector-mediated catalase expression prevents other types of oxidative stress-induced liver injury, CCl_4 was intraperitoneally injected into mice pretreated with catalase-expressing Ad vectors. CCl_4 is well known to produce CCl_3 radical, leading to acute liver injury. Serum ALT and AST levels were highly elevated following CCl_4 treatment in mice pretreated with PBS or LacZ-expressing Ad vectors (Fig. 6). However, serum ALT and AST levels were markedly reduced by pretreatment with Ad-CAT and AdK7-CAT. AdK7-CAT mediated a 4.9-fold and 3.9-fold reduction in serum ALT and AST levels, respectively, compared with PBS. Ad-CAT and AdK7-CAT also mediated a dramatic improvement of CCl_4-

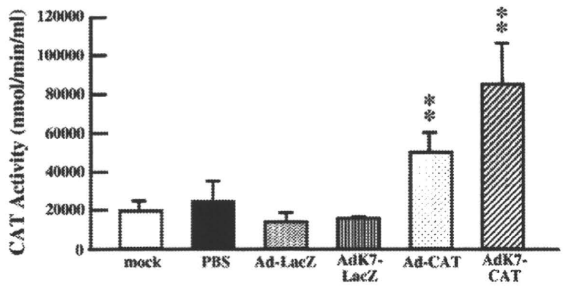

Fig. 3. Catalase activity in the liver following intravenous administration of catalase-expressing Ad vectors. Ad vectors were administered to mice at the dose of 1×10^{10} VP/mouse. The livers were recovered 48 h after injection, and catalase activities in mouse liver homogenates were determined. The data are expressed as the means ± S.D. ($n=5$). **Significantly different from the mock-infected group at $p<0.01$.

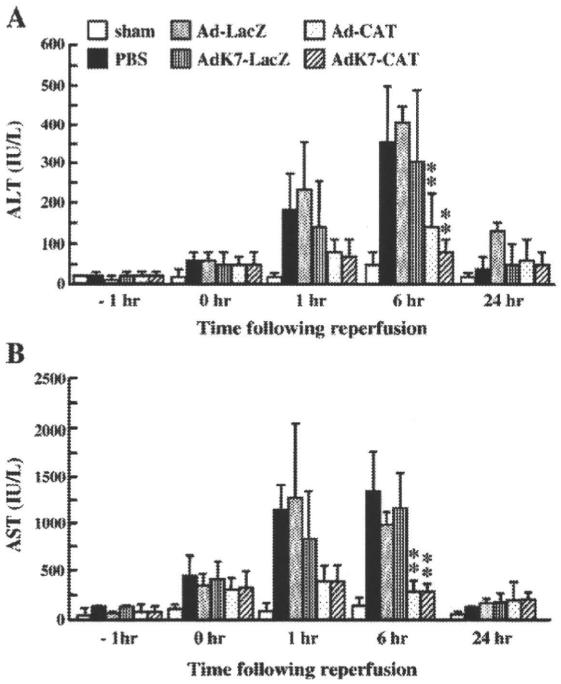

Fig. 4. Effects of pre-administration of catalase-expressing Ad vectors on serum ALT (A) and AST (B) levels in mice following hepatic ischemia/reperfusion injury. Ad vectors were intravenously administered to mice at a dose of 1×10^{10} VP/mice. Forty-eight hours after Ad vector injection, mice were subjected to a 1 h period of ischemia followed by hepatic reperfusion. Serum samples were taken at 1 h before ischemia, and 0, 1, 6, and 24 h after reperfusion. The data are expressed as the mean ± S.E. ($n=3$–8). **Significantly different from the PBS-injected group at $p<0.01$.

induced gross abnormality in the liver (data not shown). These results indicate that catalase-expressing Ad vectors possess preventive effects on oxidative stress-induced injury that are distinct from their effects on I/R injury.

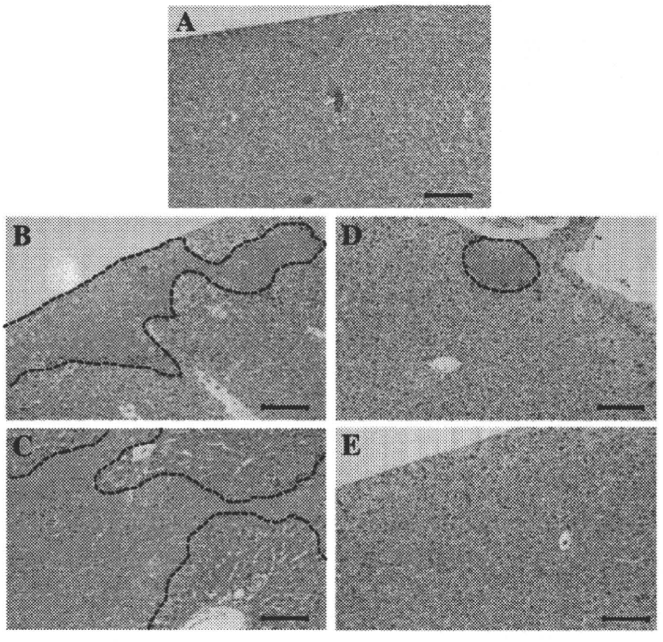

Fig. 5. Representative images of liver sections of mice 24 h following hepatic ischemia/reperfusion. A) Sham, B) PBS, C) AdK7-LacZ, D) Ad-CAT, and E) AdK7-CAT. Mice were subjected to hepatic I/R 48 h after Ad vector injection, as described in Fig. 4. Livers were recovered 24 h after I/R treatment, and liver sections stained with hematoxylin and eosin were observed under a microscope. A dashed line indicates the necrotic area. The scale bar represents 100 μm.

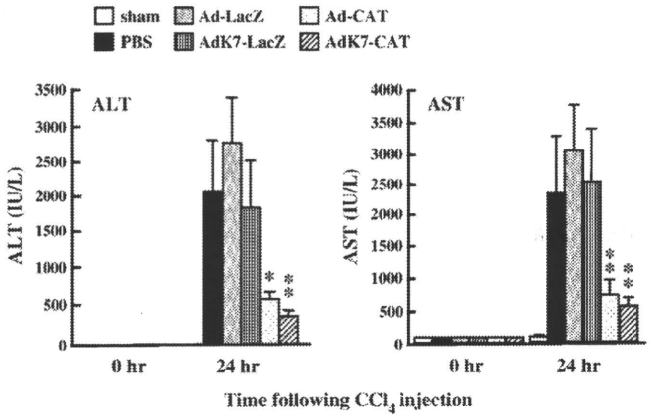

Fig. 6. Preventive effect of catalase-expressing Ad vectors on CCl_4-induced acute liver failure. CCl_4 (1 ml/kg) was intraperitoneally injected to mice 48 h following Ad vector injection. Serum samples were collected 24 h after CCl_4 administration. The data are expressed as the means ± S.D. ($n = 4$). *Significantly different from the PBS-injected group at $p<0.05$; ** at $p<0.01$.

3.5. Improvement of survival rates of mice subjected to partial hepatectomy and hepatic ischemia/reperfusion by catalase-expressing Ad vectors

To examine whether over-expression of catalase improves the remnant liver function in mice subjected to both partial hepatectomy and I/R treatment, partial hepatectomy and subsequent I/R treatment were conducted 48 h after pre-administration of Ad vectors. Partial hepatectomy is often performed under hepatic ischemia, and the remaining liver suffers from I/R injury after partial hepatectomy in clinical settings. Mice pre-administered with AdK7-CAT showed a dramatic improvement in survival rate (Fig. 7). Seventy percent of mice survived for 7 days after these treatments. The body weights of the mice pre-injected with AdK7-CAT were not significantly reduced 7 days after surgery, compared with those before surgery (data not shown), suggesting that the general health of the mice was not substantially compromised after the surgery. On the other hand, the survival of the mice was not prolonged by pre-administration of Ad-LacZ or PBS. These results indicate that catalase-expressing Ad vectors are able to protect the liver from more serious stress induced by partial hepatectomy and I/R, and to improve the remnant liver function.

4. Discussion

Hepatotoxins, drugs, and I/R can injure the liver via oxidative stress. With the aim of efficiently preventing oxidative stress-induced hepatic injury, we pre-administered Ad vectors expressing an antioxidative enzyme, catalase, to mice. The results of our study demonstrated that Ad vector-mediated over-expression of catalase in the liver effectively prevents hepatic injury caused by not only I/R but also CCl_4. Furthermore, the survival rates of mice subjected to both partial hepatectomy and I/R treatment were prolonged by over-expression of catalase.

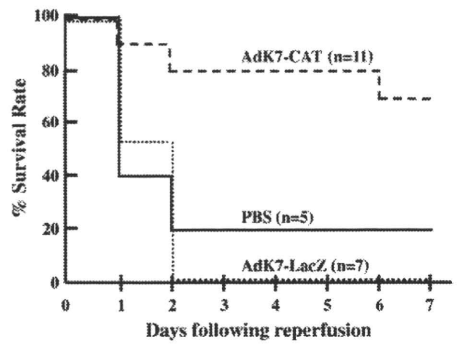

Fig. 7. Survival rates of mice subjected to partial hepatectomy and hepatic I/R following Ad vector administration. Solid line: PBS ($n = 5$); dotted line: AdK7-LacZ ($n = 7$); dashed line: AdK7-CAT ($n = 11$). Ad vectors were intravenously administered to mice as described in Fig. 4. Forty-eight hours after Ad vector administration, mice were subjected to two-thirds partial hepatectomy, followed by an 8 min-period of ischemia

Superoxide anion is the primary oxidant species generated during hepatic I/R by a xanthine oxidase system and/or decoupling of the electron transport system in mitochondria. Superoxide anion is readily converted to H_2O_2 by SOD or a spontaneous reaction. H_2O_2 itself is a weak oxidizing agent; however, hydroxyl radical is produced by H_2O_2 in the presence of transition-metal ion. Hydroxyl radical has the most oxidative ability and the strongest toxicity among the various ROS. Reduction/elimination of hydroxyl radical is considered to be the most effective strategy for prevention of hepatic I/R injury. Therefore, catalase, which prevents generation of hydroxyl radical by converting H_2O_2 to H_2O and O_2, was selected as the antioxidant enzyme in the present study. Catalase derivatives also exhibited higher preventive effects on the elevation of serum ALT and AST levels induced by hepatic I/R, compared with SOD derivatives [5,6]. In addition, over-expression of catalase in the liver might increase endogenous expression of SOD, which is another advantage of catalase gene transfer. He et al. demonstrated that delivery of catalase gene alone to the liver induced SOD activity in the liver [17].

Partial hepatectomy is often performed under hepatic ischemia. Previous studies have shown that oxidative stress induced by hepatic I/R affects hepatocyte cell death and inhibits liver regeneration [42,43]. Beyer et al. reported that ROS are directly responsible for the impairment of insulin/insulin-like growth factor 1 signaling, which is crucial for liver regeneration [44]. Furthermore, hepatocytes without catalase activity have been found in regenerating livers after partial hepatectomy [45], suggesting that hepatocytes in the regenerating livers might be susceptible for ROS-mediated injury. The present study demonstrated that over-expression of catalase in the liver dramatically improved the survival rates of mice subjected to partial hepatectomy and I/R, suggesting that the remnant livers would be protected from ROS-mediated injury by over-expression of catalase. Furthermore, over-expression of catalase might play an important role in maintenance of the regenerative capacity of hepatocytes. Recently, removal of ROS by antioxidant enzymes was demonstrated to be crucial for maintenance of the self-renewal capacity of progenitor/stem cells in an *in vitro* culture system [46,47]. In this study, the liver/body weight ratio of mice pre-injected with AdK7-CAT 1 week after partial hepatectomy and I/R was 4.7 ± 0.55%, which is not significantly different from that of naïve mice (data not shown).

Oxidative stress is also generated in the liver through metabolism of a variety of drugs, chemicals, and toxins, such as thioacetamide, lipopolysaccharide, and CCl_4. In particular, CCl_4 is often used as a representative hepatotoxin causing oxidative stress in animal experiments. CCl_4 is metabolized by cytochrome P450 in the endoplasmic reticulum of hepatocytes, leading to generation of CCl_3 radical, which induces hepatic damages, although the mechanism of CCl_4-mediated liver damage has not yet been fully revealed. The present study showed that Ad vector-mediated over-expression of catalase in the liver also attenuated CCl_4-induced liver injury (Fig. 6). Over-expression of SOD has also been shown to inhibit CCl_4-induced hepatic damages [48]. Hepatic delivery of genes encoding ROS-deleting enzymes is effective in case of hepatic injury induced by oxidative stress-generating hepatotoxins and chemical compounds.

Ad vectors offer various advantages for gene delivery to the liver; however, systemic administration of Ad vectors often induces inflammatory cytokine production and hepatic damage [24,49–51]. However, we found no apparent Ad vector-induced damages in the liver in this study (data not shown). Moreover, mice pre-administered Ad-LacZ or AdK7-LacZ did not exhibit higher levels of serum ALT and AST after I/R than those pre-administered PBS (Fig. 4). This was likely