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Establishment of novel cell lines latently infected with human immunodeficiency virus 1

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Summary. – Many human immunodeficiency virus 1 (HIV-1) researchers focus on the developing new anti-reservoir therapy to eradicate HIV-1 provirus from the HIV-1-infected patients. HIV-1 provirus is the major obstacle for effective HIV-1 treatment because it integrates into the host genome and can produce a virus progeny after stopping highly active antiretroviral therapy (HAART). We established two novel cell lines latently infected with HIV-1 by limiting dilution cloning of A3.01 cells infected with HIV-1. Analysis of the flanking sequence of HIV-1 proviral DNA integrated into chromosomal cellular DNA revealed that proviral DNA was inserted into different sites of different chromosomes in the two examined cell lines. In these lines, virus reactivation could be induced by a phorbol 12-myristate 13-acetate (PMA) treatment that resulted in a marked increase of the production HIV-1 p24 antigen and appearance of the infectious virus. The novel cell lines latently infected with HIV-1 represent further tool for the study of molecular mechanisms of viral latency and development of anti-reservoir therapy of HIV-1 infection.

Keywords: HIV-1 provirus; latency; NCHA cell lines; HIV integration; reactivation

Introduction

Latently HIV-1 infected resting memory CD4+ T cells have been reported as the critical source for HIV-1 proviral reactivation and rebounding of the viral load after stopping HAART therapy (Chun *et al.,* 1999). Many HIV-1 studies are focusing on the development of a new anti-reservoir therapy to eradicate provirus from the HIV-1 infected patients, what is a major obstacle for the effective treatment (Han *et al.,* 2007). The mechanisms involved in HIV-1 latency are very complex and multifactorial (Lassen *et al.,* 2004). The major obstacles for current HIV-1 latency studies are difficulties to isolate latently HIV-1 infected CD4+ T cells from uninfected resting CD4+ T cells and a very low frequency of latently HIV-1 infected cells *in vivo*, e.g. less than 1 in 10^6 of resting CD4+ T cells (Finzi *et al.,* 1997). Moreover, there are a few available latently HIV-1-infected cell lines such as ACH2 (Folks *et al.,* 1989), J1.1 (Perez *et al.,* 1991), and U1 (Folks *et al.,* 1987).

To expand the number of cell lines useful for study of the HIV-1 latency mechanism, we attempt to establish novel cell lines latently infected with HIV-1 having more stable latent state and higher reactivation efficiency. Further, we analyzed them for HIV-1 proviral integration sites by inverse PCR (Han *et al.,* 2004).

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Abbreviations: HAART = highly active antiretroviral therapy; HIV-1 = human immunodeficiency virus-1; MOI = multiplicity of infection; NCHA = novel chronic HIV-1 infected A3.01-derived cells; PMA = phorbol 12-myristate 13-acetate

Materials and Methods

Cell cultures and establishment of the new cell lines. A3.01 cells as parental uninfected cell lines, ACH2 cells as latently HIV-1 infected cell lines, and HIV-1 pNL4-3 strain were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 5% penicillin–streptomycin, and 2 mmol/l glutamine (37°C, 5% CO_2) and maintained at a concentration of 1×10^6 cells/ml. The A3.01 cells were infected with HIV-1 pNL4-3 strain at the multiplicity of infection (MOI) of 0.3 at 37°C. After 1hr exposure to the virus, a fresh culture medium was added. The number of live cells was counted using the cell counting kit-8 (Dojindo Molecular Technologies). Latently HIV-1 infected cells from A3.01 cells were cloned by limiting dilution, when the viability of HIV-1 infected cells was less than 5% at 50 days post infection. Total genomic DNA was isolated using Blood & Cell Culture DNA kit (Qiagen) and the HIV-1 integration into the host genome was identified using HIV-1 Gag PCR with GeneAmplimer® HIV-1 Control Reagents (Applied Biosystems).

Inverse PCR for HIV-1 integration sites. The HIV-1 integration sites were analyzed according to the Han's inverse PCR method (Han *et al.,* 2004). Genomic DNA obtained from the cells was digested with *Pst* I (New England Biolabs). DNA fragments were ligated using T_4 DNA ligase (Roche Diagnostics) and the HIV-1 flanking sequence on circular DNA was amplified by PCR using two sets of primers containing LTR and Gag sequences. PCR bands were eluted and sequenced using the ABI PRISM 3730xl DNA analyzer and BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

PMA induction assay. Latently HIV-1-infected ACH2 cells were used as a positive control and the parental uninfected cell line A3.01 cells as a negative control. All the cell lines were washed twice with PBS to remove the p24 antigen present in the supernatants and PMA (Sigma) was applied at a concentration of 20 ng/ml to stimulate 1×10^5 cells. The supernatants were collected at 0, 24, 48, and 72 hrs after PMA treatment to measure the concentration of p24 antigen secreted by each cell line. The concentration of p24 antigen was measured using an HIV-1 p24 antigen capture assay kit (Vironostika®, Biomerieux) according to the manufacturer´s protocol.

Re-infectivity assay with HIV-1 progeny. A3.01 cells were used to investigate HIV-1 infectivity of the progeny generated by latently HIV-1-infected cell lines. The p24 antigen needed as a positive control was obtained from the A3.01 cells infected at MOI of 1. The number of target cells was 2×10^5 and the amount of p24 antigen was 10 ng. The volume of cell supernatant from each cell line was calculated and added to the A3.01 cells. The supernatants were collected 0, 24, 48, and 72 hrs after addition of p24 antigen and the concentration of p24 antigen was measured by HIV-1 p24 antigen capture assay kit.

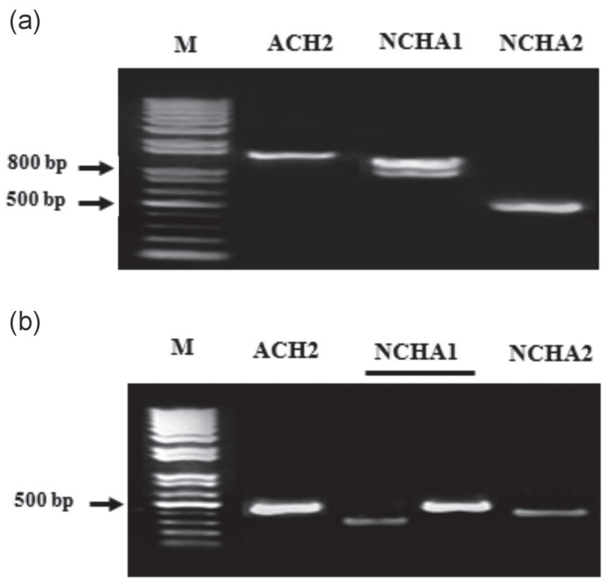

Fig. 1

Identification of integration sites of HIV-1 proviral DNA in NCHA 1, 2 cell lines

(a) Amplification of HIV-1 integration sites by inverse PCR and (b) direct HIV-1 DNA PCR. M is 100 bp DNA ladder.

Results and Discussion

This research was focused on the establishment of new cell lines related to HIV-1 latency. We established latently infected NCHA 1, 2 cell lines by limiting dilution cloning after the infection of A3.01 cells with HIV-1 pNL4-3 strain. The A3.01 cells are human T-cell line developed for the study of AIDS-associated retrovirus, which mimic normal peripheral blood lymphocytes in susceptibility to the viral cytopathic effect without cell activation or conditioned medium (Folks *et al.,* 1985). As HIV-1 integration into the host chromosome is a critical step for HIV-1 life cycle, HIV-1 integration assay is very important for the understanding of HIV-1 latency mechanisms (Vatakis *et al.,* 2007). Several methods have been used for the HIV-1 integration assays as follows: inverse PCR, Alu-gag PCR, and DNA walking PCR with linker (Liszewski *et al.,* 2009).

Inverse PCR described by Han *et al.* (2004) was used to warrant the accuracy of HIV-1 integration assay for NCHA cell lines. The optimal initial amount of genomic DNA extracted from cell lines was 50 μg in the inverse PCR. Genomic DNA was digested with PstI (New England Biolabs) at 37°C for 8 hrs and ligated using T4 DNA ligase (Roche Diagnostics) at 16°C overnight, and inactivated at 70°C for 10 mins and at 4°C for 5 mins. To analyze the junction sequences between the 5′-end of the HIV-1 viral genome and host cell DNA, a circular DNA was used for the first inverse PCR with outer LTR and *gag* primers reported by Han *et al.* (2004). The circular DNA was amplified with PCR mixture containing EF-Taq PCR buffer (SolGent), dNTP (10 mmol/l), each 10 pmol first inverse PCR primers, and EF-Taq polymerase (SolGent). PCR conditions for the first inverse PCR were

Table 1. HIV-1 proviral integration sites in NCHA cell lines

Cell line	Accession No.	Junction sequence[a]	Chromosome locus	Gene	Characteristics	Orientation	Integration site
A 3.01	ND	ND	ND	ND	ND	ND	
ACH2	NM 016489	TTCCAATTAC	7 p14.3	NT5C3	5′-nucleotidase, cytosolic III	+	33025924
NCHA1	NT022184	TTCCAGCTGC	2 p16.3	BC007278	UN	–	48258174
	UN	TTCCAAAAGA	X p11.4	UN	UN	+	38555660
NCHA2	UN	TTCCACTTTT	11q13.1	AK096155	cell cycle checkpoint protein Mrad9	+	66851424

The host nucleotide number at the junction was determined using the UCSC Bioinformatics Human Genome Database (http://www.genome.ucsc.edu). [a]First five nucleotides of 5′-end of the HIV-1 LTR followed by those of host cell DNA. ND = not detected; UN = unknown.

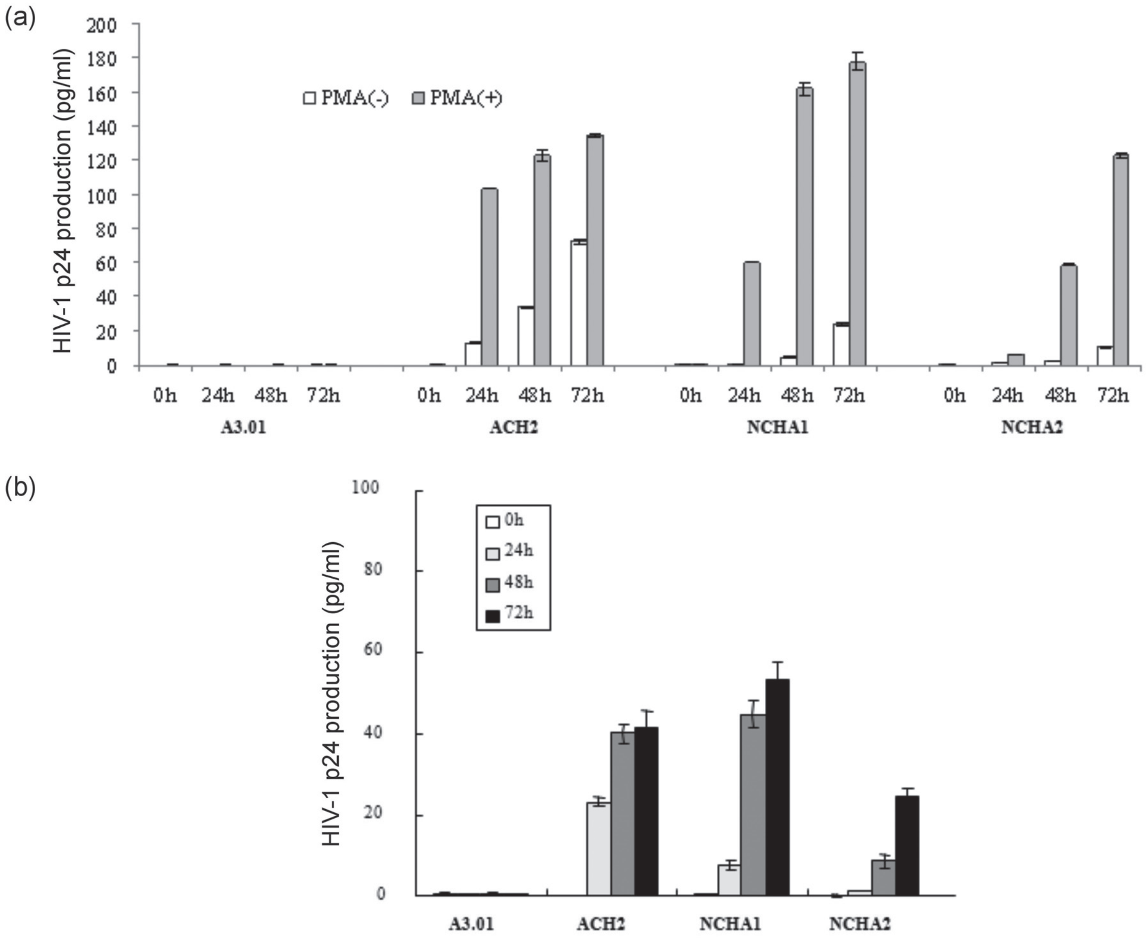

Fig. 2

Virus reactivation in NCHA 1, 2 cell lines following PMA treatment

Detection of virus reactivation based on viral antigen p24 (a) and virus infectivity (b). The white and gray filling shows the amounts of p24 antigens before and after PMA treatment, respectively. A3.01 and ACH2 represent negative and positive control, respectively. Data are representative of three independent experiments. The bars indicate ± SD from mean.

3 mins of pre-denaturation at 94°C, 30 cycles of 30 secs at 94°C, 1 min at 65°C, 2 mins at 68°C, and 4 minutes final extension at 68°C. Second inverse PCR was carried out with the inner LTR and *gag* primers reported by Han *et al.* (2004) and PCR conditions were the same as in the first inverse PCR. The PCR products were analyzed on 1% agarose gel electrophoresis, eluted, and then directly sequenced (Fig. 1a). In addition, HIV-1 DNA PCR was carried out with new prim-

ers based on the flanking sequences obtained by the inverse PCR as follows (Fig. 1b): NT5C3 gene primer, 5′-GCCC TTTAGACTCTCTAACCCATTAC-3′ for ACH2 cells, BC007278 gene primer, 5′-GGAGCCATGTGCTAATACAC TTTAG-3′, and unknown gene primer, 5′-GAGCATTCA GT TTGAAACTGGACATGA-3′ for NCHA1 cell line, AK096155 gene primer, 5′-GTGATGGGATTGCTGATTCAGACT TT-3′ for NCHA2 cell line.

The chromosomal locus and genes for HIV-1 integration sites of latently infected NCHA 1, 2 cell lines were identified using the UCSC Bioinformatics Human Genome Database (http://www.genome. ucsc.edu). Consequently, HIV-1 proviral integration sites for ACH2 cells and NCHA 1, 2 cell lines were very unique as follows (Table 1): NT5C3 gene on chromosome 7 for ACH2 cells, BC007278 gene on chromosome 2 and unknown gene on chromosome X for NCHA1 cell line, and AK096155 gene on chromosome 11 for NCHA2 cell line. ACH2 cells have been already reported to contain a single copy of HIV-1 provirus (Poli *et al.,* 1990). Ishida *et al.* (2006) firstly identified the flanking sequence of HIV-1 provirus integrated into ACH2 cells by inverse PCR and this sequence corresponded to the sequence of NT5C3 gene on the chromosome 7 by NCBI blast program. Our investigation of ACH2 cells was also consistent with their results. It suggested that our HIV-1 integration assay for NCHA cell lines was accurate and the cell lines harboring integrated HIV-1 proviral DNA were established. It has been reported that HIV-1 integration sites in resting CD4+ T cells from infected individuals and latently HIV-1 infected cell lines were generally located within the introns of active cellular genes (Schroder *et al.,* 2002). In this HIV-1 integration assay we also found that the majority of the integration sites for NCHA cell lines were within the introns of the detected cellular genes.

Next, we examined whether the NCHA cell lines could be reactivated by PMA stimulation and were able to produce intact HIV-1 progeny. Both NCHA cell lines showed the increased HIV-1 p24 antigen production after the PMA treatment similar to ACH2 cells (Fig. 2a). However, their patterns of HIV-1 replication kinetics were different from ACH2 cells. The expression of HIV-1 p24 antigen without PMA treatment was lower in NCHA cell lines than in ACH2 cells that showed rapidly increased levels of antigen with time (Fig. 2a). However, reactivation of HIV-1 provirus caused by PMA treatment in both NCHA cell lines was more effective than in ACH2 cells after 72 hrs. Both PMA-treated NCHA cell lines showed 6-12 times higher p24 antigen expression, whereas PMA-treated ACH2 cells showed only about 2 times higher expression. To investigate HIV-1 infectivity of the progeny generated by NCHA cell lines, the same concentration of progeny from each cell line was added to the A3.01 cells. HIV-1 progeny from NCHA cell lines showed different infectivity than that from ACH2 cells (Fig. 2b). These results showed that HIV-1 progeny from reactivated NCHA cell lines was intact HIV-1 virus and NCHA cell lines may be more stable than ACH2 cells due to the lower p24 expression in the non-stimulated state (Jordan *et al.,* 2003).

Taken together, the novel NCHA cell lines could be a useful tool in the understanding of molecular mechanism of viral latency and in the development of anti-reservoir therapy.

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