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Biological Characterization of HIV-1 Isolates from Long-term non-progressors (LTNP) and Rapid Progressors (RP) in Korea

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=Abstract=

To analyze the correlation between biological phenotypes of HIV-1 isolates and disease progression, we selected 9 long-term non-progressors (LTNP) and 12 rapid progressors (RP) from HIV-1 infected Korean. We isolated HIV-1 isolates by culture of PBMC of LTNP and RP with normal PBMC and measured HIV-1 p24 antigen production. The HIV-1 isolation rate from LTNP was 55.6% (5/9). And 4 HIV-1 LTNP isolates were non-syncytium inducing (NSI) phenotype and showed slow/low replication. The HIV-1 isolation rate from RP was 91.7% (11/12) which was higher than that from LTNP. Besides 3 RP HIV-1 isolates which showed syncytium inducing (SI) phenotype, 8 RP HIV-1 isolates showed NSI phenotype in normal PBMC and MT-2 cell line. All RP HIV-1 isolates replicated more rapidly than LTNP HIV-1 isolates. Comparing the replication kinetics and syncytium forming capacity of HIV-1 isolates from LTNP and RP, we suggest that the difference of biological phenotype of HIV-1 isolates could be related with disease progression of HIV-1 infected persons.

Key Words: Long-term non-progressor (LTNP), Rapid progressor (RP), Syncytium inducing (SI) phenotype, Non-syncytium inducing (NSI) phenotype

INTRODUCTION

Approximately half of HIV infected individuals are known to develop acquired immunodeficiency syndrome (AIDS) within 10 years after seroconversion [33, 36]. AIDS is the final stage of infection with human immunodeficiency virus (HIV) [28], and the consequence of depletion of CD4+ T lymphocytes counts and immune dysregulation [12, 32]. However, the clinical course of HIV-1 infection varies owing to differences in the depletion rate of CD4+ T lymphocytes counts in different patients [9, 33, 36]. In a small proportion of HIV-1 infected persons (approximately 5%), long-term non-progressors (LTNP), both the clinical and immunological states remain normal for more than 10 years [23, 29]. On the other hand, rapid progressors (RP), a rapid decline in CD4+ T lymphocytes (depletion of 200~400 cells/mm^3 of CD4+ T lymphocyte counts per year) and disease progression are observed in small group of patients [7]. Such variability is multifactorial [37] and may be influenced by the virulence of the virus itself [1,

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6] and host immunopathological response [47].

HIV-1 strains can be divided into two phenotypes, syncytium inducing (SI) phenotype and non-syncytium inducing (NSI) phenotype [1, 6, 14, 44]. NSI strains (mainly slow/low isolates [S/L]) are present throughout the asymptomatic phase [1, 42], but subsequently, rapid/high (R/H) replicating SI variants emerge during the course of infection in 50 to 60% of HIV-1 infected individuals, and this is related to rapid CD4+ T lymphocytes depletion and progression to AIDS [25]. The cross-sectional and longitudinal studies have indicated that disease progression is strictly associated with an increasing viral load and viral activity in plasma, peripheral blood mononuclear cells (PBMC), and lymph node mononuclear cells (LNMC) [20, 40].

Although most HIV-1 infected individuals are initially infected with NSI, monocyte-tropic viral strains, the onset of disease has been shown to correlate with the appearance of SI, T-cell tropic viruses [1, 6, 25, 34, 42~45]. Several studies have indicated that differences in the amino acid sequence of gp120 plays an important role in determining the viral phenotype and pathogenesis of HIV-1. These studies have identified the V3 loop region of gp120 as primary determinant of cell tropism and viral phenotype [10, 15, 18, 45]. An increase in the positive charge density of the V3 loop [15, 27, 35], coupled with changes in the V2 domain, has been suggested to cause the viral phenotype to change from NSI to SI [16].

MT-2 cell line, HTLV-I-transformed T-cell line is mainly used to differentiate biological phenotype of HIV-1 isolates between SI and NSI. When HIV-1 infects MT-2 cells, MT-2 cells show very sensitive and specific cytopathic effect, the formation of syncytium [26].

Because each countries has different HIV-1 isolates prevailing and different racial backgrounds, the natural history of HIV-1 infection is various. To reason why they differ in virological point, in this study we aimed to understand the correlation between the biological phenotypes of HIV-1 isolates and disease progression in LTNP and RP by isolating and characterizing HIV-1 isolates from Korean.

MATERIALS AND METHODS

Subjects

We selected 9 long-term non-progressors (LTNP) and 12 rapid progressors (RP) from HIV-1 infected persons and AIDS patients in Korea. A LTNP was defined as a HIV-1 infected person who has maintained clinically healthy and immunologically normal condition (CD4+ T lymphocytes: > 500 cells/mm^3) more than 7 years and no history of taking antiviral drugs. A RP was defined as a AIDS patients whose CD4+ T lymphocytes has decreased less than 200 cells/mm^3 within 5 years. Table 1 shows the epidemiological data of LTNP and RP.

Isolation and culture of HIV-1

1. Separation of PBMC from HIV-1 infected persons and AIDS patients

Peripheral blood mononuclear cells (PBMC) were separated from blood in heparinized tube within 24 hrs after bleeding. After gentle mixing, the heparinized blood was centrifuged at 2,500 rpm for 10 min. Plasma were aliquoted and stored in -70℃ deep freezer. The lower phase diluted with PBS was overlayed onto Histopaque-1077 (Sigma, USA) and centrifuged gently at 2,000 rpm for 30 min. Buffy coat layer was harvested and washed three times with PBS by centrifugation at 1,800 rpm for 10 min. The lymphocytes counts were measured by Coulter counter (Coulter T-660, Coulter Co., USA). 5×10^6 cells of PBMC suspended in 1 ml of freezing medium (90% FCS, 10% DMSO) were rapidly frozen in freezing container (Nalgene Cryo 1 C, USA) at -70℃ for further isolation and culture of HIV-1.

2. Stimulation of normal PBMC

The leucocyte concentrate (LC) from donor who is HIV antibody negative and HBV antigen negative was purchased from Central

Table 1. Epidemiological data of LTNP and RP

Subjects	Gender	Age*	Mode of transmission	Place of infection	Subtype
LKNIH 1	Male	37	Heterosexual	Outside Korea	ND
LKNIH 2	Male	37	Heterosexual	Outside Korea	C
LKNIH 3	Male	47	Heterosexual	Outside Korea	A
LKNIH 4	Male	40	Heterosexual	Korea	B
LKNIH 5	Male	32	Heterosexual	Korea	ND
LKNIH 6	Male	28	Homosexual	Korea	B
LKNIH 7	Male	32	Heterosexual	Outside Korea	ND
LKNIH 8	Male	40	Heterosexual	Outside Korea	ND
LKNIH 9	Male	44	Heterosexual	Outside Korea	ND
RKNIH 1	Male	28	Homosexual	Korea	B
RKNIH 2	Male	35	Heterosexual	Outside Korea	G
RKNIH 3	Male	34	Heterosexual	Outside Korea	B
RKNIH 4	Male	32	Heterosexual	Korea	B
RKNIH 5	Male	31	Heterosexual	Korea	B
RKNIH 6	Male	34	Heterosexual	Outside Korea	A
RKNIH 7	Male	31	Heterosexual	Korea	ND
RKNIH 8	Female	26	Transfusion	Korea	ND
RKNIH 9	Male	36	Heterosexual	Korea	ND
RKNIH 10	Male	44	Heterosexual	Korea	ND
RKNIH 11	Female	26	Heterosexual	Korea	ND
RKNIH 12	Male	52	Heterosexual	Korea	B

*Age at the sample collection, ND: not done

Blood Transfusion Service, Korea National Red Cross. The leucocyte concentrate diluted with PBS was overlayed onto Histopaque-1077 and centrifuged gently at 2,000 rpm for 30 min. Buffy coat layer was harvested and washed three times with PBS by centrifugation at 1,800 rpm for 10 min. The lymphocyte counts were measured by Coulter counter. 2×10^6 cells/ml of normal PBMC were stimulated with phytohemagglutinin (PHA)-p stimulation medium in 5% CO_2 humidified incubator at 37℃ for 48~72 hrs. PHA-p stimulation medium was RPMI-1640 (Gibco BRL) supplemented with 2μg/ml of PHA-p, 2μg/ml of polybrene (Sigma, USA), 2mM/ml of L-glutamine, penicilin (100 unit/ml)-streptomycin (100μg/ml) and 20% FCS.

3. Isolation and culture of HIV-1

RPMI-1640 (IL-2 medium) supplemented with 5% natural human T cell growth factor (TCGF, IL-2; Cellular Products Inc., USA), 2μg/ml of polybrene, 2mM/ml of L-glutamine, penicilin (100unit/ml)-streptomycin (100μg/ml) and 20% FCS was used for HIV-1 isolation. Using warm water at 37℃, frozen PBMC from subjects was rapidly thawed and washed with PBS by centrifugation. PBMC from subjects of this study was cultured with 5×10^6 cells of normal PBMC stimulated with PHA-p stimulation medium. Every week culture supernatant and cells were harvested and stored at -70℃ while PHA-p stimulated normal PBMC in fresh IL-2 medium were replaced to continue the culture for HIV-1 isolation [17]. HIV-1 isolation was confirmed by measurement of HIV-1 p24 antigen in culture supernatant. After confirmation of HIV-1 p24 antigen production, the culture of HIV-1 isolates was continued with 2×10^7 cells of normal PBMC stimulated with PHA-p. When the absorbance of the culture supernatant was over 4.0 by HIV-1 p24 antigen detection ELISA [30], we stored cells and culture supernatant in -70℃ for characterization of bio-

Table 2. HIV-1 isolation and characterization of biological phenotypes of HIV-1 isolates from LTNP

Subjects	Sample collection		Culture with normal PBMC	Biological phenotype in MT-2 cells (days)*
	Time after infection (months)	CD4+ T cell (mm^3)	HIV-1 p24 antigen (weeks)*	
LKNIH 1	104	1,364	− (10)	
LKNIH 2	129	566	+ (1)	NSI (39)
LKNIH 3	110	697	− (10)	
LKNIH 4	85	605	− (10)	
LKNIH 5	93	530	− (10)	
LKNIH 6	94	593	+ (1)	NSI (34)
LKNIH 7	88	401	+ (1)	NSI (39)
LKNIH 8	101	839	+ (2)	NA
LKNIH 9	118	634	+ (1)	NSI (34)

Note. *: period of detection; NSI: non-syncytium inducing; NA: not available

logical phenotype and genetic analysis. If HIV-1 p24 antigen was not detected, culture with PHA-p activated normal PBMC was continued for 5～10 weeks. In case HIV-1 p24 antigen was not detected even after 5～10 weeks culture, we concluded that HIV was not isolated from the subject.

4. HIV-1 p24 antigen detection ELISA

HIV-1 p24 antigen was measured by HIV-1 p24 antigen ELISA test system (Coulter, USA). Cell culture supernatant was assayed for HIV-1 p24 antigen ELISA test after removal of cells by centrifugation at 13,000 rpm for 5 min. 200μl of cell culture supernatant, negative control, and positive control were added to each well of 96 wells microwell plate coated with murine monoclonal antibody against HIV-1. To quantify HIV-1 p24 antigen, positive control was diluted with specimen diluent to the concentration of 100, 50, 25, 12.5, and 0 pg/ml. After addition of 20μl of lysis buffer to each wells, the plate was covered with cover foil and incubated at 37℃ for 1hr. The plate was washed 6 times with washing solution. 200μl of anti-HIV-1 (human) biotin reagent was added to each well. The plate was covered with cover foil and incubated at 37℃ for 1hr. After washing 200μl of streptavidin-horseradish peroxidase conjugate was added and the plate was incubated for 30 min at 37℃. After washing again 200μl of tetramethylbenzidine substrate was added to each well and stored in dark place for 30 min at room temperature. As soon as the reaction was stopped by addition of 50μl of 4N sulfuric acid, absorbance was measured by ELISA reader (Molecular Devices) at 450nm for test wavelength with 550nm for reference.

Analysis of biological phenotypes of HIV-1 isolates

1. Cell line

MT-2 cell line is Human T cell line which harbors HTLV-I. MT-2 cell line which forms syncytium by HIV-1 infection was donated from Japan National Institute of Infectious Diseases. MT-2 cells were cultured in RPMI-1640 supplemented with 10% FCS, penicilin (100unit/ml)-streptomycin (100μg/ml) and L-glutamine (2mM/ml). Cells were passaged every 3～4 days and maintained $3.0 \sim 4.0 \times 10^5$ cells/ml in 5% CO_2, 37℃ humidified incubator.

2. Analysis of biological phenotype

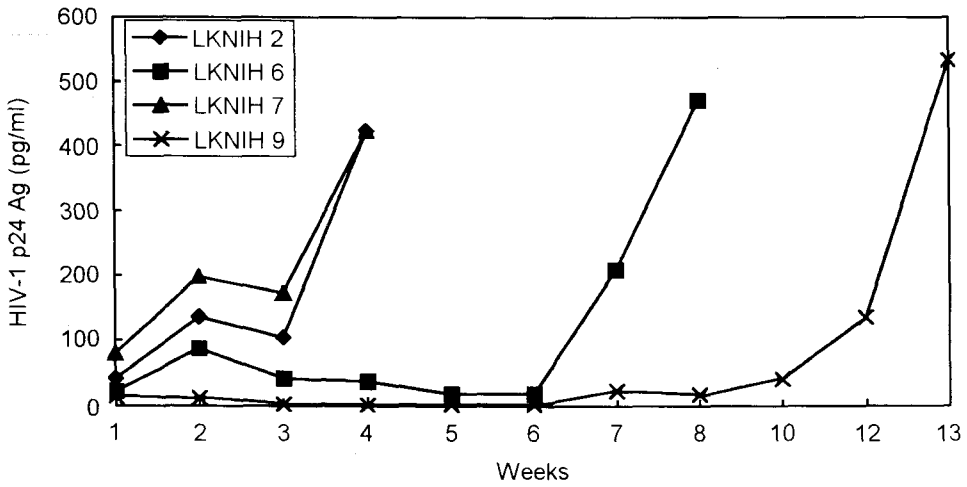
By the ability of syncytium formation, HIV-1 can be grouped to syncytium inducing (SI) phenotype and non-syncytium inducing (NSI) phenotype. The biological phenotypes of HIV-1 isolates were examined under a inverted microscope by cocultivation of PBMC of subjects with normal PBMC. Also biological phenotypes of HIV-1 isolates were examined in MT-2 cells after HIV-1 infection [19]. 1×10^6 cells of MT-

2 cells in 5 ml were infected with 2.5ml of HIV-1 isolates and incubated in 5% CO_2, 37℃ humidified incubator. The culture was passaged every 3～4 days and examined the formation of syncytium. By measurement of HIV-1 p24 antigen in culture supernatant, replication kinetics of HIV-1 was quantified.

RESULTS

Biological characterization and phenotypes of HIV-1 isolates from LTNP

We isolated and characterized HIV-1 isolates from 9 persons who belonged to LTNP (LKNIH 1 - LKNIH 9). Sample was collected at 85～129 months (mean 101 months) after suspected infection date and CD4+ T lymphocyte counts were 401～1,354/mm^3 (mean 605/mm^3). During culture of PBMC from LTNP and normal PBMC, we tested the culture supernatant for the production of HIV-1 p24 antigen by HIV-1 p24 antigen detection ELISA every week. HIV-1 was isolated from 55.6% of LTNP (5/9) after 2 weeks culture. But it was impossible to isolate HIV-1 from 4 persons even after 10 weeks culture (Table 2).



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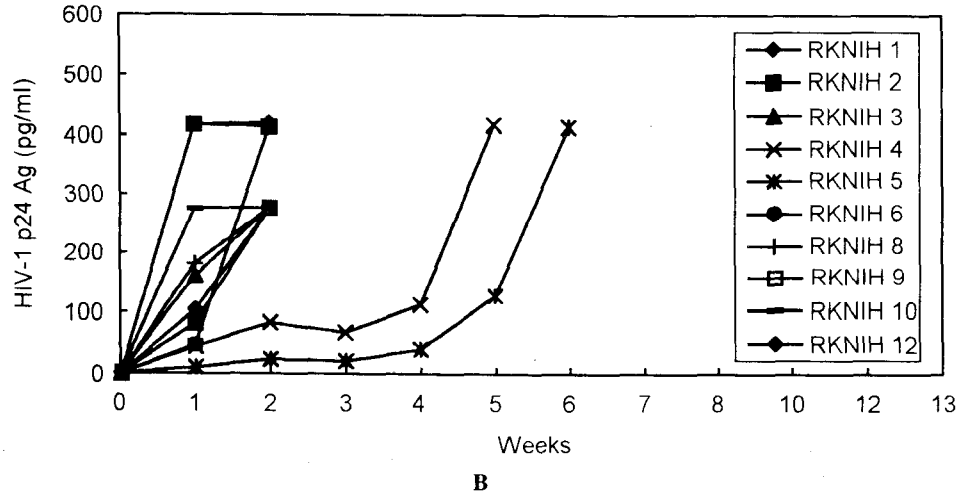

B

Figure 1. The replication kinetics of HIV-1 isolates from LTNP (**A**) and RP (**B**) cultured with normal PBMC.

In Figure 1 (A), the absorbance of culture supernatant of isolate LKNIH 2 and LKNIH 7 reached to higher than 4.0 on the third week. The absorbance of culture supernatant of isolate LKNIH 6 and LKNIH 9 reached to higher than 4.0 on the 7th week and the 12th week, respectively. For biological phenotype, 4 HIV-1 isolates LKNIH 2, LKNIH 6, LKNIH 7, and LKINH 9 did not form syncytium after culture for 34-39 days and were determined as NSI. As time went by, the amount of HIV-1 p24 antigen produced in MT-2 cell line by HIV-1 isolates LKNIH 2, LKNIH 6, and LKNIH 7 decreased. HIV-1 isolate LKNIH 8 showed weak positive result on the second week by HIV-1 p24 antigen detection ELISA. But it turned into negative and we did not analyze it further.

Biological characterization and phenotypes of HIV-1 isolates from RP

We isolated and characterized HIV-1 isolates from 12 persons who belonged to RP (RKNIH 1 - RKNIH 12). Sample was collected at 11～73 months (mean 41.5 months) after suspected infection date, and CD4+ T lymphocytes counts were 1～251/mm^3 (mean 133.5/mm^3). 50% of RP subjects (6/12) had taken AZT for AIDS therapy. During culture of PBMC from RP with normal PBMC, we tested the culture supernatant every week for HIV-1 p24 antigen by HIV-1 p24 antigen detection ELISA. While HIV-1 was isolated from 91.7% of RP (11/12) after 1 week culture, it was impossible to isolate HIV-1 from RKNIH 7 even after 10 weeks culture (Table 3). In Figure 1 (B), the absorbance of culture supernatant of 8 RP reached to higher than 4.0 on the first week culture. The absorbance of culture supernatant of isolates RKNIH 4 and RKNIH 5 reached to higher than 4.0 on the 4th week and the 5th week, respectively. 3 HIV-1 isolates of RKNIH 1, RKNIH 2, and RKNIH 3 formed syncytium in PBMC and MT-2 cells were determined as SI. Isolates RKNIH 1 and RKNIH 3 formed syncytium on the 7th day of culture and RKNIH 2 formed syncytium on the 4th day. 7 HIV-1 isolates did not form syncytium after the culture for 27-45 days and were determined as NSI. As time went by, the amount of HIV-1 p24 antigen produced by 4 NSI of isolate RKNIH 4, RKNIH 5, RKNIH 9 and RKNIH 12 in MT-2 cells decreased (Figure 2). It is understood as there was no viral replication. Culture of isolate RKINH 11 was contaminated with bac-

Table 3. HIV-1 isolation and characterization of biological phenotype of HIV-1 isolates from RP

Subjects	Sample collection			Culture with normal PBMC		Biological phenotype in MT-2 cells (days)*
	Time after infection (months)	CD4+ T cells (mm^3)	AZT treatment (months)	HIV-1 p24 antigen (weeks)*	Biological phenotype	
RKNIH 1	66	24	8	+ (1)	SI	SI (7)
RKNIH 2	73	2	46	+ (1)	SI	SI (4)
RKNIH 3	64	133	50	+ (1)	SI	SI (7)
RKNIH 4	51	251	0	+ (1)	NSI	NSI (31)
RKNIH 5	40	172	7	+ (1)	NSI	NSI (27)
RKNIH 6	37	144	0	+ (1)	NSI	NSI (34)
RKNIH 7	42	159	0	- (10)		
RKNIH 8	40	138	19	+ (1)	NSI	NSI (45)
RKNIH 9	30	183	0	+ (1)	NSI	NSI (34)
RKNIH 10	69	1	19	+ (1)	NSI	NSI (45)
RKNIH 11	11	49	0	+ (1)	NA	NA
RKNIH 12	41	43	0	+ (1)	NSI	NSI (34)

Note. *: period of detection; SI: syncytium inducing; NSI: non-syncytium inducing; NA: not available

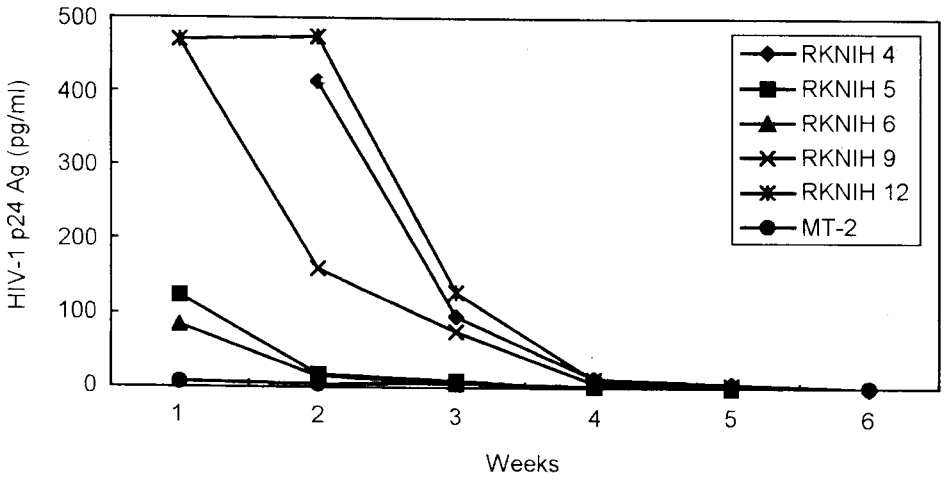

Figure 2. The replication kinetics of RP HIV-1 isolates with NSI phenotype in MT-2 cells.

Table 4. Comparison of HIV-1 isolation rate between LTNP and RP

Groups	Rate of HIV isolation (%)				Total (%)
	1st week*	2nd week	3rd week	4th week	
LTNP (n=9)	4 (44.4)	1 (11.1)	0 (0)	0 (0)	5 (55.6)
RP (n=12)	11 (91.7)	0 (0)	0 (0)	0 (0)	11 (91.7)

*: When the HIV-1 p24 antigen was positive, we identified with HIV-1 isolation

teria and discarded.

Correlation between HIV-1 isolation rate and disease progression

We analyzed the isolation rate of HIV-1 from 9 LTNP and 12 RP according to the progression of disease (Table 4). As the disease progresses, HIV-1 isolation rate became higher. HIV-1 isolation rate was the lowest in LTNP by 55.6% (5/9). HIV-1 was isolated from 4 among 5 LTNP on the first week of culture and from 1 LTNP on the second week. For RP, HIV-1 isolation rate was the higher by 91.7% (11/12). As HIV-1 was isolated from 11 RP on the first week, it represents that HIV-1 isolate from RP replicates very fast.

DISCUSSION

A small population of HIV-1 infected persons and AIDS patients are grouped into LTNP and RP. Among the some criteria to define long-term non-progressors (LTNP) and rapid progressor (RP), we selected the criteria of Lifson *et al.* [31] and Zanussi *et al.* [46] for LTNP and RP, respectively. LTNP are persons who survive longer than 7 years without AIDS symptom, maintain more than 500 cells/mm^3 of CD4+ T lymphocyte count, have no experience of AZT treatment. RP are persons whose CD4+ T lymphocyte count decreases to less than 200 cell/mm^3 within 5 years after infection. Disease progression is considered to be determined by host factors and viral factors. In this study we selected LTNP and RP from HIV-1 infected persons and AIDS patients in Korea and isolated HIV-1 to understand the correlation between biological phenotypes of virus and disease progression. By September 1998, the cumulative number of HIV infected persons including AIDS patients was 844. The previous data of HIV-1 subtypes in Korea

have reported that the nucleotide sequences of *env* and *nef* gene of prevalent HIV-1 subtype B strains was very different with those of other subtype B isolates [21, 24]. Therefore, we needed to understand the natural history of HIV-1 infection of Korean. Even though all of the subjects of this study were not grouped into Korean subtype, we studies the biological phenotype of HIV-1 isolates from LTNP and RP. Because biological difference of HIV-1 isolates is known to play an important role in pathogenesis of AIDS and decrease of CD4+ T lymphocytes count [41, 44]. Viruses isolated from patient with low CD4+ T lymphocytes counts often replicate in MT-2 cell line, and have been named rapid/high, T-cell tropic, or SI [1, 14]. In contrast, viruses isolated early in the course of the disease usually replicate slowly in primary cell culture. These viruses do not infect MT-2 cells and have been called slow/low, macrophage-tropic or NSI [26]. As time goes after infection, SI HIV is detected more frequently. It is very interesting that the switch of biological phenotype from NSI to SI happens even when SI HIV is transmitted [39]. A switch in HIV-1 biological phenotype from NSI/MT-2-negative to SI/MT-2-positive occurs in about 50% of HIV-1 infected individuals who progress to AIDS, usually at a CD4+ T lymphocytes level at around 200-400 cells/mm^3 [22] and appears to be followed by a faster CD4+ T lymphocytes decline and disease progression [38]. In fact, the NSI viral phenotype has been associated with protection against disease progression. There are some reports about the SI virus is linked with an increased prevalence of AIDS [13], a lower CD4+ T lymphocytes count [3, 25, 38], a higher viral load [13] or increased p24 antigenaemia [3], increased β_2-microglobulinaemia [3]. Therefore NSI virus gave a distinct survival advantage over SI virus. In case NSI virus was isolated, we can suppose that the HIV-1 infected person will show slow disease progression. In this study we grouped HIV-1 isolates into syncytium inducing (SI) phenotype and non-syncytium inducing (NSI) phenotype and investigated the characteristics of viral multiplication in two groups by culture with PBMC and MT-2 cells [19].

HIV-1 isolation rate from LTNP was 55.6% (5/9) (Table 2 and 4). In the test of HIV-1 p24 antigen detection, absorbance of culture supernatant of isolate LKNIH 2 and LKNIH 7 reached higher than 4.0 in 3 weeks culture after virus isolation. This rate was a little bit lower than 63% of the previous report [2]. That of isolate LKIH 6 and LKNIH 9 reached same level in 7 weeks and 12 weeks, respectively (Figure 1 (A)). The biological phenotypes of HIV-1 isolates from LTNP was NSI type. The lowest HIV-1 isolation rate, long time culture for virus isolation, slow replication, and NSI type can explain that LTNP survive longer. For RP, HIV-1 isolation rate was 91.7% (11/12) in 1 week culture (Table 3 and 4). Absorbance of culture supernatant of isolate RKNIH 4 and RKNIH 5 reached higher than 4.0 (Figure 1 (B)) in 4 weeks and 5 weeks, respectively. The biological phenotype of isolate RKNIH 1, RKNIH 2, and RKNIH 3 was SI in both normal PBMC and MT-2 cell line. Because HIV-1 isolates from some RP had SI phenotype, they are considered to be highly pathogenic and fast disease progression of RP was explained by their biological characteristics mentioned above. However, other 3 HIV-1 isolates from RP showed rapid growth but showed NSI phenotype. In addition, RKNIH 7 was not able to isolate by 10 weeks culture. Therefore we need further study to understand the factors affected on the HIV growth.

Recent discovery of several chemokine receptors which were also coreceptors for HIV-1 entry into cells has focused interest on these molecules. Macrophage-tropic NSI HIV-1 strains primarily use the CC-chemokine receptor 5 (CCR-5) which is a kind of β-chemokine receptor, as a coreceptor [11]. T-cell-tropic SI HIV-1 strains use CXC-chemokine receptor 4 (CXCR-4), α-chemokine receptor [5]. Thus,

HIV-1 generally uses the CCR-5 as a coreceptor in the early stages of disease and expands its coreceptor use to additionally include CXCR-4 as the disease progresses and a switch from NSI to SI viral phenotype occurs [4, 8].

In order to study characteristics of HIV-1 isolates further, cell tropism and molecular biological analysis of the HIV-1 genes have to be understood intensively. Finally the research about coreceptors of HIV-1 and characterization of HIV-1 isolates related with disease progression has to be performed.

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