Journal of NeuroVirology, 13: 11–22, 2007

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280601086064

informa
healthcare

Simian fetal brain progenitor cells for studying viral neuropathogenesis

Naoko Iwata,[1] Hiroaki Yoshida,[1,2] Minoru Tobiume,[1] Fumiko Ono,[3] Takuya Shimazaki,[4] Tetsutaro Sata,[1] and Noriko Nakajima[1]

[1]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan; [2]Department of Applied Biochemistry, Tokai University, Kanagawa, Japan; [3]Tsukuba Primate Center, National Institute of Biomedical Innovation, Tsukuba, Japan; and [4]Department of Physiology, Keio University School of Medicine, Tokyo, Japan

The pathogenesis of neurologic dysfunctions caused by human immunodeficiency virus type 1 (HIV-1) infection is not yet well understood. Simian immunodeficiency virus (SIV) infection of macaques is an important animal model for HIV-1 infection. This is the first report to characterize brain progenitor cells (BPCs) isolated from embryonic brain of cynomolgus monkeys (*Macaca fascicularis*) by neurosphere assay and utilize BPC-derived cell culture for studying SIV infection. The self-renewal and multilineage differentiation properties of BPCs are convenient for planning viral infection experiments. The BPC-derived culture does not contain macrophage/microglial cells, fibroblasts, or endothelial cells. Thus, this culture is appropriate for studying direct relation between SIV infection and neuronal and glial cells. First, the authors characterized undifferentiated and differentiated simian BPCs by immunocytochemistry, flow cytometry analysis, real-time polymerase chain reaction (PCR), and reverse transcriptase (RT)-PCR. The BPCs induced to differentiate by the addition of 1% fetal bovine serum (FBS) were composed of heterogeneous cells expressing nestin, glial fibrillary acidic protein (GFAP), and/or tubulin beta III isoform (Tuj). None of them expressed the monocyte/macrophage/microglial marker. mRNA expression of CD4, CXCR4, CCR5, GPR1, STRL33, and APJ in both undifferentiated and differentiated BPCs were shown by RT-PCR method, suggesting that SIV would infect and replicate in this culture system. Then, it was confirmed that the neurotropic SIV strain, SIV17/E-Fr, replicated productively in BPC-derived cells. The SIV/17E-FrΔnefGFP was inoculated to identify the infected cells and immunocytochemistry analysis revealed that green fluorescent protein (GFP)-expressing cells were mostly GFAP positive and coexpressed with SIV p27 antigen. Thus, BPC-derived cell culture system is applicable for studying SIV infection in glial and neuronal cells. *Journal of NeuroVirology* (2007) **13**, 11–22.

Keywords: encephalitis; macaque; neurosphere assay; SIV

Address correspondence to Noriko Nakajima, MD, PhD, Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan. E-mail: tenko@nih.go.jp

The authors thank Dr. M. Christine Zink (John Hopkins University School of medicine) for kindly providing pSIV17/E-Fr and appreciate for a critical reading. Fetal brains of Cynomolgus macaque (*Macaca fascicularis*) were kindly provided by the Non-human Primate Reagent and Resource Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation. This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare for AIDS research (16100601).

Received 16 June 2006; revised 29 September 2006; accepted 23 October 2006.

Introduction

Human immunodeficiency virus type 1 (HIV-1) induces encephalitis, dementia, or a milder form of neurologic dysfunction called minor cognitive/motor disorder (MCMD) in the era of highly active antiretroviral therapy (HAART) (Gonzalez-Scarano and Martin-Garcia, 2005). The pathogenesis of HIV encephalitis (HIVE) is not yet well understood. There is a consensus that the principle central nervous system (CNS) cell types productively infected by HIV

are perivasucular macrophages and microglial cells and that the neurological abnormalities are caused by some inflammatory cytokines, chemokines, and viral antigens released from these cells (Fischer-Smith *et al*, 2001; Kim *et al*, 2003). On the other hand, there is no consensus that cells of neural origin (neurons, astrocytes, oligodendrocytes) support a productive infection of HIV-1. With more sensitive detection techniques, such as *in situ* polymerase chain reaction (PCR) (Bagasra *et al*, 1996; Nuovo *et al*, 1994) and microdissection-PCR (Fassunke *et al*, 2004; Torres-Munoz *et al*, 2001), HIV RNA and HIV DNA were detected also in neurons or astrocytes, though so far productive infection in these cells has not yet been reported. It was reported that HIV infections in astrocytes were supposedly restricted to early gene products and were not productive both *in vivo* and *in vitro* infection (Gorry *et al*, 1999; Messam and Major, 2000; Tornatore *et al*, 1994). The contribution of this restricted infection in astrocytes to neuropathogenesis remains unknown. simian immunodeficiency virus (SIV) infection of macaques is an important animal model for studying the neuropathogenesis of HIV infection (Gonzalez *et al*, 2000; Kestler *et al*, 1990; Zink *et al*, 1998). As for SIV infection, neurotropic strains of SIV had been reported by the John Hopkins university laboratory (Flaherty *et al*, 1997; Mankowski *et al*, 1997; Sharma *et al*, 1992; Zink *et al*, 1997). The molecular clone of infectious SIV, SIV/17E-Fr, were shown to replicate productively in primary rhesus macaque astrocytes (Overholser *et al*, 2003, 2005). Primary fetal astrocyte cultures have been generally used for investigating the mechanism of SIV infection in CNS, though it was difficult to establish a culture consisting of more than 99% astrocytic marker–positive cells and the cultures often had the risk of being contaminated with microglial cells, in which SIV might replicate. In addition, they have limited in the number of experiments that can be performed on them. On the other hand, primary neural stem cells (NSCs) and brain progenitor cells (BPCs) isolated from embryonic brains by neurosphere assay have the capacity to proliferate and to differentiate into precursor cells expressing neuronal or glial phenotypes (Reynolds *et al*, 1992; Reynolds and Weiss, 1992). The differentiated BPC cultures do not contain any monocyte/macrophage, microglial and endothelial phenotypes and it is possible to analyze SIV infection in neuronal or glial cells directly. Recently, NSCs and BPCs have been used for studying mouse cytomegalovirus (CMV) infection (Kosugi *et al*, 2000), HIV-1 infection (Lawrence *et al*, 2004), and JC virus (JCV) infection (Messam *et al*, 2003). Thus we planned to use these cultures for studying SIV infection. There have been extensive studies on rodent or human BPCs (Davis and Temple, 1994; Roy *et al*, 2000; Uchida *et al*, 2000; Westerlund *et al*, 2003), but relatively fewer reports exist on nonhuman primates, and most studies were about progenitor cells derived from embryonic stem (ES) cells (Asano *et al*, 2002; Calhoun *et al*, 2003; Vrana *et al*, 2003).

In this study, we characterized NSCs/BPCs isolated from embryonic brain of cynomolgus monkeys (*Macaca fascicularis*) by neurosphere assay. Then, we confirmed the utility of BPC-derived cell culture system for studying SIV infection in glial and neuronal cells.

Result

Isolation and differentiation of multipotential simian progenitor cells

The cells isolated from simian fetal brain were cultured in serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Only growth factor–responsive cells proliferated as floating clusters termed neurospheres within a few days (Figure 1A). Under these neurosphere-generating conditions, both NSCs and progenitor cells survived. We treated these cells together as BPCs. They easily adhered to the plastic and began to extend processes, so the flasks were gently knocked every day to prevent attachment. The spheres were dissociated by trypsinization twice a week. A single dissociated cell is 10 to 15 μm in diameter (Figure 1B). The primary neurosphere-derived BPCs proliferated again to form secondary spheres. *In vitro* differentiation was induced by culturing the dissociated cells with medium containing 1% fetal bovine serum (FBS) without growth factors. Cells with apparent morphological changes appeared at 1 day post induction of differentiation (dpid). At 14 dpid, these cells consisted of small cells with neurite-like processes and elongated cells with a polygonal appearance (Figure 1C).

Characterization of BPC phenotypes by immunocytochemistry

Immunocytochemistry was performed on BPCs before and after induction of differentiation using various cell type–specific phenotypic markers for progenitor cells, astrocytes, neurons, oligodendrocytes, and microglia. Nestin is an intermediate filament protein and is expressed in the developing CNS (Lendahl *et al*, 1990). The prospective phenotypic marker of BPCs in the neurosphere cultures is unknown, though nestin is used as a retrospective phenotypic marker of stem cells and progenitor cells (Messam *et al*, 2000, 2002).

Before induction of differentiation, almost all BPCs expressed nestin (Figure 1D) and the astrocytic marker glial fibrillary acidic protein (GFAP) (Figure 1E, H). Some BPCs expressed the neuronal marker beta-III-tubulin (Tuj) (Figure 1G). Double-immunofluorescence staining showed colocalization of nestin and GFAP (Figure 1F). Unexpectedly some cells coexpressed GFAP and Tuj (Figure 1I).

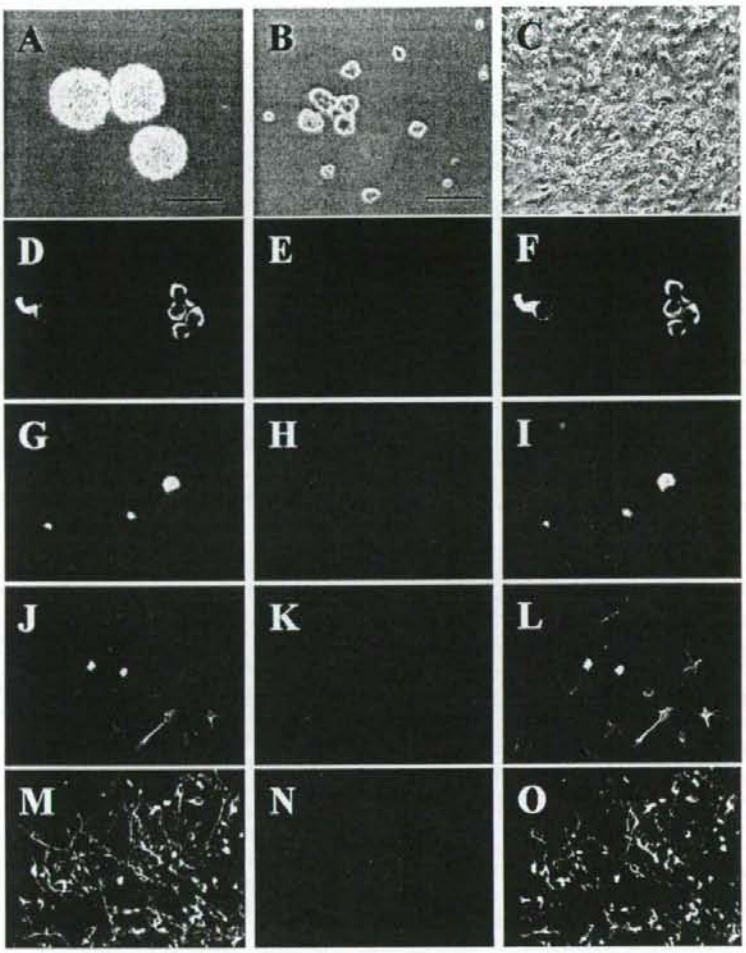

Figure 1 (**A–C**) Phase-contrast photographs of fetal macaque brain progenitor cells (BPCs). (**A**) The BPCs form neurospheres in the presence of EGF and bFGF. (**B**) A single BPC and dividing BPCs. (**C**) The BPCs 14 days post induction of differentiation (dpid). These BPC-derived cells consist of small cells and elongated cells. (**D–O**) Immunofluorescence staining of BPCs before (**D–I**) and after (**J–O**) induction of differentiation. Double-immunofluorescence stainings show GFAP (**E, H, K, N**) immunoreactivity *(red)* and nestin (**D, J**) or Tuj (**G, M**) immunoreactivity (*green*) colocalization in some cells (**F, I, L, O,** *yellow*). Nucleic acids are counterstained with topro-3 *(blue)*. Cells were imaged using an LSM 410 inverted laser-scanning microscope. (**A, B**) Scale bar, 50 μm. Original magnifications: (**A, B, J–O**) ×200, (**C**) ×100, (**D–I**) ×630.

At 14 dpid, the cells still expressed nestin (Figure 1J) and some of them expressed also GFAP (Figure 1L). Most cells expressed GFAP or Tuj (Figure 1M, N). The expression of another neuronal marker, mitogen-activated protein (MAP)-2, was also detected (data not shown). Morphologically, the elongated polygonal cells were positive for GFAP, and the small cells with neurite-like processes were positive for Tuj. The cells coexpressing GFAP and Tuj were hardly observed at this time point (Figure 1O). We failed to detect cells expressing the oligodendrocyte marker, O4. The BPCs, neither undifferentiated nor differentiated, expressed the monocyte/macrophage/microglial marker (HAM56) (data not shown).

Characterization of BPC phenotypes by flow cytometry

We examined the expression levels of cellular markers in BPCs before and after induction of differentiation by flow cytometry. The FSC/SSC (forward scatter/side scatter) profiles showed that the BPCs were composed of heterogeneous cell populations

Table 1 Percentage of analyzed cells expressing nestin, GFAP, and Tuj by flow cytometric analysis

	Percentage of analyzed cells expressing each phenotypic marker (%)								
Gate*	Nestin	GFAP	Tuj	N+/G+	N+/G−	N−/G+	T+/G+	T+/G−	T−/G+
R1	96.1	98.2	89.5	75.8	18.8	3.6	68.5	16.2	12.2
R2	91.9	99.0	59.4	91.4	4.0	4.1	61.3	2.5	35.1
R3	89.8	93.6	29.2	81.3	1.9	13.4	14.9	1.0	80.3
R4	18.9	54.4	54.4	11.1	8.9	55.7	29.3	27.8	18.5
R5	41.9	91.6	93.1	19.3	6.4	25.2	58.0	31.5	5.3
R6	34.3	87.7	73.9	22.6	1.8	44.4	46.1	14.9	31.2
R7	36.3	96.8	41.1	30.6	0.7	60.1	29.3	3.0	65.5
R8	30.5	99.2	44.3	8.9	0.4	84.6	13.7	0.7	83.9

*Gates defined in Figure 2. R1 to R3 are gates of BPCs before induction of differentiation. R4 to R8 are gates of BPC-derived cells 28 days post induction of differentiation.
N: nestin; G: GFAP; T: Tuj.

(Figure 2). According to these profiles, we set up three gates (R1, R2, R3) for the undifferentiated BPCs (Figure 2A) and five gates (R4, R5, R6, R7, R8) for the differentiated BPCs (Figure 2B). The expression levels of cellular markers in the cells of each gate were examined and summarized in Table 1.

Firstly, the expression level of nestin, GFAP, and Tuj in BPCs of each gate were examined with single-color flow cytometry by using specific antibodies as described previously in Materials and Methods. Before induction of differentiation, nestin was expressed in 96.1%, 91.9%, 89.8%, and GFAP was expressed in 98.2%, 99.0%, 93.6% of cells in fraction R1, R2, R3, respectively. Tuj was expressed in 89.5% in cells of a smaller size (R1) and 29.2% in cells of a larger size (R3). After induction of differentiation, at 28 dpid, the percentage of nestin-positive cells decreased to 32.3% from 92.6% on average. GFAP was expressed in 99.2% in larger cells (R8) and Tuj was expressed in 93.1% of smaller cells (R5).

Secondly, coexpression of GFAP and nestin, GFAP, and Tuj was examined by two-color flow cytometry. Before induction, the percentages of nestin(+)/GFAP(+) double-positive cells were 75.8%, 91.4%, and 81.3% and for Tuj(+)/GFAP(+) double-positive cells were 68.5%, 61.3%, and 14.9% of cells in fraction R1, R2, R3, respectively. These percentages of double positive cells decreased at 28 dpid. The percentage of nestin(+)/GFAP(−) cells was higher in smaller cells (18.8%, R1) and decreased to 8.9% (R4) at 28 dpid. The percentage of nestin(−)/GFAP(+) cells was higher in larger cells (13.4%, R3) and increased to 84.6% (R8) at 28 dpid. The percentage of Tuj(+)/GFAP(−) cells was higher in smaller cells (16.2%, R1) and increased to 31.5% (R5) at 28 dpid. In summary, double-positive cells and nestin-positive cells decreased and GFAP- or Tuj-positive cells increased after induction of differentiation. GFAP-positive cells were mainly larger cells (R8), and Tuj-positive cells were mainly smaller cells (R4, R5).

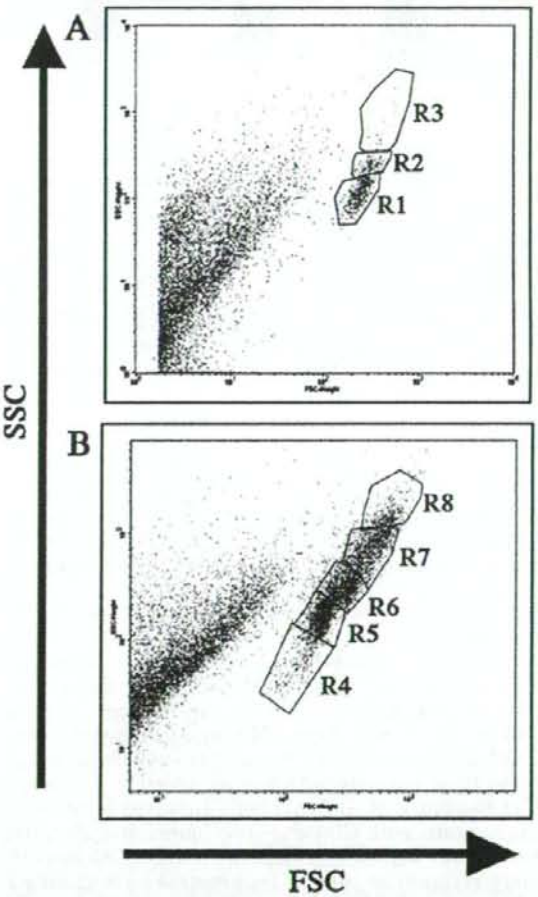

Figure 2 Flow cytometric analysis of BPCs before and after induction of differentiation. FSC/SSC (forward scatter/side scatter) profiles show the heterogeneity of the cells. Conventionally, three gates were set (R1, R2, and R3) for the undifferentiated BPCs (**A**) and five gates (R4, R5, R6, R7, and R8) for the differentiated BPC-derived cells (**B**). The expression levels of cellular markers in the cells of each gate were examined and summarized in Table 2.

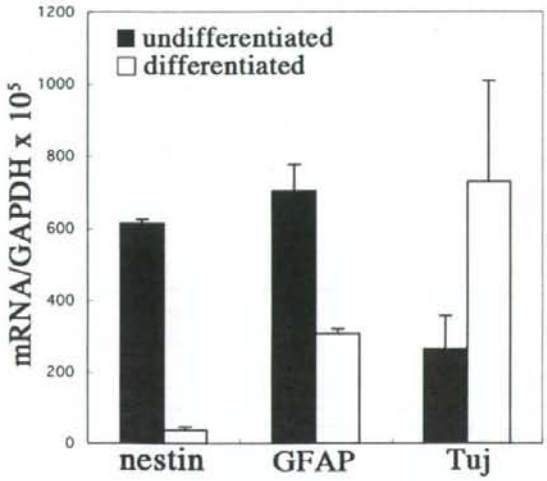

Figure 3 mRNA expression levels of nestin, GFAP and Tuj in undifferentiated BPCs and differentiated BPCs (14 dpid) were analyzed by real-time PCR. In undifferentiated BPCs *(black bars)*, mRNA expression levels of nestin and GFAP were higher than that of Tuj, while in differentiated BPCs (14 dpid) *(white bars)*, mRNA expression levels of nestin and GFAP decreased significantly, and that of Tuj increased. This study was performed three times with similar results.

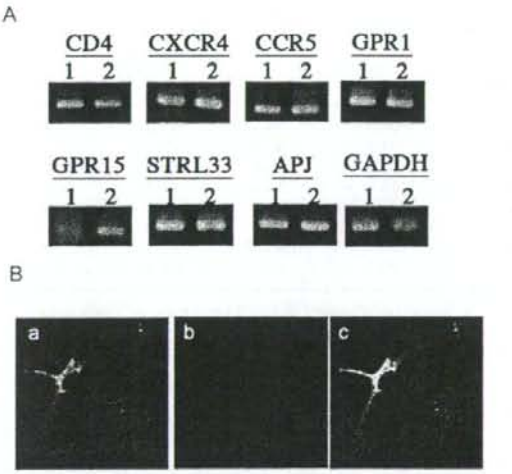

Figure 4 (**A**) mRNA expression of CD4, CXCR4, CCR5, GPR1, GPR15, STRL33, and APJ, examined by RT-PCR analysis with 1 μg of total RNA isolated from BPCs before and after induction of differentiation. Amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide. GAPDH served as an internal control for the standardization of each product. CD4 and the examined chemokine receptors except GPR15 mRNAs were all expressed in undifferentiated and differentiated BPCs. These results were representative for at least three independent experiments and individual samples were analyzed three times by RT-PCR. Lane 1: undifferentiated BPCs. Lane 2: differentiated BPCs. (**B**) Double-immunofluorescence stainings show CCR5 (**a**) immunoreactivity (*green*) and GFAP (**b**) immunoreactivity (*green*) colocalization (**c**, *yellow*) in the differentiated BPCs. Original magnifications: (**a**, **b**, **c**) ×400.

mRNA expression levels of Nestin, GFAP, and Tuj in BPCs before and after differentiation

To confirm the results of immunofluorescence analysis further, we examined mRNA expression levels of nestin, GFAP, and Tuj in BPCs cultures before and after induction of differentiation by real-time PCR (Figure 3). The mRNA expression levels of nestin in BPCs decreased significantly after induction of differentiation. GFAP mRNA was expressed at high levels before induction and the levels decreased at 14 dpid. mRNA expression levels of Tuj increased after induction.

Expression of CD4 and chemokine receptors on simian BPCs

In the CNS, chemokines and their receptors are involved in the migration, differentiation, and activation of some cells and in the proliferation of glial and neuronal cells. Reportedly, many members of the CXCR family and the CCR family are expressed in the brain (Ji *et al*, 2004; Klein *et al*, 1999). It is known that the entry of SIV into cells involves a series of interactions with CD4 and coreceptors and also that several SIV strains are capable of using CCR5 as a primary receptor to infect CD4-negative cells (Edinger *et al*, 1997, 1999; Overholser *et al*, 2005). The receptor or the coreceptor can be one of a variety of seven transmembrane G-protein-coupled chemokine receptors. We examined the mRNA expression of CD4 and several chemokine receptors that are candidates for coreceptors of SIV; CXCR4, CCR5, GPR1, GPR15, STRL33, and APJ (Choe *et al*, 2000; Edinger *et al*, 1998; Westmoreland *et al*, 2002) in BPCs before and after induction of differentiation. Reverse transcriptase (RT)-PCR analysis confirmed the expression of CD4 and the examined chemokine receptors except GPR15 in both undifferentiated and differentiated BPCs. The expression of GPR15 was turned to be positive after induction of differentiation (Figure 4A). As for CD4 and CCR5, immunocytochemistry was performed on the differentiated BPCs. As shown in Figure 4B, some of the GFAP-positive cells coexpressed with CCR5 (Figure 4B), though we failed to detect CD4-positive cells (data not shown).

SIV infection in differentiated simian BPC-derived cell culture

We examined whether the differentiated simian BPC cultures supported SIV infection and the infection was productive or restricted. When the dissociated BPCs were plated on poly-O-coated 6-well plate with medium containing 1% FBS (MHM1%FBS) for induction of differentiation, they were inoculated with SIVmac239 and SIV/17E-Fr. The clone SIV17EΔnefGFP was also inoculated to identify infected cells. As shown in Figure 5A, the neurotropic SIV/17E-Fr replicated most efficiently, but the parental SIVmac239 replicated hardly in the

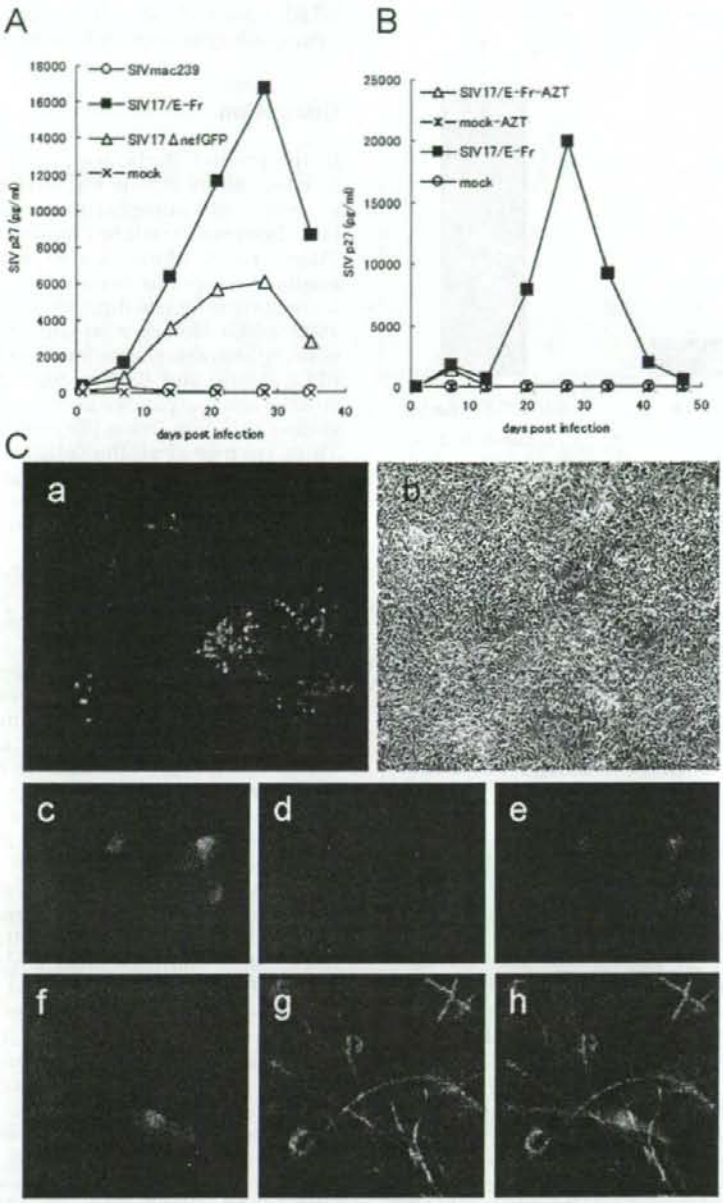

Figure 5 SIV infection of differentiated BPCs. The dissociated BPCs were plated on poly O-coated plates and were incubated with each SIV stock (100 ng of SIV p27) for 16 h in MHM1%FBS. After infection, the cells were washed 3 times extensively and fresh MHM1%FBS was added and the culture supernatants were sampled at various days post infection (dpi) and assayed for SIV p27. (**A**) SIV/17E-Fr replicates most efficiently, though the parental SIVmac239 replicates at very low levels. The SIV17EΔnef GFP also replicates efficiently, more than SIVmac 239 but less than SIV/17E-Fr. Results shown are representative of several independent experiments with BPC cultures from several macaques. (**B**) SIV p27 production depended on reverse transcription (RT). To examine whether the peak of the SIV p27 value depended on the RT step, 10 mM AZT (RT inhibitor) was added to the media every 3 days after overnight infection. SIV17/E-Fr replicates hardly in the presence of AZT in media. (**C**) (**a**) The expression of GFP in the cells infected with SIV17EΔnef GFP were observed under a fluorescent microscope (14 dpi). (**b**) Phase-contrast photographs of (**a**). (**c, d, e**) A cell infected with SIV17EΔnef GFP expressing GFP (**c**, *green*) and immunostained with mouse monoclonal anti-SIVp27 antibody using Alexa568-conjugated secondary antibody (**d**, *red*). Marged image (**e**, *yellow*). (**f, g, h**) A cell infected with SIV17EΔnef GFP (**f**, *green*) identified as GFAP-positive by immunostaining with rabbit polyclonal anti-GFAP antibody using Alexa568-conjugated secondary antibody (**g**, *red*). Marged image (**h**, *yellow*). Original magnifications: (**a, b**) ×100, (**c–h**) ×400.

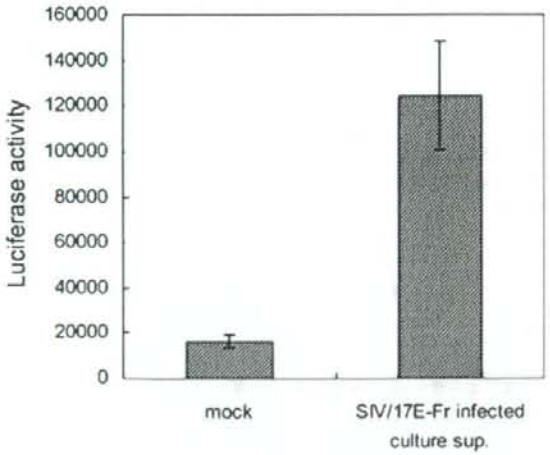

Figure 6 Luciferase activity in LuSIV cells at 72 h post infection with the supernatant of BPC-derived cell culture infected with SIV/17E-Fr and mock. The fold induction of luciferase activity over background was 7.57 ± 0.64.

differentiated BPC cultures. The SIV17EΔnefGFP also replicated more efficiently than SIVmac 239 but less than SIV/17E-Fr. The experiments were performed twice independently and the same results were obtained (Figure 5A).

To investigate the infectivity of SIV released from the SIV/17E-Fr–infected BPC-derived cell culture, the culture supernatant was assayed by the LuSIV system (Roos *et al*, 2000; Overholser *et al*, 2003). The culture supernatants were collected from the other infection experiment on 36 dpi. Though the assayed supernatant contained only 1.86 ng of SIV p27, the fold induction of luciferase activity over background was 7.57 ± 0.64 (Figure 6).

To examine whether the peak of SIV p27 value in the culture supernatant infected with SIV17/E-Fr depended on the reverse transcription step or not, 10 mM of AZT (reverse transcriptase inhibitor) was added to the medium every 3 days after overnight infection. As shown in Figure 5B, SIV17/E-Fr replicated hardly in the presence of AZT in the medium. As for SIV17EΔnefGFP infection, GFP expression was observed under fluorescence microscopy every day post infection. GFP-positive cells appeared at 4–6 days post infection and variable shapes of GFP-positive cells were observed at 14 days post infection (Figure 5C, a). The number of GFP-positive cells increased in parallel with the SIV p27 antigen titer in the supernatant (Figure 5C, a, b). The GFP-positive cells were confirmed to express SIV p27 gag antigen by immunostaining (Figure 5C, c to e). We examined the phenotype of the GFP-positive cells by immunostaining with antibodies for GFAP and Tuj. Most of these cells were positive for GFAP (Figure 5C, f to h). These results suggested that the simian fetal BPC-derived cell culture can be used for studying neuropathogenesis of SIV in the CNS.

Discussion

In the present study, we attempted to establish an *in vitro* culture system for investigating neuropathogenesis of SIV encephalitis using primary BPCs isolated from embryonic brains of Cynomolgus macaque (*Macaca fascicularis*) by neurosphere assay. The neurosphere assay has been developed primarily as a valuable tool for isolating NSCs (Reynolds and Weiss, 1992, 1996). However, recently, the NSCs isolated by neurosphere assay were found to be mostly non-stem BPCs (Maric and Barker, 2004; Suslov *et al*, 2002) and the assay expanded both stem and non-stem progenitor cell population (Reynolds and Rietze, 2005). Thus, we treated all the cells isolated by the neurosphere assay as BPCs in this report.

Primary BPC-derived cell culture has an advantage over conventional primary brain cell culture. The self-renewal and multilineage differentiation properties of BPCs are very convenient for planning viral infection experiments. It is possible to increase cells with mitogens and to prepare cell cultures by inducing differentiation by adding appropriate reagents to the culture medium according to each purpose (Caldwell *et al*, 2001). Therefore, there are no limitations for repeated experiments and the valuable simian embryonic brain can be used effectively. It is generally difficult to completely exclude any monocyte/macrophage/microglial cells, fibroblasts, and endothelial cells from conventional primary brain cell culture, though BPC-derived culture never contain such cells.

We induced differentiation of simian BPCs by culturing the cells with the medium (Dulbecco's modified Eagle's medium [DMEM]/F-12 plus hormone mix) supplemented with 1% fetal bovine serum (FBS). We characterized both undifferentiated and differentiated BPCs by examining the expression of nestin, GFAP, and Tuj in BPCs by immunocytochemistry, flow cytometry, or quantitative RT-PCR and the results obtained with these different methods were in agreement. The BPCs revealed heterogeneity both before and after induction of differentiation as shown in Figure 1 and 2. Before induction, more than 90% of cells expressed nestin or GFAP and some cells expressed Tuj. There were even nestin(+)/GFAP(+) cells and Tuj(+)/GFAP(+) cells. The mRNA expression level of GFAP was as high as the expression level of nestin before induction and the levels decreased at 14 dpi. This was because GFAP-positive cells did not always differentiate into astrocytes after induction. Several reports supported the fact that astrocytes, which are defined as GFAP-positive cells, globally possess NSC attributes and form neurospheres that give rise to both neurons and glias (Doetsch, 2003; Doetsch *et al*, 1999;

Laywell *et al*, 2000; Seri *et al*, 2001). After induction of differentiation, the number of nestin-positive cells decreased, which is shown in Figure 3. The decrease in mRNA expression level of GFAP suggested that some GFAP-positive cells changed to GFAP-negative cells after induction of differentiation. In total, the number of Tuj(+)/GFAP(+) cells decreased and nestin(−)/GFAP(+) or Tuj(+)/GFAP(−) cells increased. No detectable expression of monocyte/macrophage/microglia markers (data not shown) was confirmed.

The final aim of this study was to establish an *in vitro* culture system consisting of only neuronal and glial cells to investigate the direct relation between SIV infection and these cells. SIV infects and replicates much more efficiently in macrophages and microglial cells than in neuronal and glial cells. If the culture contains macrophages or microglial cells, it is difficult to determine whether neural damage is due to a direct effects of SIV infection or due to secondary effects of infected macrophages or microglial cells. SIV17/E-Fr is a neurotropic SIV strain that replicates productively in astrocytes (Flaherty *et al*, 1997). SIV17/E-Fr replicated also in the BPC-derived cells (Figure 5), though the value of SIV p27 gag protein in the culture supernatant was not so high. This was because the culture consisted of various cell types in neuronal and glial lineage and only a few of them were permissive to SIV infection. This heterogeneity of the culture changed and depended on the day post induction of differentiation (dpid). Thus, we did the infection experiments also at 1, 3, 7, and 14 dpid. The results of each experiment were similar among them, if the BPCs were derived from the same simian fetus. We should investigate in detail the differences of the direction of differentiation between SIV-infected BPCs and mock-infected BPCs.

To identify the permissive cells, we inoculated SIV17EΔnefGFP and observed the GFP-expressing cells under a fluorescent microscopy after infection. The SIV17EΔnefGFP also replicated efficiently in BPC-derived cells but less than SIV/17E-Fr. SIV17EΔnefGFP might require the Nef protein for efficient and optimal replication (Gorry *et al*, 1998; Overholser *et al*, 2003). The number of GFP-positive cells was less than expected. It was confirmed that GFP-expressing cells (Figure 5C, c) expressed SIV p27 gag antigen (Figure 5C, d, e) and almost all GFP-expressing cells were GFAP positive (Figure 5C, f to h) and Tuj negative (data not shown). These suggested that SIV/17E-Fr and SIV17EΔnefGFP infected astrocytes in the BPC-derived cell culture. It was reported that SIV/17E-Fr used CCR5 for entry and replication in the primary rhesus macaque astrocytes (Overholser *et al*, 2003). In our study, the coreceptor utilization was not clearly determined, though we confirmed some GFAP-positive cells co-expressed with CCR5 in the BPC-derived cell culture (Figure 4B). We also determined whether recombinant human RANTES, one of the ligands of CCR5, could block the entry of SIV/17E-Fr into the BPC-derived cell. The peak of the value of SIV p27 decresed to 21% in the supernatant of culture treated with 100 ng/ml of RANTES (Peprotech, NJ, USA) compared with that in the absence of RANTES (data not shown). Thus, RANTES partially inhibited SIV entry and replication in the BPC-derived cell culture and CCR5 was utilized by SIV/17E-Fr. The GFP-expressing cells should be characterized further in detail and should be cloned, if possible.

The number of GFP-expressing cells increased in parallel with the value of SIV p27 in the culture supernatant. Both the number of GFP-expressing cells and the value of SIV p27 were reduced by adding AZT to the culture medium (Figure 5B). This suggested that the production of SIV p27 and the expression of GFP depended on the step of reverse transcription. Thus, the value of SIV p27 in the supernatant around 28 dpi were supposed to be derived from newly produced SIV/17E-Fr. Adding this, the results of LuSIV assay showed the infectivity of SIV released from the SIV/17E-Fr-infected BPC-derived cell culture (Figure 6). In summary, BPC-derived cells supported productive infections of SIV17/E-Fr and SIV17EΔnefGFP, though the amount of viral products was low.

We showed that simian BPC-derived cell culture supported a productive infection of SIV. This culture system is easy to establish and maintain and, if necessary, it is possible to coculture with microglial cells or monocytes/macrophages (data not shown). It is applicable to investigate viral neuropathogenesis other than SIV.

Materials and methods

Cell culture

BPCs were isolated from the fetal brain of Cynomolgus macaque (*Macaca fascicularis*) at 8 to 11 weeks of gestation in accordance with institutional ethical guidelines. Fetal brains were removed, cut into 1-mm coronal sections and triturated gently with a pipette in phosphate-buffer saline (PBS). After mechanical dissociation, cells were washed in PBS, centrifuged at 600 rpm for 5 min, and resuspended in Neural Progenitor Cell Medium (NPBM; Clonetics, San Diego, USA) including epidermal growth factor (EGF) (20 ng/ml; Progen, Heidelberg, Germany) and basic fibroblast growth factor (bFGF) (20 ng/ml; Progen) as described by Reynolds and Weiss (Reynolds *et al*, 1992; Reynolds and Weiss, 1992). The cell suspension was cultivated in uncoated 75-cm^2 cell cultureflasks (Asahi Techno Glass, Tokyo, Japan) at a density of 1.0×10^6/ml at 37°C with 5% CO_2. The cells formed spheres within approximately one week. Every 5 to 7 days, the spheres were dissociated with 0.125% trypsin-EDTA (Invitrogen, CA, USA) for 10 min at 37°C. Following rinsing with 1 mg/ml trypsin inhibitor (Roche Diagnostics, Boehringer Mannheim,

Germany), single cells were centrifuged at 1000 rpm for 5 min and resuspended in fresh medium. After more than five passages of growth factor selection, the cells were used for the experiments in the present study.

For the induction of differentiation, the dissociated cells were plated on 6-well plates precoated with 30 μg/ml of poly-L-ornithine hydrobromide (poly O; Sigma-Aldrich, St. Louis, MO, USA), at a density of 3 $\times$ 10^5 cells per well. The differentiated BPC culture medium (MHM1%FBS) was composed of DMEM/F-12 (Invitrogen) supplemented with a defined hormone and salt mixture composed of insulin, transferrin, progesterone, putrescine, and sodium selenite (all from Sigma) supplemented with 1% fetal bovine serum (FBS), amphotericin B (1.25 μg/ml), penicillin (50 μg/ml), streptomycin (50 μg/ml), and neomycin (100 μg/ml) (all from Invitrogen) and lacking growth factors. The medium was changed every 7 days.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature, rinsed in PBS, permeabilized with 0.1% Triton-X (Wako Chemicals, Osaka, Japan)/PBS for 10 min at room temperature, and blocked with normal goat serum for 30 min at room temperature. Cells were then incubated at 4°C overnight with mouse monoclonal antibodies against human nestin (1:10; R&D Systems, MN, USA), tubulin beta III isoform (Tuj) (1:200; Chemicon), MAP-2 (1:200; Chemicon), oligodendrocyte marker O4 (O4) (1:50; Chemicon), human macrophage (HAM56, 1:50; DakoCytomation, Kyoto, Japan), CD4 clone 1F6 (1:10; NICHIREI Bio. Osaka, Japan), CCR5 clone 3A9 (1:100; BD PharMingen, CA, USA), and SIV p27 gag protein (1:100) or a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (1:1; DakoCytomation). After 3 washes in PBS, cells were incubated in either Alexa 488–conjugated goat anti-mouse immunoglobulin G (IgG) (1:200; Molecular Probe, Eugene, OR), Alexa 568–conjugated anti-mouse IgG (1:200; Molecular Probes), Alexa 568–conjugated anti-rabbit IgG (1:200; Molecular Probes), or fluorescein-conjugated anti-mouse IgM (Vector laboratories, Burlingame, CA) for 1 h at 37°C. Nucleic acids were counterstained with topro-3 (1:1000; Molecular Probes). Fluorescence was analyzed with an LSM 410 inverted laser-scanning microscope (Carl-Zeiss, Jena, Germany).

Flow cytometry

For flow cytometry, 0.125% trypsin-treated undifferentiated and differentiated BPCs were fixed with 4% PFA/PBS at 4°C for 30 min. After 3 washes in PBS and an incubation with 5% goat serum/PBS for 30 min on ice, cells were stained with the following primary antibodies: mouse monoclonal antibodies against human nestin (1:10; R&D), Tuj (1:500; Chemicon), or a rabbit polyclonal antibody against GFAP (1:1; DakoCytomation), for 30 min on ice at 10^6 cells/ml. The mouse IgG_{2b} isotype control (1:10; Cymbus Biothechnology) and N-universal negative control (1:1; Dako Cytomation) were used as negative controls for mouse monoclonal antibodies and the rabbit polyclonal antibody, respectively. After 3 washes in PBS, phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody (1:200; BD Biosciences Pharmingen, San Diego, CA, USA) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin-pecific polyclonal antibody (1:200; BD Bioscience Pharmingen) were added for secondary labeling. The stained cells were washed with PBS, and resuspended in 0.1% bovine serum albumin (BSA)/PBS for flow cytometry using FACS caliber (BD, NJ, USA).

RT-PCR and real-time PCR analysis

Total RNA was extracted from BPCs using Quick gene RNA cultured cell kit S and Quick gene-800 (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The concentration of total RNA was estimated by measuring the optical density. The extracted RNA samples were subjected to reverse transcription (RT) using oligo$(dT)_{15}$ and the Omniscript RT kit (Qiagen, CA, USA) according to the manufacturer's protocol. PCR was performed in 20 μl of reaction mixture containing cDNA derived from 1 μg of RNA, each primer pair (Table 2) and the TAKARA Premix (Takara Biotechnology, Tokyo, Japan) or Hi-fidelity premix kit (Roche). The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for 35 cycles (CD4, CXCR4, GPR1, STRL33, APJ, GAPDH), or denaturation at 94°C for 30 s annealing at 65°C for 30 s, extension at 72°C for 1 min for 35 cycles (CCR5, GPR15). As a negative control, the cDNA template was substituted for an equal volume of water. Amplification was performed in an i-Cycler BIO RAD (Bio-Rad Laboratories, Tokyo, Japan). The amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control in RT-PCR analyses for the standardization of each amplified product.

Real-time PCR was performed with the Quantitect probe PCR kit (Qiagen) on the ABI PRISM 7900HT sequence detector (Applied Biosystems, CA, USA). The sequence of primers and probes of nestin, GFAP, Tuj, and GAPDH were as described in previous reports (Fassunke *et al*, 2004; Li *et al*, 2005) or designed using the software Primer Express v2.0 (ABI) (Table 2). Three parallel PCRs for each sample were performed and data are shown in columns as each sample/monkey GAPDH by mean $\pm$ SD.

Viruses

SIVmac239 is a pathogenic lymphocyte-tropic molecular clone that infects macaque monkeys effectively and induces simian acquired

Table 2 Primers and probes

Genes	*Sequence*	*Product (bp)*	*Accession no./reference*
CD4	F: 5′-GTGGCACCTGGACATGC-3′ R: 5′-GGTCCCCACACTTCACA-3′	462	Mukhtar *et al*, 2002
CXCR4	F:5′-ATATACACTTCAGATAACTACACC-3′ R: 5′-CATAGGAAGTTCCCAAAGTACC-3′	311	Weatmoreland *et al*, 2002
CCR5	F: 5′-TCTCTGACCTGCTTTTCCTTCTTA -3′ R: 5′-TGCAGGTGTAATGAAGACCTTCTC-3′	314	Accession no. U77672
GPR1	F: 5′-CATGGAAGATTTGGAGGAAAC-3′ R: 5′-AGAGTTCTTGAGGGTTCGATGCCG-3′	462	Edinger *et al*, 1998
GPR15	F: 5′-CTGGTTTATCTCCTGCCTGCTGGG-3′ R: 5′-GGCAGCCAGGAGACAAGAAAGGC-3′	293	Edinger *et al*, 1998
STRL33	F: 5′-CCAGGAGGAGCATCAAGACTTCC-3′ R: 5′-AGGTCATCCTGTTGGCTTGCTGG -3′	393	Edinger *et al*, 1998
APJ	F: 5′-TACACAGACTCCAAATCCTCG-3′ R: 5′-TGCACCTTGGTGGTGTTCTCC-3′	481	Mukhtar *et al*, 2002
GAPDH	F: 5′-ATTCCATGGCACCGTCAAGGCT-3′ R: 5′-TCAGGTCCACCACTGACACGTT-3′	572	Accession no. J04038
Nestin	F: 5′-GCCCTGACCACTCCAGTTTA-3′ R: 5′-GGAGTCCTGGATTTCCTTCC-3′ P: 5′-TGGAGAATCCGGTGGGTCTAGAGTGTTCA-3′	201	Li *et al*, 2005
GFAP	F: 5′-AGAACCGGATCACCATTCCC-3′ R: 5′-TCTTGAGGTGGCCTTCTGACA-3′ P: 5′-TGCAGATCCGAGAAACCAGCCTGG-3′	96	Fussunke *et al*, 2004 Accession no. AY650314
Tuj	F: 5′-CATGGACAGTGTCCGCTCAG-3′ R: 5′-CAGGCAGTCGCAGTTTTCAC-3′ P: 5′-TGGATTCGGTCCTGGATGTGGTGC-3′	175	Li *et al*, 2005
GAPDH	F: 5′-AAGCTTGTCATCAATGGAAATCC-3′ R: 5′-CATCGCCCCACTTGATTTTG-3′ P: 5′-ACCATCTTCCAGGAGCGAGATCCC-3′	73	Accession no. J04038

F: forward primer; R: reverse primer; P: probe.

immunodeficiency syndrome (AIDS) (Kestler *et al*, 1990; Regier and Desrosiers, 1990). SIV17/E-Fr, which is a molecularly cloned macrophage-tropic and neurovirulent virus with the backbone of the clone SIVmac239, was kindly donated by Dr. M. Christine Zink (John Hopkins University School of medicine) (Flaherty *et al*, 1997). SIV17EΔnef GFP was newly constructed. A fragment of CMV-EGFP was amplified from pEGFP-C1 (BD Biosciences Clontech, Mountain View, CA, USA) by PCR using primers tagged with the *Bsp*HI site (5′-gggtcatgatag taatcaattacggggtc-3′, 5′-gggtcatgactagtacagctcgtccat gc-3′). The purified PCR product was inserted in the *Nco*I site of the nef region of pSIV17/E-Fr. Virus stocks were prepared by transfecting infectious viral plasmid DNA (pSIVmac239, pSIV17/E-Fr, and pSIV17EΔnef GFP) into 293T cells with Fugene 6 transfection reagent (Roche). Two days after transfection, supernatants were filtered through 0.45-μm filters (PALL, MI, USA) and subjected to RNase-free DNase I digestion (2 U/ml, Roche) for 30 min at 37°C in the presence of 0.01 M of $MgCl_2$. The amount of virus was standardized by the assay for p27 gag antigen with the SIV core antigen assay kit (Coulter, FL, USA).

SIV infection of differentiated stem cell cultures

The dissociated BPCs were plated on 6-well plates at a density of 3×10^5 cells/well and were incubated for 16 h in medium supplemented with 1% FBS (MHM1%FBS) and SIV stocks containing 100 ng of p27. After infection, the cells were washed 3 times with MHM extensively and fresh MHM-1%FBS was added. For 3′-azido-3′-deoxythymidine (AZT) treatment, 10 mM of AZT was also added to the media every 3 days after overnight infection. The culture medium was exchanged once a week and the culture supernatants were collected for measuring SIV p27 gag antigen with the SIV core antigen assay kit (Coulter). The expression of GFP in the cells infected with SIV17EΔnefGFP was observed under a fluorescent microscope (Olympus, Tokyo, Japan) after infection.

LuSIV assay

The infectivity of the supernatant of SIV/17E-Fr or mock-infected culture was assayed with the LuSIV system (Roos *et al*, 2000; Overholser *et al*, 2003). The LuSIV cells were maintained in RPMI supplemented with 10% FBS and 300 μg/ml of hygromycin B (Wako, Tokyo, Japan). The LuSIV cells (2×10^5 cells) were incubated in triplicate with the supernatant of SIV/17E-Fr or mock-infected culture. The value of SIV p27 in the culture supernatant collected on 36 dpi was 12380 pg/ml and 150 μl (1.86 ng of SIV p27) of the supernatant was used for LuSIV assay. After 72 h incubation, LuSIV cells were washed and assayed for the presence of luciferase activity with Luciferase Assay system (Promega). The luciferase activity was measured with Centro LB960 (Berthold). The fold induction of luciferase activity over background was calculated.

Reference

Asano T, Hanazono Y, Ueda Y, Muramatsu S, Kume A, Suemori H, Suzuki Y, Kondo Y, Harii K, Hasegawa M, Nakatsuji N, Ozawa K (2002). Highly efficient gene transfer into primate embryonic stem cells with a simian lentivirus vector. *Mol Ther* **6:** 162–168.

Bagasra O, Lavi E, Bobroski L, Khalili K, Pestaner JP, Tawadros R, Pomerantz RJ (1996). Cellular reservoirs of HIV-1 in the central nervous system of infected individuals: identification by the combination of in situ polymerase chain reaction and immunohistochemistry. *AIDS* **10:** 573–585.

Caldwell MA, He X, Wilkie N, Pollack S, Marshall G, Wafford KA, Svendsen CN (2001). Growth factors regulate the survival and fate of cells derived from human neurospheres. *Nat Biotechnol* **19:** 475–479.

Calhoun JD, Lambert NA, Mitalipova MM, Noggle SA, Lyons I, Condie BG, Stice SL (2003). Differentiation of rhesus embryonic stem cells to neural progenitors and neurons. *Biochem Biophys Res Commun* **306:** 191–197.

Choe W, Albright A, Sulcove J, Jaffer S, Hesselgesser J, Lavi E, Crino P, Kolson DL (2000). Functional expression of the seven-transmembrane HIV-1 co-receptor APJ in neural cells. *J NeuroVirol* **6(Suppl 1):** S61–S69.

Davis AA, Temple S (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* **372:** 263–266.

Doetsch F (2003). The glial identity of neural stem cells. *Nat Neurosci* **6:** 1127–1134.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97:** 703–716.

Edinger AL, Blanpain C, Kunstman KJ, Wolinsky SM, Parmentier M, Doms RW (1999). Functional dissection of CCR5 coreceptor function through the use of CD4-independent simian immunodeficiency virus strains. *J Virol* **73:** 4062–4073.

Edinger AL, Hoffman TL, Sharron M, Lee B, O'Dowd B, Doms RW (1998). Use of GPR1, GPR15, and STRL33 as coreceptors by diverse human immunodeficiency virus type 1 and simian immunodeficiency virus envelope proteins. *Virology* **249:** 367–378.

Edinger AL, Mankowski JL, Doranz BJ, Margulies BJ, Lee B, Rucker J, Sharron M, Hoffman TL, Berson JF, Zink MC, Hirsch VM, Clements JE, Doms RW (1997). CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. *Proc Natl Acad Sci U S A* **94:** 14742–14747.

Fassunke J, Majores M, Ullmann C, Elger CE, Schramm J, Wiestler OD, Becker AJ (2004). In situ-RT and immunolaser microdissection for mRNA analysis of individual cells isolated from epilepsy-associated glioneuronal tumors. *Lab Invest* **84:** 1520–1525.

Fischer-Smith T, Croul S, Sverstiuk AE, Capini C, L'Heureux D, Regulier EG, Richardson MW, Amini S, Morgello S, Khalili K, Rappaport J (2001). CNS invasion by CD14+/CD16+ peripheral blood-derived monocytes in HIV dementia: perivascular accumulation and reservoir of HIV infection. *J NeuroVirol* **7:** 528–541.

Flaherty MT, Hauer DA, Mankowski JL, Zink MC, Clements JE (1997). Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. *J Virol* **71:** 5790–5798.

Gonzalez RG, Cheng LL, Westmoreland SV, Sakaie KE, Becerra LR, Lee PL, Masliah E, Lackner AA (2000). Early brain injury in the SIV-macaque model of AIDS. *AIDS* **14:** 2841–2849.

Gonzalez-Scarano F, Martin-Garcia J (2005). The neuropathogenesis of AIDS. *Nat Rev Immunol* **5:** 69–81.

Gorry P, Purcell D, Howard J, McPhee D (1998). Restricted HIV-1 infection of human astrocytes: potential role of nef in the regulation of virus replication. *J NeuroVirol* **4:** 377–386.

Gorry PR, Howard JL, Churchill MJ, Anderson JL, Cunningham A, Adrian D, McPhee DA, Purcell DF (1999). Diminished production of human immunodeficiency virus type 1 in astrocytes results from inefficient translation of gag, env, and nef mRNAs despite efficient expression of Tat and Rev. *J Virol* **73:** 352–361.

Ji JF, He BP, Dheen ST, Tay SS (2004). Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci Lett* **355:** 236–240.

Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, Regier D, Sehgal P, Daniel M, King N, *et al* (1990). Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. *Science* **248:** 1109–1112.

Kim WK, Corey S, Alvarez X, Williams K (2003). Monocyte/macrophage traffic in HIV and SIV encephalitis. *J Leukoc Biol* **74:** 650–656.

Klein RS, Williams KC, Alvarez-Hernandez X, Westmoreland S, Force T, Lackner AA, Luster AD (1999). Chemokine receptor expression and signaling in macaque and human fetal neurons and astrocytes: implications for the neuropathogenesis of AIDS. *J Immunol* **163:** 1636–1646.

Kosugi I, Shinmura Y, Kawasaki H, Arai Y, Li RY, Baba S, Tsutsui Y (2000). Cytomegalovirus infection of the central nervous system stem cells from mouse embryo: a model for developmental brain disorders induced by cytomegalovirus. *Lab Invest* **80:** 1373–1383.

Lawrence DM, Durham LC, Schwartz L, Seth P, Maric D, Major EO (2004). Human immunodeficiency virus type 1 infection of human brain-derived progenitor cells. *J Virol* **78:** 7319–7328.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc Natl Acad Sci U S A* **97:** 13883–13888.

Lendahl U, Zimmerman LB, McKay RD (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60:** 585–595.

Li X, Xu J, Bai Y, Wang X, Dai X, Liu Y, Zhang J, Zou J, Shen L, Li L (2005). Isolation and characterization of neural stem cells from human fetal striatum. *Biochem Biophys Res Commun* **326:** 425–434.

Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Amedee AM, Murphey-Corb M, Kirstein LM, Munoz A, Clements JE, Zink MC (1997). Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. *J Virol* **71:** 6055–6060.

Maric D, Barker JL (2004). Neural stem cells redefined: a FACS perspective. *Mol Neurobiol* **30:** 49–76.

Messam CA, Hou J, Berman JW, Major EO (2002). Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells. *Brain Res Dev Brain Res* **134:** 87–92.

Messam CA, Hou J, Gronostajski RM, Major EO (2003). Lineage pathway of human brain progenitor cells identified by JC virus susceptibility. *Ann Neurol* **53:** 636–646.

Messam CA, Hou J, Major EO (2000). Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. *Exp Neurol* **161:** 585–596.

Messam CA, Major EO (2000). Stages of restricted HIV-1 infection in astrocyte cultures derived from human fetal brain tissue. *J NeuroVirol* **6(Suppl 1):** S90–S94.

Nuovo GJ, Becker J, Burk MW, Margiotta M, Fuhrer J, Steigbigel RT (1994). In situ detection of PCR-amplified HIV-1 nucleic acids in lymph nodes and peripheral blood in patients with asymptomatic HIV-1 infection and advanced-stage AIDS. *J Acquir Immune Defic Syndr* **7:** 916–923.

Overholser ED, Babas T, Zink MC, Barber SA, Clements JE (2005). CD4-independent entry and replication of simian immunodeficiency virus in primary rhesus macaque astrocytes are regulated by the transmembrane protein. *J Virol* **79:** 4944–4951.

Overholser ED, Coleman GD, Bennett JL, Casaday RJ, Zink MC, Barber SA, Clements JE (2003). Expression of simian immunodeficiency virus (SIV) nef in astrocytes during acute and terminal infection and requirement of nef for optimal replication of neurovirulent SIV in vitro. *J Virol* **77:** 6855–6866.

Regier DA, Desrosiers RC (1990). The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res Hum Retroviruses* **6:** 1221–1231.

Reynolds BA, Rietze RL (2005). Neural stem cells and neurospheres—re-evaluating the relationship. *Nat Methods* **2:** 333–336.

Reynolds BA, Tetzlaff W, Weiss S (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci* **12:** 4565–4574.

Reynolds BA, Weiss S (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255:** 1707–1710.

Reynolds BA, Weiss S (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* **175:** 1–13.

Roos JW, Maughan MF, Liao Z, Hildreth JEK, Clements JE (2000). LuSIV cell: a reporter cell line for the detection and quantitation of a single cycle of HIV and SIV replication. *Virology* **273:** 307–315

Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, Goldman SA (2000). In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. *Nat Med* **6:** 271–277.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* **21:** 7153–7160.

Sharma DP, Zink MC, Anderson M, Adams R, Clements JE, Joag SV, Narayan O (1992). Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytetropic parental virus: pathogenesis of infection in macaques. *J Virol* **66:** 3550–3556.

Suslov ON, Kukekov VG, Ignatova TN, Steindler DA (2002). Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc Natl Acad Sci U S A* **99:** 14506–14511.

Tornatore C, Meyers K, Atwood W, Conant K, Major E (1994). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68:** 93–102.

Torres-Munoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK (2001). Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. *J Neuropathol Exp Neurol* **60:** 885–892.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL (2000). Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A* **97:** 14720–14725.

Vrana KE, Hipp JD, Goss AM, McCool BA, Riddle DR, Walker SJ, Wettstein PJ, Studer LP, Tabar V, Cunniff K, Chapman K, Vilner L, West MD, Grant KA, Cibelli JB (2003). Nonhuman primate parthenogenetic stem cells. *Proc Natl Acad Sci U S A* **100(Suppl 1):** 11911–11916.

Westerlund U, Moe MC, Varghese M, Berg-Johnsen J, Ohlsson M, Langmoen IA, Svensson M (2003). Stem cells from the adult human brain develop into functional neurons in culture. *Exp Cell Res* **289:** 378–383.

Westmoreland SV, Alvarez X, deBakker C, Aye P, Wilson ML, Williams KC, Lackner AA (2002). Developmental expression patterns of CCR5 and CXCR4 in the rhesus macaque brain. *J Neuroimmunol* **122:** 146–158.

Zink MC, Amedee AM, Mankowski JL, Craig L, Didier P, Carter DL, Munoz A, Murphey-Corb M, Clements JE (1997). Pathogenesis of SIV encephalitis. Selection and replication of neurovirulent SIV. *Am J Pathol* **151:** 793–803.

Zink MC, Spelman JP, Robinson RB, Clements JE (1998). SIV infection of macaques—modeling the progression to AIDS dementia. *J NeuroVirol* **4:** 249–259.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 23, Number 5, 2007, pp. 686–692

DOI: 10.1089/aid.2006.0212

The Polymorphisms in *DC-SIGNR* Affect Susceptibility to HIV Type 1 Infection

NUANJUN WICHUKCHINDA,[1,2] YOSHIHIRO KITAMURA,[3], ARCHAWIN ROJANAWIWAT,[1] EMI E. NAKAYAMA,[2] HAIHAN SONG,[2] PANITA PATHIPVANICH,[4] WATTANA AUWANIT,[1] PATHOM SAWANPANYALERT,[1] AIKICHI IWAMOTO,[3] TATSUO SHIODA,[2] and KOYA ARIYOSHI[5]

ABSTRACT

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*) and its homologue *DC-SIGNR* (*DC-SIGN* related) have been thought to play an important role in establishing HIV infection by enhancing *trans*-infection of $CD4^+$ T cells in the regional lymph nodes. To identify polymorphisms associated with HIV-exposed seronegative (ESN) individuals in Thais, genomic DNA from 102 HIV-seronegative individuals of HIV-seropositive spouses, 305 HIV-seropositive individuals, and 290 HIV-seronegative blood donors was genotyped for two single nucleotide polymorphisms (SNPs) in *DC-SIGN* promoter (−139A/G and −336A/G), a repeat number of 69 bp in Exon 4 of *DC-SIGN* and *DC-SIGNR*, and one SNP in Exon 5 of *DC-SIGNR* (rs2277998A/G). We found that the proportion of individuals possessing a heterozygous 7/5 and 9/5 repeat and A allele at rs2277998 of *DC-SIGNR* in HIV-seronegative individuals of HIV-seropositive spouses was significantly higher than HIV-seropositive individuals [$p = 0.0373$, OR (95% CI) = 0.57 (0.32,1.01); $p = 0.0232$, OR (95% CI) = 0.38 (0.15,0.98); and $p = 0.0445$, OR (95% CI) = 0.61 (0.37,1.02), respectively]. Analysis after stratifying by gender showed that these associations were observed only in females but not in males. Moreover, HIV-seropositive females tend to have a homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance [$p = 0.0556$, OR (95% CI) = 1.79 (0.94,3.40)]. Haplotype analysis showed that the proportion of individuals possessing the 5A haplotype in HIV-seronegative females was significantly higher than HIV-seropositive females [$p = 0.0133$, OR = 0.50 (0.27,0.90)]. These associations suggest that *DC-SIGNR* may affect susceptibility to HIV infection by a mechanism that is different in females and males. Further studies are warranted to investigate the mechanisms of their function.

INTRODUCTION

SOME INDIVIDUALS, SUCH AS HIV-SERONEGATIVE COMMERCIAL SEX WORKERS and HIV-seronegative spouses of HIV-seropositive individuals, have been sexually exposed to HIV repeatedly, yet remain negative for anti-HIV antibody.[1,2] These individuals are recognized as HIV-exposed but seronegative persons (ESN). Our study in Thailand has identified a number of married couples in whom the serostatus of HIV infection was discordant between spouses and in the majority of cases, this discordance could not be explained by their sexual behavior, such as frequency of unprotected sexual contacts, or by viral load of their infected spouses (Rojanawiwat *et al.*, submitted). These observations made us speculate that these seronegative spouses were likely to have certain biological mechanisms that made them resistant to HIV infection.

A number of polymorphisms in host genes that encode products involved in HIV replication and/or immune regulation were reported to be associated with HIV infection and HIV disease progression.[3] Among these, only the homozygous *CCR5* 32-bp deletion (CCR5Δ32) was found to be consistently associated with resistance to HIV infection.[4–6] However, the CCR5Δ32 al-

[1]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.
[2]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
[3]Institute of Medical Science, University of Tokyo, Tokyo, Japan.
[4]Day Care Center, Lampang Hospital, Lampang, Thailand.
[5]Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

lele is very rare among Asians,[6–9] including Thais.[10] Thus other unidentified genes are likely to play a role in resistance to HIV infection among ESN in Thailand.

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*, also called CD209) is a receptor on dendritic cells (DCs) that binds to ICAM-3 expressed on T cells to facilitate the initial interaction between DCs and T cells. *DC-SIGN* has been considered important in HIV research because it acts as an intermediate receptor for binding to HIV-1 at mucosal sites and then enhancing *trans*-infection of $CD4^+$ T cells in regional lymph nodes.[11] A homologue of *DC-SIGN* called *DC-SIGNR* (*DC-SIGN* related or CD209L or L-SIGN; the official name is C-type lectin domain family 4, member M, CLEC4M) shares 77% amino acid identity and exhibits a similar capacity of binding to HIV-1.[12,13] *DC-SIGN* is expressed at high levels on DCs and some types of macrophages,[14,15] whereas *DC-SIGNR* is expressed on endothelial cells in liver and lymph nodes.[12,13,16] Recently, the presence of *DC-SIGNR* mRNA was demonstrated in the human vaginal and rectal mucosa by nested reverse transcriptase polymerase chain reaction (RT-PCR).[17] Because *DC-SIGN* and *DC-SIGNR* have an apparent role in DC–T cell interaction and HIV infection, the polymorphisms associated with these genes may have an impact on the transmission of HIV as shown in several studies.[18–23] The objective of this study is, therefore, to identify those reported polymorphisms in *DC-SIGN* and *DC-SIGNR* that are associated with ESN individuals in Thais. Having investigated polymorphisms of these two genes among Thai couples, we found that polymorphisms in the *DC-SIGNR* but not in the *DC-SIGN* were significantly associated with ESN females but not with ESN males.

MATERIALS AND METHODS

Patients and samples

From 6 July 2000 to 15 October 2002 we conducted the Lampang HIV couple study at the HIV clinic in the Day Care Center of the Lampang Hospital, which is a referral hospital located about 600 km to the north of Bangkok (Rojanawiwat *et al.*, submitted). For this study of genetic polymorphisms, we recruited 188 couples, including 70 HIV-serodiscordant couples with 43 HIV-seronegative females and 27 HIV-seronegative males, and 118 HIV-seroconcordant couples. All 144 HIV-seropositive females and 155/161 (96.3%) HIV-seropositive males were heterosexually infected. There were two males with a history of injecting drug use, one male with blood transfusion, two males with more than one possible risk factor, and one male with no information. In terms of age, viral load, $CD4^+$ cell count, and clinical symptoms of HIV-infected spouses, there was no significant difference between discordant couples and concordant couples in both females and males, except that the proportion of HIV-infected males with HIV-seronegative wives was higher than that of HIV-infected males with HIV-infected wives (Table 1).

To increase the number of HIV seronegative spouses of HIV-infected individuals, we also included two HIV-seronegative individuals (one male, one female) who did not bring their HIV-seropositive spouses and 30 HIV-seronegative widows whose husband died of HIV/AIDS. These widows were included because they were significantly exposed to HIV: the median [interquartile range (IQR)] frequency of sexual contact was four (2, 6) times per month and 26/30 (86%) of them had never used a condom before they were aware of HIV. Furthermore, we believe that the level of HIV exposure was very high because their index case has already progressed and died: in 14/30 (46%) women, their husband died within 1 year of disclosure and in 21/30 women (70%) within 2 years of disclosure. There was one HIV-seropositive female who previously had an HIV-positive husband and remarried an HIV-negative husband after her first husband died. Therefore in total, 102 HIV-seronegative individuals (74 females and 28 males) at high risk for HIV infection and 305 HIV-seropositive individuals (144 females and 161 males) were enrolled.

The median age (IQR) of both HIV-seronegative and HIV-seropositive individuals was 32 (29, 36) years. Among 74 HIV-seronegative female spouses, one of them had known her HIV status before the marriage. In the other 73 females, the median (IQR) duration of marriage before they knew the HIV status of their husbands was estimated to be 5 (3, 8) years. The median (IQR) frequency of sexual contacts before the disclosure of the HIV status of their husband was five (3, 8) times per month. Of females, 64 (87.7%) reported that they had never used a condom during those sexual contacts and only four (5.5%) reported that they used a condom at every contact. Among 28 HIV-seronegative male spouses, nine of them had known the HIV status of their wives before the marriage. In the other 19 HIV-seronegative males, the median (IQR) duration of marriage before the disclosure of HIV status of their wife was estimated to be 1 (0, 2) year. The median (IQR) frequency of sexual contact before the disclosure of the HIV status of their wife was six (4, 24) times per month, and all of them reported that they had never used a condom during sexual contacts.

For a control HIV-seronegative group representing the Thai general population, 290 blood samples were collected from blood donors (171 males and 119 females) at the blood bank of the Lampang Hospital. All participants gave written informed consent. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

EDTA-treated blood samples were separated for plasma and buffy coat, then stored at −80°C until used. Genomic DNA was extracted and purified from the frozen buffy coat using a kit (QIAamp mini blood kit; QIAGEN GmbH, Hilden, Germany).

HIV-1 serology was screened by an ELISA kit (Enzynost anti-HIV-1/2 plus; Dade Behring Marburg GmbH, Marburg, Germany). The positive samples were then confirmed by another ELISA kit (Genscreen HIV 1/2 Diagnostic Pasture Ltd., France) and a gel particle agglutination test (Serodia HIV-1; Fujirebio Inc., Tokyo Japan).

Polymorphism genotyping

DC-SIGN promoter: −139A/G (rs2287886) and −336A/G (rs4804803): *DC-SIGN −139A/G* was genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Amplification of fragments containing this single nucleotide polymorphism (SNP) was performed using forward primer: 5′-GTCGGAATCCAAGGCCTCTC-3′ and

reverse primer: 5'-CAGGAAAGCCAGGAGGTCAC-3'. PCR was performed in a total of 25 μl containing 0.1 μM each primer, 100 μM dNTPs, 2.5 mM $MgCl_2$, and 0.5 unit of heat-activated DNA polymerase (IMMOLASE, Bioline USA Inc., MA) in 1× buffer supplied with the enzyme. The thermal profile was 7 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 20 sec at 58°C, and 1 min at 72°C. The 574-bp PCR products were incubated with *Spe*I restriction enzyme. The product, which contained the −139A allele, was cut into 318-bp and 256-bp fragments, and determined by electrophoresis in 2% agarose gel.

For *DC-SIGN −336A/G* genotyping, we used an allelic discrimination (AD) assay by 7500 real-time PCR with SDS analysis software (Applied Biosystems, CA). Two primers (forward primer: 5'-TGTGTTACACCCCCTCCACTAG-3'; reverse primer: 5'-GGACAGTGCTTCCAGGAACT-3') were used to amplify 68-bp products containing the *DC-366A/G* SNP site, which was detected by two probes labeled with different fluorescent dyes: 5'-VIC-TACCTGCCTACCCTTG-MGB-3' to detect the "A" allele and 5-FAM-CTGCCCACCCTTG-MGB to detect the "G" allele. Real-time PCR was performed in a 10-μl reaction mixture containing 5 μl of 2 × TaqMan universal master mix, 1× primers and probes mix, and 10 ng of DNA sample. Thermocycling consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C.

The 69-bp repeat number in Exon 4

The 69-bp repeat number was determined by the length of the PCR products. Primers and the thermal profile for *DC-SIGNR* 69-bp repeat number genotyping was performed as previously described.[13] For *DC-SIGN* 69-bp repeat number genotyping, forward primer: 5'-CCTTGGCTCTCACAAATGATGTCC-3' and reverse primer: 5'-CACCCACTGCAGCCTTCAGCTG-3' were used in the PCR condition as described above. The thermal profile was 7 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 64°C, and 1 min at 72°C. The PCR products were analyzed by electrophoresis in 2% agarose gel.

DC-SIGNR Exon 5 (rs2277998)

A/G SNP of rs2277998 was genotyped by PCR-RFLP as previously described.[18]

Data management and sample analysis

We conducted this study in a blinded manner between field investigators and laboratory investigators. The proportions of individuals possessing a certain allele/genotype among different groups were compared by a chi-square test or Fisher's exact test. Odds ratio (OR) and 95% confidence interval (95% CI) were used to measure the strength of the genetic influence associated with susceptibility to HIV infection. All statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *DC-SIGNR* haplotypes were constructed and estimated for their frequencies by the expectation-maximization algorithm (Arlequin version 3.01, Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

Genotyping of polymorphisms in DC-SIGN and DC-SIGNR

We genotyped the five polymorphisms (three in *DC-SIGN* and two in *DC-SIGNR*) in 102 HIV-seronegative individuals, 305 HIV-seropositive individuals, and 290 blood donors (Table 2). We then compared the proportions of individuals possessing a specific genotype between HIV-seronegative and HIV-seropositive individuals (Table 3).

As for the two SNP sites in the *DC-SIGN* promoter, we did not find any significant differences in the proportion of individuals possessing the G allele (genotype G/G and A/G). The *DC-SIGN* 69-bp repeat number was highly conserved; the genotype 7/7 repeat was found almost exclusively at a frequency of 0.99. In contrast, we found some significant differences in genetic polymorphisms in *DC-SIGNR* associated with HIV infection.

TABLE 1. CHARACTERISTICS OF HIV-SEROPOSITIVE INDIVIDUALS

	Male		*Female*	
	With HIV −ve spouse (n = 43)	*With HIV +ve spouse (n = 118)*	*With HIV −ve spouse (n = 27[a])*	*With HIV +ve spouse (n = 118)*
Median age; years (IQR)[b]	34 (31, 38)	33 (30, 37)	31 (28, 35)	30 (27, 35)
Median viral load,[c] $\log_{10}$ copies/ml (IQR)	5.257 (4.915, 5.757)	5.305 (4.773, 5.756)	4.939 (4.358, 5.579)	4.993 (4.214, 5.477)
Median CD4 count,[c] cells/mm^3 (IQR)	28 (13, 72)	38 (11, 246)	321 (157, 524)	277 (148, 427)
HIV-1-related symptoms,[c] % (*n*)	79.1 (34)	59.5 (69)	33.3 (9)	27.4 (32)
Diagnosis of AIDS,[c] % (*n*)	65.1 (28)	38.8 (45)	11.1 (3)	12.8 (15)

[a]This includes a female who was counted as a concordant couple with her previous HIV +ve husband.
[b]IQR, interquartile-range.
[c]Data not available on two concordant couples.

TABLE 2. DISTRIBUTION OF GENOTYPES [NO. (%)] OF POLYMORPHISMS IN DC-SIGN AND DC-SIGNR AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		HIV-seronegative			*HIV-seropositive*			*Blood donor*
		Male (n = 28)	*Female (n = 74)*	*Total (n = 102)*	*Male (n = 161)*	*Female (n = 144)*	*Total (n = 305)*	*Total (n = 209)*
DC-SIGN								
Pomoter region								
−139 (rs2287886)	A/A	16 (57.14)	31 (41.89)	47 (46.08)	64 (39.75)	74 (51.39)	138 (45.25)	146 (50.34)
	A/G	9 (32.14)	35 (47.30)	44 (43.14)	86 (53.42)	56 (38.89)	142 (46.56)	120 (41.38)
	G/G	3 (10.71)	8 (10.81)	11 (10.78)	11 (6.83)	14 (9.72)	25 (8.20)	24 (8.28)
−336 (rs4804803)	A/A	22 (78.57)	59 (79.73)	81 (79.41)	119 (73.91)	121 (84.03)	240 (78.69)	237 (81.72)
	A/G	6 (21.43)	13 (17.57)	19 (18.63)	40 (24.84)	21 (14.58)	61 (20.00)	51 (17.59)
	G/G	0 (0.00)	2 (2.70)	2 (1.96)	2 (1.24)	2 (1.39)	4 (1.31)	2 (0.69)
Exon 4	7/5	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
69-bp repeat numbers	7/7	28 (100.00)	73 (98.65)	101 (99.02)	160 (99.38)	144 (100.00)	304 (99.67)	288 (99.31)
	8/7	0 (0.00)	1 (1.35)	1 (0.98)	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.69)
DC-SIGNR								
Exon 5 (rs2277998)	G/G	19 (67.86)	46 (62.16)	65 (63.73)	111 (68.94)	115 (79.86)	226 (74.10)	215 (74.14)
	A/G	9 (32.14)	28 (37.84)	37 (36.27)	45 (27.95)	25 (17.36)	70 (22.95)	66 (22.76)
	A/A	0 (0.00)	0 (0.00)	0 (0.00)	5 (3.11)	4 (2.78)	9 (2.95)	9 (3.10)
Exon 4	5/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	4 (2.78)	8 (2.62)	11 (3.79)
69-bp repeat numbers	6/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	0 (0.00)	4 (1.31)	3 (1.03)
	6/6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	7/5	6 (21.43)	21 (28.38)	27 (26.47)	33 (20.50)	19 (13.19)	52 (17.05)	47 (16.21)
	7/6	3 (10.71)	6 (8.11)	9 (8.82)	12 (7.45)	8 (5.56)	20 (6.56)	23 (7.93)
	7/7	9 (32.14)	22 (29.73)	31 (30.39)	52 (32.30)	62 (43.06)	114 (37.38)	133 (45.86)
	8/5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	8/7	1 (3.57)	1 (1.35)	2 (1.96)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
	9/5	3 (10.71)	7 (9.46)	10 (9.80)	8 (4.97)	4 (2.78)	12 (3.93)	8 (2.76)
	9/6	1 (3.57)	0 (0.00)	1 (0.98)	4 (2.48)	1 (0.69)	5 (1.64)	0 (0.00)
	9/7	5 (17.86)	14 (18.92)	19 (18.63)	39 (24.22)	40 (27.78)	79 (25.90)	56 (19.31)
	9/9	0 (0.00)	3 (4.05)	3 (2.94)	4 (2.48)	5 (3.47)	9 (2.95)	7 (2.41)
	10/7	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.69)	1 (0.33)	0 (0.00)

TABLE 3. COMPARISON OF PROPORTIONS [ODDS RATIO (95% CI) AND p VALUE] OF INDIVIDUALS POSSESSING CERTAIN GENOTYPE BETWEEN HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		Total		*Male*		*Female*	
Polymorphism	*Genotype*	*OR (95% CI)*	p	*OR (95% CI)*	p	*OR (95% CI)*	p
DC-SIGN							
−139 (rs2287886)	G/G and A/G	1.03 (0.64, 1.66)	0.8838	2.02 (0.84, 4.91)	0.0856	0.68 (0.37, 1.25)	0.1839
−336 (rs4804803)	G/G and A/G	1.04 (0.58, 1.89)	0.8769	1.29 (0.47, 4.17)	0.6012	0.75 (0.34, 1.64)	0.4283
69-bp repeat numbers	7/7	0.33 (0.00, 26.34)	0.4389	Undefined	1.0000	0.00 (0.00, 20.04)	0.3394
DC-SIGNR							
Exon 5 (rs2277998)	A/A and G/G	0.61 (0.37, 1.02)	0.0445	0.95 (0.37, 2.46)	0.9088	0.41 (0.21, 0.81)	0.0049
69-bp repeat numbers	5/5	Undefined	0.2097	Undefined	1.0000	Undefined	0.3022
	6/5	Undefined	0.5760	Undefined	1.0000	—	—
	6/6	—	—	—	—	—	—
	7/5	0.57 (0.32, 1.01)	0.0373	0.95 (0.34, 3.9)	0.9105	0.38 (0.18, 0.82)	0.0061
	7/6	0.73 (0.30, 1.79)	0.4412	0.67 (0.16, 3.97)	0.4699	0.67 (0.19, 2.44)	0.5613
	7/7	1.37 (0.82, 2.28)	0.2022	1.01 (0.40, 2.60)	0.9870	1.79 (0.94, 3.40)	0.0556
	8/5	—	—	—	—	—	—
	8/7	0.16 (0.00, 3.21)	0.1562	0.17 (0.00, 13.73)	0.2750	0.00 (0.00, 20.04)	0.3394
	9/5	0.38 (0.15, 0.98)	0.0232	0.44 (0.10, 2.73)	0.2112	0.27 (0.06, 1.13)	0.0477
	9/6	1.68 (0.19, 80.39)	1.0000	0.69 (0.06, 35.12)	0.5556	Undefined	1.0000
	9/7	1.53 (0.84, 2.78)	0.1369	1.47 (0.50, 5.28)	0.4619	1.65 (0.79, 3.47)	0.1513
	9/9	1.00 (0.24, 5.88)	1.0000	Undefined	1.0000	0.85 (0.16, 5.64)	1.0000
	10/7	Undefined	1.0000	—	—	Undefined	1.0000

As for the *DC-SIGNR* 69-bp repeat number, we found a variation from 5 to10 repeats with 13 genotypes. The most common genotype in our study population was the 7/7 repeat, followed by the 9/7 and 7/5 repeats. We found that HIV-seronegative individuals had a significantly higher frequency of possessing the heterozygous 7/5 or 9/5 repeat than HIV-seropositive individuals ($p = 0.037$ and 0.023, respectively). Interestingly, we did not find any individuals with three or four repeats, which were found occasionally in whites. Instead we found one individual with 10 repeats that had not been reported elsewhere. For the SNP in *DC-SIGNR* Exon 5 (rs2277998), the proportion of individuals possessing the A allele (genotype A/A and A/G) was significantly higher in HIV-seronegative than that in HIV-seropositive individuals ($p = 0.0445$).

We further analyzed these associations after stratifying the study population into male and female groups (Table 3). Interestingly, we found that these associations remained significant in females ($p = 0.0061$ for the 7/5 repeat, $p = 0.0477$ for the 9/5 repeat, and $p = 0.0049$ for the A allele at the SNP rs2277998A) but not in males ($p = 0.9105$ for the 7/5 repeat, $p = 0.2112$ for the 9/5 repeat, and $p = 0.9088$ for the A allele at the SNP rs2277998A). Moreover, we found that HIV-seropositive females tend to have the homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance ($p = 0.0556$). Again, this association was not found in males.

TABLE 4. DISTRIBUTION OF DC-SIGNR HAPLOTYPE [NO. (%)] AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS AND BLOOD DONORS

	HIV-seronegative			*HIV-seropositive*			*Blood donor*
DC-SIGNR haplotype[a]	*Male (n = 56)*	*Female (n = 148)*	*Total (n = 204)*	*Male (n = 322)*	*Female (n = 288)*	*Total (n = 610)*	*(n = 580)*
5A	9 (16.07)	28 (18.92)[b]	37 (18.14)	51 (15.84)	30 (10.42)[b]	81 (13.28)	77 (13.28)
6A	0 (0.00)	0 (0.00)	0 (0.00)	3 (0.93)	3 (1.04)	6 (0.98)	1 (0.17)
7A	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.31)	1 (0.35)	1 (0.16)	2 (0.34)
5G	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.62)	1 (0.35)	3 (0.49)	5 (0.86)
6G	4 (7.14)	6 (4.05)	10 (4.90)	17 (5.28)	6 (2.08)	23 (3.77)	26 (4.48)
7G	33 (58.93)	86 (58.11)	119 (58.33)	188 (58.39)	190 (65.97)	379 (62.13)	390 (67.24)
8G	1 (1.79)	1 (0.68)	2 (0.98)	1 (0.31)	1 (0.35)	2 (0.33)	1 (0.17)
9G	9 (16.07)	27 (18.24)	36 (17.65)	59 (18.32)	55 (19.10)	114 (18.69)	78 (13.45)
10G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.35)	1 (0.16)	0 (0.00)

[a]Haplotypes were constructed from repeat number of 69-bp (Exon 4) and A/G allele at rs227799 (Exon 5).
[b]HIV-seronegative female versus HIV-seropositive female; $p = 0.0133$, OR (95% CI) = 0.50 (0.27, 0.90).

We also analyzed frequencies of the *DC-SIGNR* haplotypes constructed from a 69-bp repeat number in Exon 4 and an allele at rs227799 in Exon 5 in relation to HIV infection as summarized in Table 4. We found nine haplotypes in our study population. The 7G was the most common haplotype in all groups. The 5A was the second most common haplotype in the HIV-seronegative group whereas the 9G was the second most common haplotype in the HIV-seropositive and blood donor groups. We found that the proportion of individuals possessing the 5A haplotype was significantly higher in HIV-seronegative females than HIV-seropositive females [$p = 0.0133$, OR (95% CI) = 0.50 (0.27,0.90)].

DISCUSSION

We identified the polymorphisms at the two loci in *DC-SIGNR* that showed a statistically significant association with Thai HIV-seronegative individuals of HIV-seropositive spouses, especially among females. Our data on their marital history and sexual behavior indicated that most of these seronegative females were exposed to HIV repeatedly. There were four HIV-seronegative females reporting condom use at every contact. However, three of the four women had had a child with their HIV-infected index case, indicating that there was a possibility of HIV exposure. Thus we included these women in our analysis. We have also done the analysis excluding this group, but the conclusions remain the same. Therefore we think that *DC-SIGNR* may play an important role in conferring resistance to HIV infection.

The previous study from the United States showed that the heterozygous 7/5 repeat in *DC-SIGNR* was associated with resistance to HIV infection, whereas the homozygous 7/7 repeat was associated with susceptibility to HIV infection.[19] Our study is the first conducted in Asia showing consistent results with the American study, although in our study the significance of the association between the homozygous 7/7 repeat and HIV infection was marginal and was found only in females. In another published study, however, such associations of repeated number with susceptibility to HIV infection were not shown.[20] We think this is because the comparison was made only with the HIV-negative general population but not with ESN. In our study population as well, we did not find a significance difference between HIV-seropositive individuals and HIV-negative blood donors. Gramberg *et al.*[21] investigated the effect of polymorphisms in the *DC-SIGNR* neck domain on the interaction with HIV the envelope protein in *in vitro* experiments, but they found that coexpression of seven repeats with five repeats did not decrease the interaction with HIV compared with seven repeats only. However, there remains a possibility that they did not show the inhibitory effect because their experiments were conducted under high level expression of the gene.

Our study is the first showing associations of the other two polymorphisms that are heterozygous 9/5 repeat and A allele at the SNP site rs2288997 in Exon 5 of *DC-SIGNR* with HIV-seronegative individuals. The A-to-G change at this SNP site is particularly interesting as it causes an aspatate-to-asparagine substitution in the carbohydrate recognition domain (CRD). This amino acid change may affect the binding affinity of CRDs to HIV-1 gp120 and/or ICAM-3. But the relevance of this genetic polymorphism to HIV infection has not yet been investigated in *in vitro* experiments. Since we found a significant linkage between the A allele in Exon 5 and five repeats in Exon 4, the association of the 7/5 and 9/5 repeat with HIV-seronegative individuals may merely be due to a confounding effect by the A allele in Exon 5 and it may be the polymorphism in the CRD coding region that truly affects susceptibility to HIV infection. It is also possible that these two polymorphisms reported here are in linkage disequilibrium with another variant elsewhere in this region that is actually responsible for the observed protective effect.

In our study population, we found that these associations with polymorphisms in *DC-SIGNR* were not observed when only males were included in the analysis. Instead, the stronger associations were observed when only females were analyzed than when males were combined. We have two possible reasons for this difference. First, there might be a different mechanism of HIV infection between female and male, and *DC-SIGNR* plays a role only in female HIV infection. Second, according to our information on marital history, the duration of marriage before the disclosure of HIV status was much longer in females than in males and one-third of HIV-negative males had known the HIV status of their wives before marriage. Therefore, a considerable proportion of HIV-seronegative males was unlikely to have been highly exposed to HIV; thus they may still be susceptible to HIV infection. In fact, our follow-up data of their serostatus showed a three times higher seroconversion rate in male seronegative individuals than female seronegative individuals (data not shown).

We found that the repeat number of *DC-SIGN* in Thais was highly conserved in the homozygous 7/7 repeat and was not associated with susceptibility to HIV infection as showed in the previous study.[22] We did not find any association between polymorphisms in the *DC-SIGN* promoter (−139A/G and −336A/G) and susceptibility to HIV infection in our study group, whose risk for acquiring HIV infection was heterosexual contact. This finding confirmed the previously reports, which showed an association of −336G with risk for parenteral risk, but not mucosal risk for HIV infection.[23]

Although *DC-SIGN* and *DC-SIGNR* are quite similar in amino acid sequences and both have a binding ability to carbohydrate ligands, there are differences in their characteristics, including expression distribution,[16,24] carbohydrate binding profiles,[25–29] alternatively splicing,[17,30] and level of polymorphism in repeat numbers.[13,19,21,29] Thus it is plausible that they may play a different role in HIV infection. *DC-SIGNR* expression at mucosal sites (vaginal and rectal) has been found to have an alternative splicing that produces predicted soluble isoforms of *DC-SIGNR* molecules.[17] This soluble isoform may modulate the efficiency of viral transmission and dissemination.[17] Our experiment in monocyte-derived DCs cultured *in vitro* revealed the expression of *DC-SIGNR* by nested RT-PCR (data not shown). The 375-bp nested PCR product had 100% identity to the *DC-SIGNR* mRNA isoform I [variant 1 (NM_04257) and variant 2 (NM_214675)] and isoform II [variant 3 (NM_214676)]. Further *in vivo* and *in vitro* studies are warranted to investigate the mechanisms of their functions.

ACKNOWLEDGMENTS

We are grateful to all the participants of the Lampang cohort and blood donors. We also thank Ms. Wimala Inunchot,

Ms. Suthira Kasemsuk, Ms. Sriprai Senecwong-na-ayuthaya, Ms. Anong-nard Suyasarojna, Ms. Nutira Boonna, and Mr. Prapan Wongnamnong for their technical assistance. This study was supported by The Ministry of Public Health Thailand, The Japan International Cooperation Agency, and The Japanese Foundation for AIDS Prevention.

REFERENCES

1. Fowke KR, Nagelkerke NJ, Kimani J, *et al.*: Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet 1996;348:1347–1351.
2. Mann JM, Quinn TC, Francis H, Nzilambi N, Bosenge N, Bila K, *et al.*: Prevalence of HTLV-III/LAV in household contacts of patients with confirmed AIDS and controls in Kinshasa, Zaire. JAMA 1986;256:721–724.
3. O'Brien SJ and Nelson GW: Human gene that limit AIDS. Nat Genet 2004;36:565–573.
4. Dean M, Carrington M, Winkler C, *et al.*: Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science 1996;273:1856–1862.
5. Liu R, Paxton WA, Choe S, *et al.*: Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 1996;86:367–377.
6. Samson M, Libert F, Doranz BJ, *et al.*: Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 1996;382:722–725.
7. Huang Y, Paxton WA, Wolinsky SM, *et al.*: The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 1996;2:1240–1243.
8. Martinson JJ, Chapman NH, Rees DC, Liu YT, and Clegg JB: Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet 1997;16:100–103.
9. Jiang JD, Wang Y, Wang ZZ, *et al.*: Low frequency of the CCR5delta32 HIV-resistance allele in mainland China: Identification of the first case of CCR5delta32 mutation in the Chinese population. Scand J Infect Dis 1999;31:345–348.
10. Ruchusatsawat N, Vongsheree S, Thaisri H, and Phutiprawan T: The first report of CCR5 delta 32 mutant in Thai injecting drug users. Asian Pac J Allergy Immunol 2000;18:93–98.
11. Geijtenbeek TB, Kwon DS, Torensma R, *et al.*: DC-SIGN, a dendritic cell specific HIV-1-binding protein that enhances trans-infection of T-cell. Cell 2000;100:587–597.
12. Pohlmann S, Soilleux EJ, Baribard F, *et al.*: DC-SIGNR, a DC-SIGN homologue expressed in endothelial cells, binds to human and simian immunodeficiency viruses and activates infection in trans. Proc Natl Acad Sci USA 2001;98:2670–2675.
13. Bashirova AA, Geijtenbeek TB, van Duijnhoven GC, *et al.*: A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. J Exp Med 2001;193:671–678.
14. Soilleux EJ, Morris LS, Leslie G, *et al.*: Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. J Leukoc Biol 2002;71:445–457.
15. Granelli-Piperno A, Pritsker A, Pack M, *et al.*: Dendritic cell-specific intercellular adhesion molecule 3-grabbling nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. J Immunol 2005;175:4265–4273.
16. Soilleux EJ, Morris LS, Rushbrook S, Lee B, and Coleman N: Expression of human immunodeficiency virus (HIV)-binding lectin DC-SIGNR: Consequences for HIV infection and immunity. Hum Pathol 2002;33:652–659.
17. Liu H, Hladik F, Andrus T, *et al.*: Most DC-SIGNR transcripts at mucosal HIV transmission sites are alternatively spliced isoforms. Eur J Hum Genet 2005;13;707–715.
18. Kobayashi N, Nakamura HT, Goto M, *et al.*: Polymorphisms and haplotypes of the CD209L gene and their association with the clinical courses of HIV-positive Japanese patients. Jpn J Infect Dis. 2002;55(4):131–133.
19. Liu H, Carrington M, Wang C, Holte S, *et al.*: Repeat-region polymorphisms in the gene for the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related molecule: Effects in HIV-1 susceptibility. J Infect Dis 2006;193:698–702.
20. Lichterfeld M, Nischalke HD, van Lunzen J, *et al.*: The tandem-repeat polymorphism of the DC-SIGNR gene does not affect the susceptibility to HIV infection and the progression to AIDS. Clin Immunol 2003;107:55–59.
21. Gramberg T, Zhu T, Chaipan C, *et al.*: Impact of polymorphisms in the DC-SIGNR neck domain on the interaction with pathogens. Virology 2006;347:354–363.
22. Liu H, Hwangbo Y, Holte S, *et al.*: Analysis of genetic polymorphism in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. J Infect Dis 2004;190:1055–1058.
23. Martin MP, Lederman MM, Hutcheson HB, *et al.*: Association of DC-SIGN promoter polymorphism with increased risk for parenteral, but not mucosal, acquisition of human immunodeficiency virus type 1 infection. J Virol 2004;78:14053–14056.
24. Soilleux EJ: DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) and DC-SIGN-related (DC-SIGNR): Friend or foe? Clin Sci 2003;104:437–446.
25. Feinberg H, Mitchell DA, Drickamer K, and Weis WI: Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. Science 2001;294:2163–2166.
26. Mitchell DA, Fadden AJ, and Drickamer K: A novel mechanism of carbohydrate recoginition by the C-type lectins DC-SIGN and DC-SIGNR. J Biol Chem 2001;276:28939–28945.
27. Guo Y, Feinberg H, Conroy E, *et al.*: Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat Struct Mol Biol 2004;11:591–598.
28. Van Liempt E, Imberty A, Bank CM, *et al.*: Molecular basis of the differences in binding properties of the highly related C-type lectins DC-SIGN and L-SIGN to Lewis X trisaccharide and Schistosoma mansoni egg antigens. J Biol Chem 2004;279:33161–33167.
29. Davis CW, Nguyen HY, Hanna SL, Sanchez MD, Doms RW, and Pierson TC: West Nile virus discrimination between DC-SIGN and DC-SIGNR for cellular attachment and infection. J Virol 2006; 80:1209–1301.
30. Mummidi S, Catano G, Lam L, *et al.*: Extensive repertoire of membrane-bound and soluble dendritic cell-specific ICAM-3-grabbing nonintegrin 1 (DC-SIGN1) and DC-SIGN2 isoforms. J Biol Chem 2001;276:33196–33212.

Address reprint requests to:
Nuanjun Wichukchinda
National Institute of Health
Department of Medical Sciences
Ministry of Public Health
88/7 Tiwanond Road
Muang Nonthaburi, 11000
Thailand

E-mail: nuanjun@health.moph.go.th

Published online 16 April 2007

Nucleic Acids Research, 2007, Vol. 35, No. 9 ***2955–2964***
doi:10.1093/nar/gkm181

All APOBEC3 family proteins differentially inhibit LINE-1 retrotransposition

Masanobu Kinomoto[1], Takayuki Kanno[1], Mari Shimura[2], Yukihito Ishizaka[2], Asato Kojima[1], Takeshi Kurata[1], Tetsutaro Sata[1] and Kenzo Tokunaga[1,*]

[1]Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan and
[2]Department of Intractable Diseases, International Medical Center of Japan, Tokyo, 162-8655, Japan

Received January 29, 2007; Revised February 25, 2007; Accepted March 13, 2007

ABSTRACT

Approximately 17% of the human genome is comprised of long interspersed nuclear element 1 (LINE-1, L1) non-LTR retrotransposons. L1 retrotransposition is known to be the cause of several genetic diseases, such as hemophilia A, Duchene muscular dystrophy, and so on. The L1 retroelements are also able to cause colon cancer, suggesting that L1 transposition could occur not only in germ cells, but also in somatic cells if innate immunity would not function appropriately. The mechanisms of L1 transposition restriction in the normal cells, however, are not fully defined. We here show that antiretroviral innate proteins, human APOBEC3 (hA3) family members, from hA3A to hA3H, differentially reduce the level of L1 retrotransposition that does not correlate either with antiviral activity against Vif-deficient HIV-1 and murine leukemia virus, or with patterns of subcellular localization. Importantly, hA3G protein inhibits L1 retrotransposition, in striking contrast to the recent reports. Inhibitory effect of hA3 family members on L1 transposition might not be due to deaminase activity, but due to novel mechanism(s). Thus, we conclude that all hA3 proteins act to differentially suppress uncontrolled transposition of L1 elements.

INTRODUCTION

Human APOBEC3G (hA3G) is known to be a powerful innate antiretroviral factor, which can suppress Vif (virion infectivity factor)-deficient human immunodeficiency virus type 1 (HIV-1) infection by deaminating viral minus-strand DNA during reverse transcription, resulting in G-to-A hypermutation (1–4). This cytidine deaminase targets not only on retroviruses, such as simian immunodeficiency virus (5–8), primate foamy virus (9,10), human T-cell leukemia virus type I (11) and murine leukemia virus (MuLV (2,7,12)), but also on Hepatitis B virus (13,14) which has a reverse transcription step. Recent findings have revealed that hA3G also restricts transposition of murine MusD and intracisternal A-particle long terminal repeat (LTR) retrotransposons (15). hA3G is one of the APOBEC3 (A3) family containing A3A to A3H in human. Vertebrates from fish to birds do not encode any of A3 proteins (16), while mammals before primates encode a single or two to three A3 proteins (17). Primates have acquired seven tandem A3 genes throughout 33 million years of evolution (18), implying the ancient history of battles between primates and retroelements.

In human genome, retrotransposons have accumulated up to ~42%, divided into two classes, which are non-LTR, and LTR retrotransposons (19). Non-LTR types are subdivided into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). The most common human LINE, called LINE-1 (L1), accounts for ~17% of the human genome, corresponding to 500 000 copies, out of which 100 copies are known to be full-length and transposition-competent (19–21). This non-LTR retrotransposon is 6-kb long, and consists of two open reading frames (ORFs) called ORF1 and ORF2 (22). ORF1 encodes an RNA-binding protein ORF1p (23), and ORF2 encodes an enzymatic protein ORF2p like Pol of retroviruses, carrying an endonuclease (24) and a reverse-transcriptase (25,26), both of which are enzymatically active. Importantly, L1 elements can cause several genetic diseases by *de novo* L1 insertion at dispersed positions in the germinal chromosomes, such as the Factor VIII gene in patients with hemophilia A (27,28), the Factor IX gene in patients with hemophilia B (29), the dystrophin gene in patients with Duchene muscular dystrophy (30,31), and with X-linked dilated cardiomyopathy (32), the β-globin gene in patients with β-thalassaemia (33), the CYBB gene in patients with chronic granulomatous disease (34), and the RP2 gene in patients with type-2 retinitis pigmentosa (35). The fact that colon cancer is caused by L1 insertion into the APC genes of somatic chromosomes (36), indicates that, L1 could be

*To whom correspondence should be addressed. Tel: +81 3 5285 1111; Fax:+81 3 5285 1189; Email: tokunaga@nih.go.jp