化誘導能を持つことが、熱力学的変化の測定により確認されている(Schon A, *Biochemistry*, 45:10973-80, 2006)。このことは、FACS解析でNBD-556がsCD4と同様にCD4i抗体の結合を増強することで確認し昨年度報告した。そこで、今年度はHIV-1のエンベロープに立体構造変化を起こさせる低分子化合物NBD-556が、gp120のどの部位と結合しているかを我々が新規に樹立したin vitro耐性誘導システムを用いて、化合物に耐性のウイルスをとり、sCD4の耐性ウイルスとシークエンスを比較検討した。PM1/CCR5細胞にIIIBウイルスを感染させ、NBD-556またはsCD4をそれぞれ1mM及び1mg/ml存在下に培養を開始した。NBD-556は50mM (21 passage)、sCD4は20mg/ml (5 passage)まで培養を続け、パッセージごとに感染細胞のproviral DNAからPCRによりシークエンスを行い、変異部位の同定を行った。それぞれの耐性誘導で得られた変異部位を比較すると、NBD-556は50mM存在下でEnvのC3領域(S375N)とC4領域(A433T)に変異がみられた。一方、sCD4による耐性誘導では、経過中gp120に6つの変異が確認でき(P212L、V255E、N280K、S375N、G380R、G431E)、最終的に20mM存在下でV255E、G380R、G431Eの3つが残った。それぞれの変異部位をgp120の3次元結晶解析図上にプロットすると、NBD-556とsCD4の変異部位がそれぞれの結合部位を取り巻くように位置していることがわかった。次に、これらの変異が実際にNBD-556とsCD4に対する耐性に関与しているかどうかを検索するために、S375N、A433T、V255E変異Envを持つpseudovirusを作成し、NBD-556とsCD4に対する感受性をsingle-round assayで調べた。その結果、NBD-556はすべての変異ウイルスに対して耐性となり、sCD4はV255Eを持つウイルスに対しては高度耐性であるが、NBD耐性Env clonesに対してはIC_{50}の値で数倍耐性を示した。NBD-556はsCD4に比べると、gp120に対する結合がやや弱い可能性が示唆された。

D.考察

V3に対する中和抗体の抗ウイルス効果を増強するような立体構造変化を引き起こす機能を持つ分子量350前後の低分子化合物NBD-556は、in vitro耐性誘導実験の結果sCD4の結合部位に近い場所に結合していることが分かった。このことは、NBDのどの部位が立体構造の変化に強く関与しているかを知る上で、非常に大きな知見となった。現在進行中であるNBD compoundの誘導体の設計の上で大変有用な情報となることは確かである。実際、現在10以上のNBD誘導体を今回の結果をもとに合成している。今後、これらの中からより低濃度で、強力に中和抗体の感受性を増強し、細胞毒性も低いものを追求していく。

E. 結論

本年度の研究成果として、耐性誘導の結果から、NBD-556はsCD4と非常に近い形で結合していることが示唆された。今後、毒性が低くより効果の強い誘導体の開発を目指していく予定である。

F. 健康危惧情報

なし

G. 研究発表

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H.知的所有権の出願・取得状況

厚生労働科学研究費補助金（創薬等ヒューマンサイエンス総合研究事業）

分担研究報告書

非B型HIV-1感染小児のエイズ発症に影響を及ぼす因子に関する研究
－非B型HIV-1感染小児におけるHIV-1エンベロープ遺伝子の変化－

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研究要旨

HIV-1感染者の約半分でHIV-1の使用するコレセプターがCCR5からCXCR4に変化する。本研究では、このようなHIV-1のコレセプター使用の変更が抗レトロウイルス療法（ART）によって促進されるかどうかを明らかにすることを目的とした。2000年からケニアで追跡調査中のHIV-1感染小児81人から年2回採取・保存された血漿検体を用い、ウイルスEnv-V3アミノ酸配列を経時的に解析した。ART加療中の小児41人中35人の治療前のウイルスはCCR5をコレセプターとして使用していた。そのうち7人（20%）で追跡期間中にHIV-1のコレセプター使用の変更がみられた。ART開始からコレセプター使用変更までの期間は平均2.6年（0.5-5.2年）であった。未治療小児40人中32人の追跡開始時のウイルスはCCR5をコレセプターとして使用しており、3人（9.4%）で追跡期間中にHIV-1のコレセプター使用変更がみられた。コレセプター使用変更時の平均年齢は7.3歳（ART群）と9.7歳（未治療群）であった。コレセプター使用の変更率（p=0.38）、変更時の年齢（p=0.31）には両群間で有意な差は認められなかった。HIV-1感染小児のうち10人が5歳までにARTを開始していたが（早期進行群）、23人は10歳まではARTを必要としなかった（遅進行群）。早期進行群では遅進行群に比べ高率にHIV-1のコレセプター使用の変更がみられた（40% vs. 8.7%, p=0.053）。以上の結果より、HIV-1感染小児ではウイルスのCCR5からCXCR4へのコレセプター使用の変更はARTではなく、病態促進に関与する因子に影響を受けていることが示唆された。

A. 研究目的

HIV-1の使用するコレセプターの種類はウイルスの細胞指向性や病原性に重要な役割を果たしており、HIV-1感染者の自然経過では約半分でHIV-1の使用するコレセプターがCCR5からCXCR4に変化することが知られている。また、有効な抗レトロウイルス療法（ART）を受けている患者でウイルスエンベロープ遺伝子の有意な進化がみられることが報告されている。

今回の研究では、非Bサブタイプ HIV-1に感染しているケニアの母子感染児においてHIV-1のコレセプター使用の変更がARTによって促進されるかどうかを明らかにする事を目的とした。

B. 研究方法

対象：ケニアのN孤児院で生活しているHIV-1母子感染小児95人を追跡調査している。2000年から6ヶ月に一度採血を行い、血漿を保存している。今回、2007年8月までに最低3回追跡調査出来た81人を対象とした。41人がART（核酸系逆転写阻害剤2剤と非核酸系逆転写阻害剤1剤、平均治療期間：7.6年）を受けており、残り40人は未治療であった。

方法：これらの児から経時的に採取された血漿中のHIV-1 *env*-C2V3領域をRT-PCRで増幅し、直接またはクローニングした後、塩基配列およびアミノ酸配列を解析した。HIV-1のコレセプター使用は、*env*-V3領域のアミノ酸配列により予想した。V3ループの電荷が＋6以上をR5ウイルス（CCR5をコレセプターとして使用するHIV-1）、＋4以下をX4ウイルス（CXCR-4をコレセプターとして使用するHIV-1）、＋5の場合、V3ループの11番目と25番目のアミノ酸がアルギニンまたはリジンでなければR5ウイルスとした。

倫理面への配慮

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C. 研究結果

HIV-1コレセプター使用

ART加療中の小児41人中35人の治療前のウイルスはCCR5をコレセプターとして使用していた。平均年齢、血中ウイルス量、$CD4^+T$細胞数はそれぞれ5.5歳(1-12歳)、

5.2 x10^6コピー/ml、537/mm^3であった。そのうち7人（20%）で追跡期間中にCCR5からCXCR4へのHIV-1コレセプター使用の変更がみられた。ART開始からコレセプター使用変更までの期間は平均2.6年（0.5-5.2年）であった（表1）。加療中の残りの6人のウイルスは治療前からCXCR4をコレセプターとして使用していた。

未治療小児40人中32人の追跡開始時のウイルスはCCR5をコレセプターとして使用していた。平均年齢、血中ウイルス量、$CD4^+$T細胞数はそれぞれ8.0歳(3-19歳)、4.8 x10^6コピー/ml、684/mm^3であった。そのうち3人（9.4%）で追跡期間中にHIV-1のコレセプター使用変更がみられたが、29人のウイルスはCCR5のままであった。残りの8人のウイルスのコレセプター使用は追跡開始時からCXCR4のままであった（表1）。

コレセプター使用変更時の平均年齢は7.3歳（ART群）と9.7歳（未治療群）であった。コレセプター使用の変更率（p=0.38）、変更時の年齢（p=0.31）には両群間で有意な差は認められなかった。

コレセプター使用変更がみられたHIV-1感染小児10人のウイルスのenv-V3領域のアミノ酸配列の経時的変化を図1に示した。

病態進行速度の違いとHIV-1コレセプター使用の変更

HIV-1感染小児のうち10人が5歳までにARTを開始していたが（早期進行群）、23人は10歳まではARTを必要としなかった（遅進行群）。早期進行群では遅進行群に比べ高率にHIV-1のコレセプター使用の変更がみられた(40% vs. 8.7%, p=0.053)（表2）。

HIV-1感染サブタイプ

児に感染しているHIV-1のサブタイプ（*env*-C2V3領域）は、A1（65例）、A2（4例）、C（2例）、D（9例）とCRF02_AG（1例）であった。HIV-1のsubtype/CRFとコレセプター使用の間には有意な相関は認められなかった。

D. 考察

非B亜型HIV-1感染小児ではウイルスのCCR5からCXCR4へのコレセプター使用の変更は抗レトロウイルス療法ではなく、病態促進に関与する因子に影響を受けてい

ることが示唆された。

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F. 知的財産権の出願・登録状況

特に予定なし。

Table 1: Characteristics of the study children at baseline, ART start and co-receptor switch

	Co-receptor usage	Baseline			ART Start			Co-receptor switch			
		Mean (range)			Mean (range)			Mean (range)			
		Viral Load[a]	CD4+[b]	Age[c]	Viral Load[a]	CD4+[b]	Age[c]	Viral Load[a]	CD4+[b]	Age[c]	Duration of ART (Years)
ART	R5►R5 (n=28)	5.3 (3.9-6.1)	472 (6-1566)	5.7 (1-12)	5.3 (3.9-6.1)	447 (93-1340)	6.6 (1-12)				
	R5►X4 (n=7)*	4.8 (3.6-5.4)	479 (178-1760)	5.0 (1-8)	4.7 (3.6-5.4)	357 (147-1442)	4.7 (2.5-7)	4.8 (4.2-5.4)	677 (157-1439)	7.3 (3-12)	2.6 (0.5-5.2)
	X4►X4 (n=6)**	3.5 (2.3-5.0)	590 (17-1620)	11.2 (7-18)							
NO ART	R5►R5 (n=29)	4.8 (2.3-6.0)	697 (70-1637)	8.1 (2-19)							
	R5►X4 (n=3)	4.4 (3.3-5.2)	573 (338-700)	7.7 (5-11)				4.8 (4.6-5.1)	462 (411-550)	9.7 (7-12)	
	X4►X4 (n=8)	4.4 (2.9-5.3)	716 (345-1570)	8.6 (6-13)							

* One of the 7 children had already received ART at baseline, ** All the 6 children in this group had received ART at baseline.

[a]Log (copies/ml), [b]CD4+T cell count (cells/µl), c: years old, R5: CCR5, X4: CXCR4.

TABLE 2. HIV-1 co-receptor usage in association with the rate of disease progression

	Change in Co-receptor usage	Number of Children (on ART)	Mean Age (range) at ART start/recruitment	Mean Age (range) at switch	Children with co-receptor switch
Rapid Progressor	R5 → R5	6 (6)			
	R5 → X4	4 (4)	3.0 (1-4)	4.8 (3-6)	40%*
Slow Progressor	R5 → R5	21 (6)			
	R5 → X4	2 (0)	7.5 (6-9)	12.0 (10-14)	8.7%*

R5: CCR5, X4:CXCR4, *p=0.053

Child ID	Date of sample collection	V3 amino acid sequence 11 25	Viral load (log/ml)	net charge	11/25 amino acid	predicted phenotype
36m*	aug,02	CTRPGNNTRE[S]VRIGPGQAFYATN[D]VIGDIRQAHC	4.9	+3	S/D	R5
	apr,03	.I..S.....[.].....[.].....	5.8	+3	S/D	R5
	feb,04	.....R..[.].....[.].....	5.6	+4	S/D	R5
	Oct,05	......K..R..[.].....RV..T.N[V]IR.....	5.1	+7	S/V	X4
38m*	mar,03	....ST...N[.].....G[E]IT.....	4.6	+5	S/E	R5
	dec,02	....S....N[.].....G[E]IT.....	4.8	+5	S/E	R5
	feb,04	....SSP..TR.A..R.....S[R]IT.T..K.Y.	4.6	+6	R/A	X4
	sep,05	....S.P..RR.A.....S[R]IX.T..T.Y.	4.9	+6	R/A	X4
51m*	apr,03	....N....K[G].H.....S.FT.G[R]I.....K.Y.	5.3	+5	G/N	R5
	nov,04	....N....K[G].H.....S.FT.G[R]I.....K.Y.	5.2	+5	G/N	R5
	apr,05	....N....K[G].H.....SLFT.G[R]I.....K.Y.	5.5	+5	G/N	R5
	oct,05	....N....K[G].H.....SLFT.G[R]I..N..K.Y.	5.4	+6	G/N	X4
69m*	mar,03	.I..N....Q[G]H.....WV.N[.]...E....Y.	5.4	+4	G/D	R5
	may,04	.I..N....Q[G]H....R..WV..[K].V.IK...Y.	5.1	+7	G/K	X4
85f*	feb,03	....N....N[.].....T....G[.]IT.....	4.5	+4	S/D	R5
	dec,02	....N....K[.]H....RT....G[.]IT.....	3.5	+5	S/D	R5
	apr,04	....N....K[.]H....RT....G[.]IT.....	4.9	+5	S/D	R5
	apr,05	....N....K[.].....T....G[.]IT.....	4.3	+5	S/D	R5
	sep,05	....N....K[.]H....RT....G[.]I..N.....	4.5	+6	S/D	X4
89f*	feb,04	..S.T...SR[G]HM...RS....D[.]I..N.....	5.2	+5	G/D	R5
	jul,04	..SNTSS.SR[G]HM...RS....D[.]I..N.....	5.0	+5	G/D	R5
	mar,05	..S.T...SR[G]HM...RS....D[.]I..N.....	5.5	+5	G/D	R5
	oct,05	..SRT...SR[G]MM..LRS....D[R]I..N.....	4.8	+6	G/R	X4
91f*	mar,05	....N....K[.]HF.....L.T.D[N]I..N....Y.	4.9	+4	S/N	R5
	mar,06	....N....R[.]H.....L.T.N[R]I..N.....	4.2	+6	S/R	X4
	aug,06	....N....K[G]HF.....L.T.N[R]I....KK.Y.	4.0	+6	G/R	X4
21f**jul,03		....S....K[.]HL...R.....G[.]I.....	4.2	+5	S/D	R5
	dec,04	....N....K[.]HL...R.....G[R]I..N.....	4.7	+7	S/R	X4
	apr,06	....S....K[.]HL.A.R.....G[R]I.....	4.2	+6	S/R	X4
49f**aug,02		.S..S....N[.].....G[.]IV.....	4.2	+5	S/D	R5
	jun,03	.S.....N[.].....G[.]IV.....	4.5	+5	S/D	R5
	feb,04	.S.....N[.].....V...G[R]IV.....	4.1	+5	S/A	R5
	mar,05	.S.....N[.].H.....G[R]IV....R...	4.6	+6	S/A	X4
72f**Jul,02		.I.VN....Q[.]L.....MG[.]I..N..D...	5.2	+3	S/D	R5
	Jun,03	.I..Y.....G[T]HM...K.YFT...[.]I.....D...	5.1	+4	G/D	R5
	feb,04	.I..N....Q[.]N.....MG[.]I.....D...	NT	+2	S/D	R5
	sep,04	....N....N[.]HF.....L.TNM[T]I..N..D...	5.1	+6	S/I	X4

Figure 1: Changes in the HIV-1 V3 amino acid sequences during follow-up of the 10 children whose infected viruses had a switch from CCR5 to CXCR4 co-receptor usage. A net charge of less than and more than +5 in the V3 region was considered as CCR5-using (R5) and CXCR4-using (X4) variants, respectively, and a net charge of +5 was considered as R5 variants, unless this was accompanied by appearance of either an arginine or lysine residue at position 11 or 25 of the V3 amino acid sequences. *, **: Children whose HIV-1 showed a switch in co-receptor usage from CCR5 to CXCR4 with treatment (*) and without (**) treatment. NT: not tested.

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Comparison Between Sendai Virus and Adenovirus Vectors to Transduce HIV-1 Genes Into Human Dendritic Cells

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Immuno-genetherapy using dendritic cells (DCs) can be applied to human immunodeficiency virus type 1 (HIV-1) infection. Sendai virus (SeV) has unique features such as cytoplasmic replication and high protein expression as a vector for genetic manipulation. In this study, we compared the efficiency of inducing green fluorescent protein (GFP) and HIV-1 gene expression in human monocyte-derived DCs between SeV and adenovirus (AdV). Human monocyte-derived DCs infected with SeV showed the maximum gene expression 24 hr after infection at a multiplicity of infection (MOI) of 2. Although SeV vector showed higher cytopathic effect on DCs than AdV, SeV vector induced maximum gene expression earlier and at much lower MOI. In terms of cell surface phenotype, both SeV and AdV vectors induced DC maturation. DCs infected with SeV as well as AdV elicited HIV-1 specific T-cell responses detected by interferon γ (IFN-γ) enzyme-linked immunospot (Elispot). Our data suggest that SeV could be one of the reliable vectors for immuno-genetherapy for HIV-1 infected patients. ***J. Med. Virol. 80:373–382, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: viral vector; immuno-genetherapy; AIDS

INTRODUCTION

Introduction of highly active anti-retroviral therapy (HAART) has improved the clinical course of patients infected with human immunodeficiency virus type 1 (HIV-1) dramatically. However, there are many obstacles to the long-term administration of anti-retroviral drugs, such as metabolic disorders, emergence of drug resistant viruses, and high medical expenses. The combination of therapeutic vaccines and HAART could not only reduce the adverse effects of HAART but also decrease the medical expenses especially in developing countries.

Cellular immune responses play a crucial role in controlling the replication of HIV-1 [Yang et al., 1997;

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Matano et al., 1998; Brander and Walker, 1999; Jin et al., 1999; McMichael and Rowland-Jones, 2001]. One of the possible strategies for treating HIV-1 infection is to enhance the cellular anti-viral capacity. Since dendritic cells (DCs) have high ability of antigen presentation, they have been used as stimulators of T-cell responses by inserting HIV-1 antigens [Engelmayer et al., 2001; Stubbs et al., 2001; Tsunetsugu-Yokota et al., 2003; Mwau et al., 2004].

Sendai virus (SeV), one of the members of *Paramyxoviridae*, is an enveloped virus with a nonsegmented negative-strand RNA genome. It causes severe respiratory disease in mice but is nonpathogenic for humans. SeV vector has been developed and shown to have high gene transduction efficiency and protein expression in different cell lineages [Kato et al., 1996; Kawana-Tachikawa et al., 2002]. In addition, the cytoplasmic replication of SeV precludes the integration of its genetic information into cellular genome. Recently, the second generation SeV vector deficient in F gene (dF-SeV) has been developed [Li et al., 2000]. The dF-SeV has been proved not to cause secondary infection. SeV and dF-SeV vectors could be promising systems to introduce HIV-1 genes into DCs for stimulating HIV-1 specific T-cell responses in primates and humans [Kano et al., 2002; Takeda et al., 2003; Kato et al., 2005].

Adenovirus (AdV), vaccinia virus, and retrovirus have been used as viral vectors for gene transfer into DCs [Engelmayer et al., 1999; Rea et al., 1999; Bonini et al., 2001; Rouas et al., 2002]. In this study, we compared the efficiency of inducing green fluorescent protein (GFP) and HIV-1 gene expression in human monocyte-derived DCs between AdV vectors and new viral vectors based on SeV. We showed that SeV vector transduced DCs efficiently and elicited HIV-1 specific T-cell responses. Our results suggest the potential use of SeV vector for immuno-genetherapy for HIV-1 infected patients.

MATERIALS AND METHODS

Viral Vectors

SeV carrying GFP (SeVGFP) [Agungpriyono et al., 2000], HIV-1 NL4-3 Gag (SeVGag), and HIV-1 SF2 Env (SeVEnv) [Yu et al., 1997] were propagated in 10-day-old embryonated chicken eggs. The fusion protein (F)-defective SeV (dF-SeV) [Li et al., 2000] was propagated in the monkey kidney cell line expressing Sendai virus F gene product (LLC-MK2/F7) [Li et al., 2000] because the replication capacity of dF-SeV was incompetent. SeV particles were purified by 50% sucrose (w/v)/10 mM Tris–HCl and 30% sucrose (w/v)/10 mM Tris–HCl density centrifugation, dialyzed against Dulbecco's phosphate buffered saline (PBS) (Sigma, St. Louis, MO), and stored at −80°C. The titers of the vector stocks, determined on LLC-MK2 [Kato et al., 1996], were as follows; SeVGag: 5.2×10^8 CIU/ml, SeVEnv: 6.4×10^7 CIU/ml, SeVGFP: 5.2×10^8 CIU/ml, GFP/dF-SeV: 3.1×10^9 CIU/ml, SeV without inserts: 5.2×10^8 CIU/ml.

Recombinant AdV used in this experiment was derived from AdV type 5 and was replication-deficient with deletion of E1 and E3 genes. The AdV carrying HIV-1 genes (*gag-pol*, *env*, *rev*, and RRE) was generated with AdV Expression Kit (TakaraBio, Shiga, Japan) according to the manufacturer's protocol. Each HIV-1 gene was inserted into the expression cassette of pAxCAwt cosmid vector equipped with CAG promoter and rabbit beta-globin polyadenylation signal. All HIV-1 sequences were derived from SF2 strain [Levy et al., 1986]. 5′ half and 3′ half of *Eco*RI fragments from SF2 provirus were subcloned pUC19, generating pUC19-9B/R7 and pUC19-9B/R6, respectively. HIV-1 *gag-pol* gene was obtained from pUC19-9B/R7 after deleting the sequence upstream of *gag*. *Nar*I site was introduced next to gag initiation codon by PCR using primers *gag*/*Nar*I-S (5′-CAGGCGGCAAGGAGAGAGATGGGTGC-GAG-3′) and *gag*/*Apa*I-AS (5′-CCTTTTTCCTAGGGG-CCCTGC-3′) (restriction sites are underlined). PCR-amplified fragment was returned to *Nar*I and *Apa* I-digested pUC19-9B/R7, generating pUC19-GP. The 4.5 kb *Nar*I-*Nde*I fragment containing the HIV-1 *gag-pol* gene was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVGP. HIV-1 *rev* responsible element (RRE) was generated from pUC19-9B/R6 by PCR using primers rre/*pfl*MI-S (5′-GCCATAGAATG-GCCAAGGCAAAGAGAAGAGTGG-3′) and rre/*Bam*HI-AS (5′-GGGATCCCAAGGCACAGCAGTGGTTGC-3′). The PCR fragment was inserted between *pfl*MI and *Bam*HI site of pUC19-GP, and thus placed downstream of *gag-pol* gene. The consequent 4.9 kb *Nar*I-*Bam*HI fragment containing the HIV-1 *gag-pol*-RRE sequences was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVGPR. HIV-1 *rev* gene was made by two-step PCR from *Stu*I-*Xho*I fragment of pUC19-9B/R6. The 1st-PCR primer set was *rev*/1st-S (5′-CTCA-GGACAGTCAGACTCATCAAGCTTCTCTATCAAAG-CAACCCGCCTCC-3′) and *rev*-AS (5′-GGCTATTCTT-TAGTTCCTGAATCCAATACTGCA-3′), and the 2nd-PCR primer set was *rev*/2nd-S (5′-GGATGGCAGGAA-GAAGCGGAGACAGCGACGAAGAGCTCCTCAGGA-CAG-3′) and *rev*-AS. The PCR fragment was digested with *Sph*I and *Spe*I, and inserted into the *Swa*I site of pAxCAwt to create AdVRev. The absence of PCR errors was confirmed for all PCR-amplified fragments by sequencing. The 2.1 kb *Mlu*I-*Xho*I fragment of pUC19-9B/R6 containing the HIV-1 *env* gene was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVEnv. Recombinant AdV, Ax1w1 [Miyake et al., 1996] bearing no insert and AdVGFP [Miyake et al., 1996] expressing GFP, were kindly provided by Dr. Izumu Saito and Yumi Kanegae (University of Tokyo, Tokyo, Japan). AdV was propagated in 293 cells [Graham et al., 1977], purified by two rounds of CsCl density centrifugation [Kanegae et al., 1994], dialyzed against PBS containing 10% glycerol and stored at −80°C. The titer of the viral stocks was determined by a plaque-forming assay on 293 cells. The titers of each virus were as follows; AdVGPR: 7.6×10^9 pfu/ml, AdVEnv: 1.6×10^{11} pfu/ml, AdVRev: 2.7×10^{11} pfu/ml, AdVGFP: 5.9×10^{10} pfu/ml, Ax1w1: 4.7×10^{11} pfu/ml. Vector stocks were tested for the

absence of replication-competent AdV as described [Ishii-Watabe et al., 2003].

Cell Lines and Media

Monkey kidney cell line (LLC-MK2) was cultured in minimal essential medium (MEM) (Sigma) supplemented with 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS). Two hundred ninety three cells were cultured in Dulbecco's modified eagle medium (DMEM) (Sigma) supplemented with 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% FCS.

Generation of DCs

Immature myeloid DCs were generated from human peripheral blood mononuclear cells (PBMCs) using previously described methods [Nagayama et al., 2003]. Briefly, PBMCs of healthy adult volunteers were collected in heparinized tubes, subjected to density centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ), and washed twice with PBS. These PBMCs were plated on 10 cm PRIMARIA tissue culture dish (Becton Dickinson Labware, FranklinLakes, NJ) and kept at 37°C for 30 min to remove nonadherent cells. Floating cells were removed gently by rinsing with 10 ml of PBS three times and the remaining adherent cells were cultured overnight in 6 ml of RPMI 1640 medium at 37°C. Cells were washed three times again on the next day with 10 ml of PBS and the remaining adherent cells were cultured for 7 days in DC medium [6 ml of RPMI 1640 supplemented with 100 U of penicillin/ml, 10 mg of streptomycin/ml, and 10% FCS, 300 ng of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) (Wako, Osaka, Japan), and 300 ng of recombinant human interleukin-4 (rhIL-4) (Wako)]. After 7 days, DCs were collected with a scraper. Tumor necrosis factor-α (TNF-α) (Wako) was added to the DC medium at a final concentration of 50 ng/ml on day 7 in some experiments and cultured for another 2 days to generate mature DCs. The purity of DCs was >95% based on the expression of CD1a and CD11c and lack of expression of T-cell, B-cell, NK-cell, and monocyte lineage markers by flow cytometry (data not shown).

Infection of DCs With Viral Vectors

5×10^5 DCs were infected with SeV or AdV vectors for 1 hr at 37°C in a final volume of at least 500 μl of serum-free RPMI 1640. After the infection, DCs were washed with serum-free RPMI 1640 medium and cultured in 24 well plates with 1 ml of the DC medium.

SDS-PAGE and Western Blot Analysis

DCs infected with SeV (SeVGag or SeVEnv) at a multiplicity of infection (MOI) of 2 or with AdV (AdVGFR, AdVEnv, AdVRev) at an MOI of 1,000 were harvested after 24 or 48 hr of infection, respectively. After washing with PBS, the cells were resuspended in RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, complete mini as 1× protease inhibitor cocktail [Roche, Basel, Switzerland]), and kept on ice for 10 min. The suspension was spun for 5 min at 9,000*g* to remove cell debris. The amount of protein in the cell lysate was determined by protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates containing 30 μg of cellular protein were loaded onto a 10–20% Ready Gels J (Bio-Rad Laboratories) and electrophoretically transferred to immobilon polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA). Western blot analysis was performed using Lumi-Light plus Western Blotting Kit (Roche) according to the manufacturer's instructions. Briefly, the membrane was blocked in 1% blocking solution at 4°C overnight. The membrane was incubated with mouse monoclonal antibodies against p24 (Advanced Biotechnologies, Inc., Columbia, MD) and gp120 (Immuno Diagnostics, Inc., Woburn, MA) of HIV-1 for 1 hr. The blots were then washed four times with 1× TBST and incubated with anti-mouse IgG conjugated with horseradish peroxidase (Roche). Proteins were illuminated by Lumilight Plus (Roche) and detected with Lumi Imager (Roche). Quantification was done by densitometric analysis with the Lumi Analyst software (Roche).

Immunostaining and Flow Cytometry

GFP expression and viability of DCs infected with SeVGFP, dF-SeVGFP, or AdVGFP were analyzed by flow cytometry. To determine the viability, 5×10^5 DCs in about 300 μl of media were stained with 10 μl of propidium iodide (PI) (50 μg/ml; SIGMA). Events were acquired on a FACS-Caliber (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson) and Flow Jo software version 4.1 (Tree Star, Asland, OK).

To determine the effects of transduction on the expression of DC surface marker, immature DCs (imDCs) were cultured with the DC medium for 48 hr, mature DCs (mDCs) were cultured with the DC medium plus TNF-α for 48 hr. DCs transduced with SeV or AdV were cultured with the DC medium for 24 or 48 hr, respectively. Those DCs were stained with antibodies at 4°C for 20 min and then washed three times with PBS. Those cells were analyzed by flow cytometry after fixing 1% paraformaldehyde. The antibodies we used were as follows: fluorescein isothiocyanate-anti-Lineage (Lin-FITC) (CD3, CD14, CD16, CD19, CD20, CD56) (Becton Dickinson), phycoerythrin (PE)-anti-CD1a (Immunotech, Marseilles, France), PE-anti-CD83 (Immunotech), PE-anti-HLA-ABC (Dako), Peridinin chlorophyll protein (PerCP) -anti-CD4 (Becton Dickinson), PerCP-anti-HLA-DR (Becton Dickinson), allophycocyanin (APC)-anti-CD14 (Immunotech), APC-anti-CD40 (PharMingen), APC-anti-CD11c (PharMingen), Biotin-conjugated anti-CD86 (Becton Dickinson). Streptavidin-FITC (Becton