6. Aiello LP, Bursell SE, Clermont A, *et al*: Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C *in vivo* and suppressed by an orally effective beta-isoform-selective inhibitor. Diabetes 46: 1473-1480, 1997.
7. Lee TS, MacGregor LC, Fluharty SJ and King GL: Differential regulation of protein kinase C and (Na,K)-adenosine triphosphatase activities by elevated glucose levels in retinal capillary endothelial cells. J Clin Invest 83: 90-94, 1989.
8. Nakamura J, Kato K, Hamada Y, *et al*: A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes 48: 2090-2095, 1999.
9. Hammes HP, Wellensiek B, Kloting I, Sickel E, Bretzel RG and Brownlee M: The relationship of glycaemic level to advanced glycation end-product (AGE) accumulation and retinal pathology in the spontaneous diabetic hamster. Diabetologia 41: 165-170, 1998.
10. Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K and Horiuchi S: The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. Diabetologia 40: 764-769, 1997.
11. Aiello LP: Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. Ophthalmic Res 29: 354-362, 1997.
12. Aiello LP, Avery RL, Arrigg PG, *et al*: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331: 1480-1487, 1994.
13. Ozaki H, Seo MS, Ozaki K, *et al*: Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. Am J Pathol 156: 697-707, 2000.
14. Ferrara N, Houck K, Jakeman L and Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 13: 18-32, 1992.
15. Jakeman LB, Winer J, Bennett GL, Altar CA and Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89: 244-253, 1992.
16. Senger DR, Perruzzi CA, Feder J and Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 46: 5629-5632, 1986.
17. Thieme H, Aiello LP, Takagi H, Ferrara N and King GL: Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. Diabetes 44: 98-103, 1995.
18. Adamis AP, Miller JW, Bernal MT, *et al*: Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118: 445-450, 1994.
19. Boulton M, Gregor Z, McLeod D, *et al*: Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management. Br J Ophthalmol 81: 228-233, 1997.
20. Okamoto N, Tobe T, Hackett SF, *et al*: Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. Am J Pathol 151: 281-291, 1997.
21. Adamis AP, Shima DT, Tolentino MJ, *et al*: Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 114: 66-71, 1996.
22. Robinson GS, Pierce EA, Rook SL, Foley E, Webb R and Smith LE: Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. Proc Natl Acad Sci USA 93: 4851-4856, 1996.
23. Aiello LP, Pierce EA, Foley ED, *et al*: Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92: 10457-10461, 1995.
24. Goldman CK, Kendall RL, Cabrera G, *et al*: Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc Natl Acad Sci USA 95: 8795-8800, 1998.
25. Hasumi Y, Mizukami H, Urabe M, *et al*: Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. Cancer Res 62: 2019-2023, 2002.
26. Kendall RL and Thomas KA: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90: 10705-10709, 1993.
27. Kong HL, Hecht D, Song W, *et al*: Regional suppression of tumor growth by *in vivo* transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. Hum Gene Ther 9: 823-833, 1998.
28. Shiose S, Sakamoto T, Yoshikawa H, *et al*: Gene transfer of a soluble receptor of VEGF inhibits the growth of experimental eyelid malignant melanoma. Invest Ophthalmol Vis Sci 41: 2395-2403, 2000.
29. Kendall RL, Wang G and Thomas KA: Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 226: 324-328, 1996.
30. Bainbridge JW, Mistry A, De Alwis M, *et al*: Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. Gene Ther 9: 320-326, 2002.
31. Honda M, Sakamoto T, Ishibashi T, Inomata H and Ueno H: Experimental subretinal neovascularization is inhibited by adenovirus-mediated soluble VEGF/flt-1 receptor gene transfection: a role of VEGF and possible treatment for SRN in age-related macular degeneration. Gene Ther 7: 978-985, 2000.
32. Shinohara M, Masuyama T, Shoda T, *et al*: A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. Int J Exp Diabetes Res 1: 89-100, 2000.
33. Kakehashi A, Saito Y, Mori K, *et al*: Characteristics of diabetic retinopathy in SDT rats. Diabetes Metab Res Rev (Epub ahead of print March 30, 2006).
34. Dudus L, Anand V, Acland GM, *et al*: Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. Vision Res 39: 2545-2553, 1999.
35. Martin KR, Klein RL and Quigley HA: Gene delivery to the eye using adeno-associated viral vectors. Methods 28: 267-275, 2002.
36. Auricchio A, Behling KC, Maguire AM, *et al*: Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. Mol Ther 6: 490-494, 2002.
37. Lotery AJ, Yang GS, Mullins RF, *et al*: Adeno-associated virus type 5: transduction efficiency and cell-type specificity in the primate retina. Hum Gene Ther 14: 1663-1671, 2003.
38. Rabinowitz JE, Rolling F, Li C, *et al*: Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 76: 791-801, 2002.
39. Yang GS, Schmidt M, Yan Z, *et al*: Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. J Virol 76: 7651-7660, 2002.
40. Yoshimura I, Mizuguchi Y, Miyajima A, Asano T, Tadakuma T and Hayakawa M: Suppression of lung metastasis of renal cell carcinoma by the intramuscular gene transfer of a soluble form of vascular endothelial growth factor receptor I. J Urol 171: 2467-2470, 2004.
41. Zhang M, Volpert O, Shi YH and Bouck N: Maspin is an angiogenesis inhibitor. Nat Med 6: 196-199, 2000.
42. Hermens WT, ter Brake O, Dijkhuizen PA, *et al*: Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther 10: 1885-1891, 1999.
43. Zolotukhin S, Byrne BJ, Mason E, *et al*: Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6: 973-985, 1999.
44. Kadof PF, Takahashi Y, Wyman M, Ferris F III: Diabetes-like proliferative retinal changes in galactose-fed dogs. Arch Ophthalmol 113: 352-354, 1995.
45. Casey R and Li WW: Factors controlling ocular angiogenesis. Am J Ophthalmol 124: 521-529, 1997.

Int. J. Cancer: **120,** 278–284 (2006)

Suppression of ovarian cancer by muscle-mediated expression of soluble VEGFR-1/Flt-1 using adeno-associated virus serotype 1-derived vector

Yuji Takei[1,2], Hiroaki Mizukami[1], Yasushi Saga[2], Ichiro Yoshimura[3], Yoko Hasumi[4], Takeshi Takayama[2], Takahiro Kohno[2], Takashi Matsushita[1], Takashi Okada[1], Akihiro Kume[1], Mitsuaki Suzuki[2] and Keiya Ozawa[1]*

[1]*Division of Genetics Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan*
[2]*Department of Obstetrics and Gynecology, Jichi Medical School, Tochigi, Japan*
[3]*Department of Urology, National Defense Medical College, Saitama, Japan*
[4]*Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan*

Vascular endothelial growth factor (VEGF) is known to play a major role in angiogenesis in a variety of tumors. A soluble form of Flt-1 (sFlt-1), a VEGF receptor, is potentially useful as an antagonist of VEGF, and accumulating evidences suggest the applicability of sFlt-1 in tumor suppression by means of anti-angiogenesis. We previously demonstrated the efficacy of *sflt-1* gene expression *in situ* to suppress tumor growth and ascites in ovarian cancer. Here, we demonstrate the therapeutic applicability of muscle-mediated expression of sFlt-1 in tumor-bearing mice. Initially, tumor suppressive action was confirmed by inoculating sFlt-1-expressing ovarian cancer (SHIN-3) cells into mice, both subcutaneously and intraperitoneally. To validate the therapeutic efficacy in a more clinically relevant model, adeno-associated virus vectors encoding *sflt-1* were introduced into mouse skeletal muscles and were subsequently inoculated with tumor cells. As a result, high serum sFlt-1 levels were constantly observed, and the growth of both subcutaneously- and intraperitoneally-inoculated tumors was significantly suppressed. No delay in wound healing or adverse events of neuromuscular damage were noted, body weight did not change, and laboratory data, such as those representing liver and renal functions, were not affected. These results indicate that sFlt-1 suppresses growth and peritoneal dissemination of ovarian cancer by the inhibition of angiogenesis, and thus suggest the usefulness of gene therapy for ovarian cancer.

Key words: sFlt-1; AAV; gene therapy; ovarian cancer; VEGF

In recent years, the incidence of ovarian cancer has been on the increase. It is currently the leading cause of death from gynecological cancer.[1] Since the early-stage ovarian cancer is generally asymptomatic, more than half of the patients are diagnosed with the condition at an advanced stage with ascites and peritoneal dissemination.[2] The standard treatment for advanced ovarian cancer is radical cytoreductive surgery followed by combination chemotherapy. Fortunately, ovarian cancer is relatively sensitive to chemotherapy, and a remission can be achieved in a majority of patients, even at the advanced stages.[3,4] Nonetheless, more than half of the patients develop recurrence, and this eventually leads to death, thereby indicating the limitations of the current therapy. Therefore, new strategies are required to improve the therapeutic outcomes.

Angiogenesis is closely related to the development of malignant tumors,[5] and it plays an important role in the growth of primary, metastatic and disseminated lesions of ovarian cancer.[6] Therefore, the inhibition of angiogenesis may suppress peritoneal dissemination, the main mode of progression of ovarian cancer, and may improve the prognosis of advanced ovarian cancer.

The significance of angiogenic factors upon clinical outcome of ovarian cancer have been intensively studied, including vascular endothelial growth factor (VEGF),[7] basic fibroblast growth factor,[8] platelet-derived endothelial cell growth factor (PD-ECGF)[9] and hepatocyte growth factor.[10] Among all, VEGF appears to be the most important and versatile, and it is also reported to be an independent prognostic factor in ovarian cancer patients.[7]

The VEGF-inhibiting factor used in this study was sFlt-1. This is a soluble form of VEGFR-1, and acts as a VEGF antagonist.[11] sFlt-1 is a secretory protein, and systemic expression through circulation can be expected. Main VEGF receptors are Flt-1 and KDR, and both are tyrosine kinase receptors. KDR shows stronger tyrosine kinase activity when the ligand, VEGF, binds, but Flt-1 has stronger VEGF-binding activity.[12,13] Accordingly, when sFlt-1 is present in circulation at a sufficient level, sFlt-1 may competitively inhibit binding of blood VEGF to Flt-1 and KDR, resulting in inhibition of VEGF action.

We have previously demonstrated the tumor suppressive activities of sFlt-1 when it was introduced into ovarian cancer cells.[14] In this study, we aimed at developing a more clinically relevant strategy through a continuous supply of sFlt-1 by muscle-mediated gene transfer using adeno-associated virus (AAV) vectors. AAV vector is derived from a nonpathogenic virus, and a long-term transgene expression can be obtained after intramuscular vector injection.[15–17] Taking these facts into consideration, we began to test the efficacy of the therapeutic approach for ovarian cancer using muscle-mediated *sflt-1* gene expression and compared with the previous strategy which utilized tumor cell transduction.

Material and methods

Cells and plasmids

The human ovarian serous adenocarcinoma cell line SHIN-3[18] was provided by Dr. Y. Kiyozuka (Hyogo College of Medicine, Japan) and used in this study, instead of the previously utilized RMG-1 cells,[14] as the latter cell line did not efficiently form tumors upon inoculation. The SHIN-3 and the human embryonic kidney 293 cell lines were maintained as described previously.[18,19] The murine *sflt-1* cDNA was isolated from the SmaI sites of plasmid psFlt-1,[14] and inserted into the SmaI site of the pCMV-IRES-bsr vector[20] to generate pCMV-sFlt-1-IRES-bsr. Luciferase (LUC)-encoding pCMV-LUC-IRES-bsr[20] was used as a control vector. p2ITR-hsFlt-1 is an sFlt-1 expression plasmid prepared by incorporating human *sflt-1* cDNA into the EcoRI site of pAAV-MCS (Stratagene, La Jolla, CA). Suppression of VEGF-driven HUVEC proliferation by conditioned medium of sFlt-1-expressing cells was already confirmed for both murine[14] and human[21] constructs.

Development of stably transduced cells

Either pCMV-sFlt-1-IRES-bsr or pCMV-LUC-IRES-bsr was introduced into the SHIN-3 cells using the standard calcium phosphate precipitation method.[22] After transfection, the cells were cultured and selected in the presence of 10 μg/ml of blasticidin S hydrochloride (Kaken Pharmaceutical, Tokyo, Japan). After 4 weeks, the blasticidin-resistant SHIN-3/sFlt-1 and SHIN-3/LUC

Grant sponsors: Ministry of Health, Labor and Welfare, Japan; Ministry of Education, Culture, Sports, Science and Technology, Japan; Japan Medical Association. The research award to JMS graduate student.
***Correspondence to:** Division of Genetics Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan. E-mail: kozawa@jichi.ac.jp
Received 18 November 2005; Accepted after revision 7 August 2006
DOI 10.1002/ijc.22307
Published online 25 October 2006 in Wiley InterScience (www.interscience.wiley.com).

cell lines were established and maintained thereafter in the presence of 10 μg/ml of blasticidin S hydrochloride.

AAV vector production

AAV vectors were produced based on the triple plasmid transfection to 293 cells using p2ITR-hsFlt-1, the helper plasmid for adenovirus genes,[23] and the helper plasmid for AAV1.[24,25] A plasmid encoding *lacZ* gene was used to prepare the control AAV vector. The vector stocks were purified using iodixanol (Invitrogen, Carlsbad, CA) density-gradient ultracentrifugation,[24,26,27] and the titer was determined by quantitative DNA dot-blot hybridization.

VEGF quantitation

SHIN-3 cells were inoculated into 10-cm dishes and cultured in a 10% FBS-supplemented DMEM medium. When the cells grew to approximately 80% confluence, the culture supernatant was replaced with serum-free culture medium. After 24 hr, the culture supernatant was recovered. The concentration of VEGF in the supernatant was determined by ELISA System (Amersham Biosciences, Piscataway, NJ).

Western blot analysis

sFlt-1 in the culture supernatant of SHIN-3/sFlt-1 was detected by Western blotting, using standard techniques as described previously.[14] Briefly, the culture supernatant was electrophoresed, transferred into nitrocellulose membrane and incubated with a 1:200 dilution of anti-sFlt-1 polyclonal antibody (provided by Dr. Shibuya). Subsequently, the membrane was reacted with a horseradish peroxidase-labeled secondary antibody, *i.e.*, anti-rabbit antibody (Amersham Biosciences). The bound antibody was visualized by chemiluminescence using an ECL kit (Amersham Biosciences).

In vitro *cell growth kinetics*

SHIN-3/sFlt-1 and SHIN-3/LUC were plated in 6-well plates at a density of 5×10^4 cells/well, and cultured in a 10% serum-supplemented DMEM/F-12 medium. For each group, the cells from a single well were dislodged using 0.05% trypsin-EDTA every 24 hr and were counted using a hemocytometer. This experiment was performed in triplicate.

Tumor cell transduction model

Subcutaneously inoculated tumor growth. Four- to five-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used in the experiment. SHIN-3/sFlt-1 or SHIN-3/LUC cells (5×10^6) were subcutaneously transplanted into the back of the mice, and tumor sizes were measured twice a week using a micrometer caliper. The tumor volume was calculated using the formula: volume = (short diameter)^2 × (long diameter) × 0.5.[28]

Angiogenesis in subcutaneous tumor. On the 21st day after the subcutaneous transplantation of 5×10^6 SHIN-3/sFlt-1 or SHIN-3/LUC cells into the back, the mice were killed and the subcutaneous tumors were excised. After fixation of the tumors in 4% paraformaldehyde, frozen sections were cut, and the endogenous peroxidases were blocked with 3% hydrogen peroxide. The sections were incubated overnight at 4°C with a 1:50 dilution of anti-CD31 antibody (Pharmingen, San Diego, CA) as the primary antibody that recognizes vascular endothelial cells. The sections were then reacted with the secondary antibody, *i.e.*, peroxidase-conjugated anti-rat antibody (Simple Stain Mouse MAX-PO, Rat; Nichirei, Tokyo, Japan) at room temperature for 30 min, followed by color development with diaminobenzidine (DAB). In addition to CD31, immunostaining of von Willbrand factor (vWF) and endothelial nitric oxide synthase (eNOS) was performed. For the specific antibodies, anti-vWF (H-300) and anti-NOS3 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 50 times, and reacted with the sections at room temperature for 2 hr, followed by color development by DAB reaction using a DAKO LSAB Kit (DAKO, Carpinteria, CA). The number of newly formed vessels was counted under a light microscope at 100× magnification.

Ascites and peritoneal tumor dissemination. SHIN-3/sFlt-1 or SHIN-3/LUC cells (5×10^6) were inoculated into mice intraperitoneally. After 23 days, the mice were killed using diethyl ether, the volume of the ascitic fluid was measured and the peritoneally disseminated lesions were weighed. The volume of ascitic fluid was calculated by subtracting 1 ml from the total volume of fluid that was recovered after the intraperitoneal injection of 1 ml of PBS. The weights of the peritoneally disseminated lesions were calculated by subtracting the weight of the intestine of age-matched, untreated mice from the total weight of the intestine and disseminated lesions removed in one block.

Survival time. SHIN-3/sFlt-1 or SHIN-3/LUC cells (5×10^6) were inoculated into mice intraperitoneally, and the mice were monitored twice daily until they died of massive ascites. Survival rates were calculated using the Kaplan–Meier method.

Therapeutic model using AAV vector

Measurement of serum VEGF and ascitic VEGF. Nude mice were subcutaneously ($n = 5$) or intraperitoneally ($n = 5$) inoculated with SHIN-3 cells (5×10^6 cells), and blood was collected from the tail vein 1, 2, and 3 weeks after inoculation. The serum VEGF level was measured using human VEGF ELISA (R&D system, Minneapolis, MN). Mice that received intraperitoneal SHIN-3 cell inoculation were killed using diethyl ether 3 weeks after inoculation ($n = 3$), ascites were collected, and the ascitic VEGF level was measured by ELISA (R&D system).

Measurement of serum sFlt-1. AAV1-sFlt-1 or AAV1-LacZ vector (2.5×10^{12} genome copy) was given in 10 separate injections into the hind limb skeletal muscles of mice, and the blood samples were obtained from the tail vein every 2 weeks. The concentration of sFlt-1 in the serum was measured using a human sVEGF-R1 ELISA (Bender MedSystems, Vienna, Austria).

Subcutaneously inoculated tumor growth. AAV1-sFlt-1 or AAV1-LacZ vector (2.5×10^{12} genome copy) was injected into the hind limb skeletal muscles of mice. After 9 days, the mice were subcutaneously inoculated with 5×10^6 SHIN-3 cells and monitored thereafter.

Peritoneal tumor dissemination. AAV1-sFlt-1 or AAV1-LacZ vector (2.5×10^{12} genome copy) was injected into the hind limb skeletal muscles of mice. After 9 days, the mice were intraperitoneally inoculated with 5×10^6 SHIN-3 cells. After 23 days later, the mice were killed, and the weights of the intraperitoneally disseminated lesions were measured.

Adverse events. AAV1-sFlt-1 or AAV1 LacZ vectors (2.5×10^{12} genome copies) were injected into the hind limb skeletal muscle of mice. The following experiments were performed to investigate wound healing, neuromuscular damage and body weight changes: Regarding wound healing, a 6-mm square incision was made with scissors in the dorsal skin in nude mice 2 weeks after AAV vector injection, and the healing process was observed with time. As for neuromuscular damage, skeletal muscle of the hind limb at the AAV vector-inoculated site was excised 5 weeks after AAV vector injection, fixed with formalin, paraffin-embedded and sectioned, and the morphology was observed by HE staining. As for body weight changes, body weight of the nude mice was measured before AAV vector injection and 2 and 4 weeks after the administration. Blood was collected 5 weeks after AAV vector injection, and complete blood counts, Alb, BUN, Cr, AST, ALT, Na, K and Cl were measured.

Statistical analysis. Intergroup differences were tested for significance using Student's *t*-test. Survival rates were analyzed by the generalized Wilcoxon and log-rank tests. p values less than 0.05 were considered to be significant.

Results

Detection of VEGF and sFlt-1 in culture supernatants

The concentration of VEGF in the culture supernatant of SHIN-3 cells was 600 pg/ml. sFlt-1 was detected by Western blotting only in the culture supernatant of SHIN-3/sFlt-1 (data not shown).

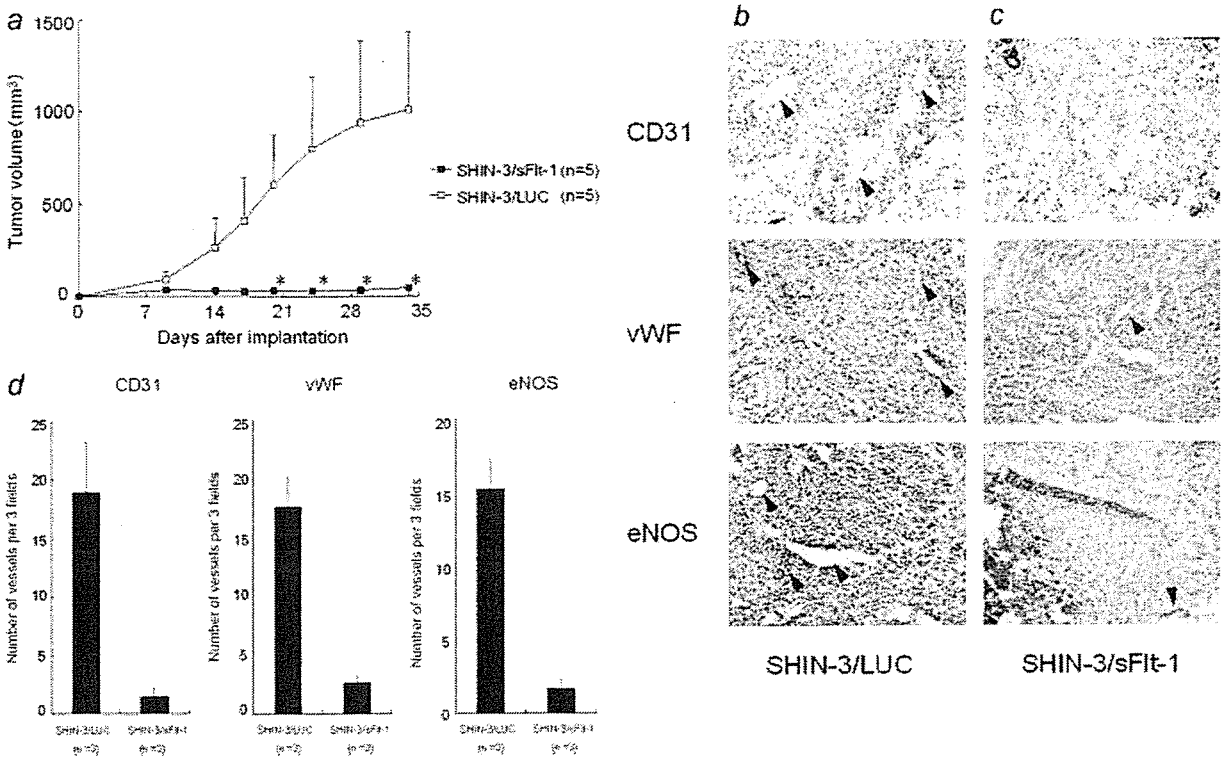


FIGURE 1 – (*a*) *In vivo* tumor growth of sFlt-1-expressing SHIN-3 cells. Tumor cells were subcutaneously injected into the back of mice, and the tumor size was measured every 3 days. The tumor size of SHIN-3/sFlt-1 (■) was significantly smaller than that of SHIN-3/LUC (□, $p < 0.01$ (*)). The tumor volume was calculated using the formula: $(\text{width})^2 \times (\text{length}) \times 0.5$ (mm^3). The data represent the mean ± SD. (*b* and *c*) Immunostaining of subcutaneous tumors of SHIN-3/LUC (*b*) and SHIN-3/sFlt-1 (*c*) with anti-CD31 antibody (upper), anti-vWF antibody (middle) and anti-eNOS antibody (bottom). Endothelial cells of newly formed vessels (arrowhead) were stained dark brown. (*d*) The numbers of new blood vessels in SHIN-3/sFlt-1 and SHIN-3/LUC subcutaneous tumors on the 21st day after inoculation. The number of new blood vessels in SHIN-3/sFlt-1 tumors was significantly smaller than that in SHIN-3/LUC tumors (anti-CD31 antibody; 1.5 ± 0.7 *versus* 19 ± 4, $p < 0.05$, anti-vWF antibody; 2.7 ± 0.6 *versus* 17.7 ± 2.5, $p < 0.01$, anti-eNOS antibody; 1.7 ± 0.6 *versus* 15.3 ± 2.1, $p < 0.01$). Each bar represents the mean ± SD.

In vitro *cell growth kinetics*

The effects of sFlt-1 gene expression on *in vitro* cell growth were examined. There were no differences in growth between SHIN-3/sFlt-1 and the control (SHIN-3/LUC), indicating that the expression of the *sflt-1* gene does not affect *in vitro* cell growth kinetics (data not shown).

Tumor cell transduction model

Subcutaneously inoculated tumor growth. As shown in Figure 1*a*, subcutaneous tumor growth was markedly suppressed in cells expressing sFlt-1. The size of subcutaneous tumors on the 34th day after the transplantation of SHIN-3/sFlt-1 cells was significantly smaller than that of control (43 ± 37 mm^3 *versus* 1004 ± 421 mm^3, $p < 0.01$).

Angiogenesis in subcutaneous tumor. Typical immunohistochemistry of tumors determined using anti-CD31 antibody, anti-vWF antibody and anti-eNOS antibody is shown in Figures 1*b* and 1*c*, and the number of new vessels is summarized in Figure 1*d*. The number of new vessels was significantly smaller in SHIN-3/sFlt-1 than that in control (anti-CD31 antibody; 1.5 ± 0.7 *versus* 19 ± 4, $p < 0.05$, anti-vWF antibody; 2.7 ± 0.6 *versus* 17.7 ± 2.5, $p < 0.01$, anti-eNOS antibody; 1.7 ± 0.6 *versus* 15.3 ± 2.1, $p < 0.01$).

Ascites and peritoneal tumor dissemination. The effects of *sflt-1* gene expression on peritoneal dissemination *in vivo* are shown in Figure 2*a*. The mean volume of ascitic fluid on the 23rd day after intraperitoneal inoculation of SHIN-3/sFlt-1 cells was significantly smaller than that of control (Fig. 2*b*; 0.17 ± 0.13 ml *versus* 1.67 ± 0.71 ml, $p < 0.01$). Similarly, the number of metastasis (Fig. 2*c*) and mean weight of the peritoneally disseminated SHIN-3/sFlt-1 tumors was significantly lower than that of control (Fig. 2*d*; 0.48 ± 0.29 g *versus* 2.74 ± 0.54 g, $p < 0.001$). Thus, *sflt-1* gene expression suppressed ascites production and peritoneal dissemination.

Survival time. The survival of mice was monitored after inoculating tumor cells intraperitoneally. In the control group, the accumulation of ascitic fluid became prominent from the 14th day after inoculation, and all mice died by the 46th day. In contrast, in the SHIN-3/sFlt-1 group, ascitic fluid accumulation was suppressed, resulting in a significantly longer survival (Fig. 2*e*, $p < 0.05$). Therefore, *sflt-1* gene expression prolonged the survival of mice with peritoneal dissemination of ovarian cancer.

Therapeutic model using AAV vector

Serum VEGF concentration and ascitic VEGF concentration. The serum VEGF level was lower than the detection limit in both the subcutaneous and intraperitoneal inoculation groups. In contrast, the ascitic VEGF level was very high (30 ± 9 ng/ml).

Serum sFlt-1 concentration. Following the injection of AAV1-sFlt-1 vector into the mouse skeletal muscles, serum sFlt-1 levels were higher than 1,000 pg/ml throughout the observation period, whereas in the control group with AAV1-LacZ vector, the levels were below the detection limit (Fig. 3*a*).

Tumor growth. The efficacy of muscle-mediated sFlt-1 expression was evaluated in both subcutaneously- and intraperitoneally-transplanted SHIN-3 tumor cell growth. As shown in Figure 3*b*, a

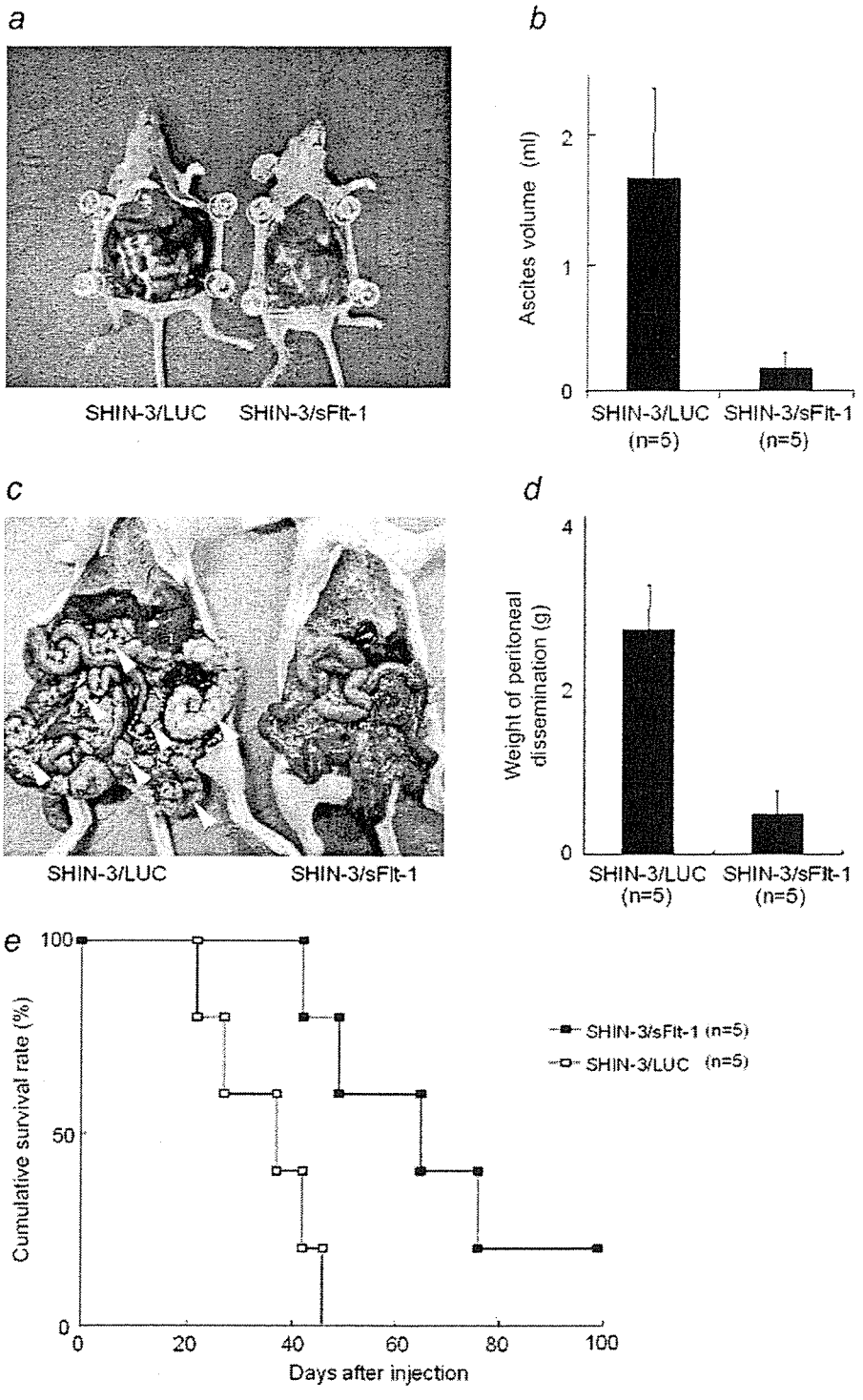


FIGURE 2 – (*a* and *b*) Ascites fluid accumulation on the 23rd day after the intraperitoneal inoculation of cancer cells. Large amounts of bloody ascitic fluid were observed in the SHIN-3/LUC group, whereas in the SHIN-3/sFlt-1 group, the accumulation of ascitic fluid was significantly suppressed (1.67 ± 0.71 ml *versus* 0.17 ± 0.13 ml, $p < 0.01$). (*c* and *d*) Peritoneal dissemination on the 23rd day after peritoneal inoculation of cancer cells. In the SHIN-3/LUC group, marked peritoneal dissemination was observed, particularly on the intestinal surface (arrowheads), whereas in the SHIN-3/sFlt-1 group, peritoneal dissemination was significantly suppressed (2.74 ± 0.54 g *versus* 0.48 ± 0.29 g, $p < 0.001$). (*e*) Kaplan–Meier analysis after intraperitoneal inoculation of cancer cells. Survival was significantly prolonged in the SHIN-3/sFlt-1 group, when compared with that in the SHIN-3/LUC group ($p < 0.05$).

significant suppression of tumor growth was observed in the sFlt-1 injected group ($p < 0.05$). In mice with peritoneal dissemination, the total weight of the peritoneally disseminated lesions on the 23rd day after inoculation was significantly lower than that of control group (Fig. 3*c*; 1.07 ± 0.87 g *versus* 3.15 ± 2.10 g, $p < 0.05$). Therefore, a therapeutic effect was observed in both models.

Survival time. The survival of mice was monitored in both groups. The animals showed accumulation of ascites, and the mean survival lengths were 28.2 and 30.1 days for AAV-LacZ- and AAV-sFlt-1-injected group, respectively. Although the survival in AAV-sFlt-1 group was longer than the controls, statistic significance was not recognized between these groups.

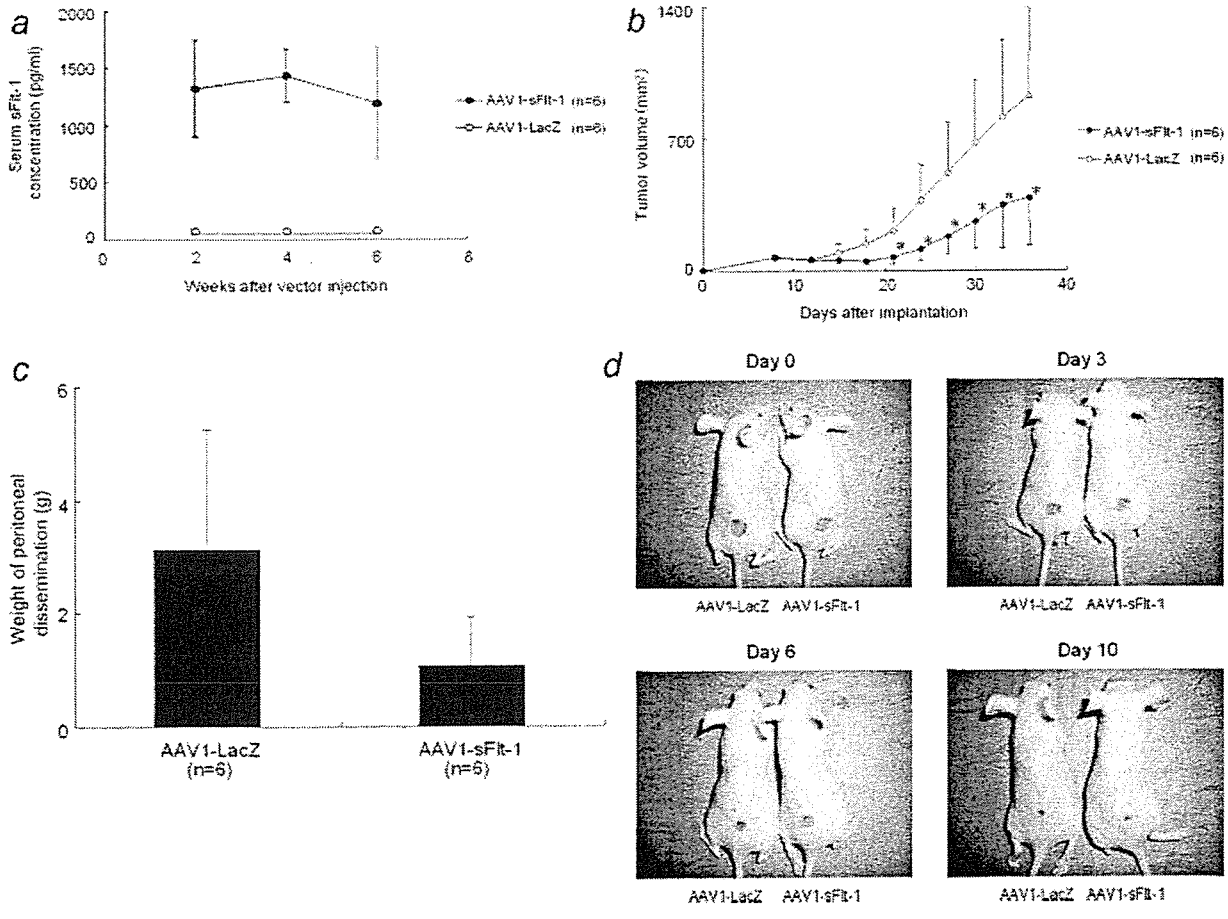


FIGURE 3 – (*a*) Serum sFlt-1 concentrations. In the mice that were intramuscularly injected with AAV1-sFlt-1, the serum sFlt-1 concentration was higher than 1,000 pg/ml. In contrast, in the mice that were intramuscularly injected with AAV1-LacZ, the serum sFlt-1 concentration was below the detection limit. (*b*) Growth of SHIN-3 subcutaneous tumors in the mice that were intramuscularly injected with AAV1-sFlt-1 or AAV1-LacZ. The growth of subcutaneous tumors was significantly suppressed in the intramuscular AAV1-sFlt-1 injection group, compared with that in the intramuscular AAV1-LacZ injection group. The sizes of subcutaneous tumors on the 36th day after the transplantation of SHIN-3 cells in the AAV1-sFlt-1 and AAV1-LacZ groups were 380 ± 250 mm^3 and 921 ± 466 mm^3, respectively (*, $p < 0.05$). (*c*) The total weight of peritoneally disseminated tumors on the 23rd day after the intraperitoneal inoculation of SHIN-3 cells in the mice that had been intramuscularly injected with AAV1-sFlt-1 or AAV1-LacZ. The peritoneal dissemination was significantly suppressed in the AAV1-sFlt-1 group, when compared with that in the AAV1-LacZ group (1.07 ± 0.87 g *versus* 3.15 ± 2.10 g, $p < 0.05$). (*d*) A 6-mm square injury was made in the dorsal region 2 weeks after intramuscular injection of AAV1-sFlt-1 or AAV1-lacZ into nude mice. Photographs of typical views immediately and 3, 6 and 10 days after skin incision are presented. No significant difference was noted between the groups.

Adverse events. Regarding wound healing, the wound was completely repaired about 2 weeks after skin incision in both the AAV1-sFlt-1 ($n = 3$) and control ($n = 3$) groups, showing no significant difference in the time required for healing between the groups. Photographs of typical views immediately and 3, 6, and 10 days after skin incision are shown in Figure 3*d*. As for neuromuscular damage, no apparent damage was noted at the histological level in the AAV1-sFlt-1 group, similar to the control group (data not shown). The body weights were 17.1 ± 1.0, 21.5 ± 0.9, and 23.3 ± 1.2 g before intramuscular AAV vector injection and 2 and 4 weeks after the injection, respectively, in the AAV1-sFlt-1 group ($n = 5$) and 16.9 ± 1.2, 20.7 ± 1.8, and 23.0 ± 1.9 g, respectively, in the control group ($n = 5$), showing no significant difference. The laboratory data are shown in Table I. There were no significant differences in the serum Alb, BUN, Cr, AST, ALT, Na, K or Cl level between the AAV1-sFlt-1 and control groups, nor were there significant differences in the complete blood counts.

Discussion

In this study, we demonstrated the efficacy of muscle-mediated sFlt-1 expression using AAV vectors in both subcutaneous and intraperitoneally disseminated tumors.

A VEGF receptor, Flt-1, consists of an intracellular tyrosine kinase domain, transmembrane domain and extracellular domain, containing 7 immunoglobulin-like domains. sFlt-1 generated by alternative splicing of the Flt-1 gene lacks the intracellular tyrosine kinase and transmembrane domains, and consists of an extracellular domain containing 6 immunoglobulin-like domains, from which the 7th immunoglobulin-like domain is deleted. The VEGF-binding site of Flt-1 is located on the 2nd and 3rd immunoglobulin-like domains in the extracellular domain.[29,30] Hence, sFlt-1 has VEGF-binding ability. However, it has no signal transduction activity because the molecule is not anchored on the cell surface, and lacks the tyrosine kinase do-

TABLE I – LABORATORY DATA

	AAV1-LacZ ($n = 3$)	AAV1-sFlt-1 ($n = 3$)	p
WBC ($10^3/\mu l$)	1.7 ± 0.3	1.4 ± 0.5	n.s.
Hemoglobin (g/dl)	12.3 ± 1.0	13.9 ± 1.3	n.s.
Platelets ($10^4/\mu l$)	75 ± 49	96 ± 42	n.s.
Albumin (g/dl)	0.5 ± 0.1	0.5 ± 0	n.s.
BUN (mg/dl)	36 ± 5	29 ± 4	n.s.
Creatinine (mg/dl)	0.25 ± 0.03	0.19 ± 0.04	n.s.
AST (IU/l)	103 ± 34	86 ± 30	n.s.
ALT (IU/l)	37 ± 5	31 ± 2	n.s.
Na (meq/l)	153 ± 2	154 ± 2	n.s.
K (meq/l)	4.1 ± 0.4	4.2 ± 0.1	n.s.
Cl (meq/l)	116 ± 2	116 ± 2	n.s.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; n.s., not significant.

main. Therefore, sFlt-1 acts as a VEGF antagonist by competing with the original VEGF receptors, Flt-1 and KDR.

Studies suggesting the therapeutic efficacy with sFlt-1-encoding adenoviral vectors for lung[31] or pancreatic[32] cancer have been reported. Our strategy is aimed at attaining long-term sFlt-1 expression not only for tumor suppression but also to prevent the potential recurrence of the tumor after surgical excision. For this purpose, AAV vector appears to be ideal, since transgene expression can be achieved for a number of years.[16,17,24] To date, various serotypes of AAV (1 through 11) have been identified, and the applicability of these serotype-derived vectors has been investigated.[25,33–39] In case of muscle transduction, AAV1-based vector was shown to be more efficient than the rest of the serotypes.[24,25] Therefore, we used AAV1 vectors for *sflt-1* gene transfer. In fact, we had previously attempted the same set of experiments using vectors AAV2 and AAV5; however, none of these experiments showed any promising results (data not shown).

In this study, we confirmed the tumor-suppressive actions of sFlt-1 by transducing tumor cells as we reported in different cell line[14] and demonstrated the therapeutic efficacy of muscle-directed *sflt-1* gene transfer in the tumor-bearing mouse model. Although the muscle transduction model is clinically more relevant, the tumor suppressive action observed in this model was less complete, as assessed by tumor growth (Figs. 1*a vs.* 3*b*), tumor volume (Figs. 2*d vs.* 3*c*) and overall survival. The difference in therapeutic outcome seems to lie in the concentration of sFlt-1 within tumor and the surrounding area. Therefore, in order to obtain more substantial therapeutic benefit, approaches to enhance the supply of sFlt-1 from the muscle by either increasing the vector dose or by transducing more muscle tissues would be necessary. Another approach, increasing the intraperitoneal concentrations of sFlt-1, would be also helpful to improve the therapeutic outcome. However, peritoneal dissemination was inhibited at a concentration of 1,000 pg/ml in this experiment. AAV vectors have an advantage of long-term gene expression, unlike adenovirus vectors, and the level could be maintained at 1,000 pg/ml or higher for as long as 6 weeks in this experiment. The persistent sFlt-1 gene expression may have contributed to the inhibition of peritoneal dissemination. For clinical application, combination of available anticancer drugs and AAV1-sFlt-1 may be recommended more than administration of AAV1-sFlt-1 alone.

Molecular targeted therapy against VEGF has been conducted by using a variety of different molecules.[40–45] Of all these molecules, bevacizumab, an anti-human VEGF monoclonal antibody, appears to be most promising, and clinical trials are ongoing for patients with cancer.[40,42,45] Significant prolongation of progression-free survival was noted in clinical trials with bevacizumab for metastatic colorectal[42] and renal[45] cancer, either in combination with chemotherapy or independently. On the other hand, this strategy requires frequent infusion of drugs, and the adverse effects due to the use of the monoclonal antibody have been noted.[45] Although difficult to predict, our approach may be safer, since it eliminates inherent problems that arise during the repetitive injection of a monoclonal antibody. However, an influence on vascular endothelial cells in normal tissues is a concern as an adverse drug reaction of angiogenesis inhibitors including sFlt-1. Actually, an anti-human VEGF monoclonal antibody, bevacizumab, has been reported to exhibit adverse events of proteinuria, hypertension, nasal bleeding and hematuria.[45] The mechanism of proteinuria is assumed to be injuries of kidney glomerular endothelial and epithelial cells due to bevacizumab-induced reduction of the blood VEGF level.[46] A delay in wound healing has also been reported.[47] However, no delay in wound healing, neuromuscular damage or body weight changes were noted in our experiment, nor were there changes in the laboratory data, and no apparent AAV1-sFlt-1-induced adverse event was observed.

Reports on gene therapy for ovarian cancer that are underway include the use of adenoviral vectors encoding tumor suppressor genes p53[48] and phosphatase and tensin homolog deleted on chromosome 10.[49] These studies aimed at destroying cancer cells by introducing a therapeutic gene into the cancer cells. However, these methods are unrealistic, since introducing a therapeutic gene into all of the peritoneally disseminated cancer cells is impractical. On the other hand, targeted therapy against VEGF is advantageous because it not only has tumor-suppressive effects but also controls the formation of ascites, since it simultaneously suppresses enhanced vascular permeability.[14,50] Theoretically, molecular targeted therapy against VEGF is effective against high VEGF-producing tumors. It is demonstrated that approximately half of the ovarian cancer patients have elevated serum VEGF level.[7] Therefore, this approach would be suitable for at least half of the patients with ovarian cancer. In case of patients who have ovarian cancer with low VEGF-producing tumors, an alternative strategy should be applied. The results of our *in vitro* study suggests that low VEGF-producing ovarian cancer cell lines frequently secrete other angiogenic factors such as PDGF, PD-ECGF and interleukin-8 (unpublished observations). Therefore, a different therapeutic strategy based on the increased level of another angiogenic factor may prove useful for treating patients with low VEGF-producing tumors.

In summary, the experiments using transduced cancer cells confirmed that sFlt-1 has angiogenesis-suppressing activity, through which it inhibits the growth of subcutaneously transplanted ovarian cancer cells and the peritoneal dissemination of tumors. In addition, the *in vivo* experiment that aimed at the clinical application of gene therapy using the AAV vector (AAV1-sFlt-1) revealed that the intramuscular injection of AAV1-sFlt-1 had similar inhibitory effects. These results suggest the possibility of implementing gene therapy using AAV1-sFlt-1 aimed at suppressing peritoneal dissemination of ovarian cancer.

References

1. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ. Cancer statistics, 2004. CA Cancer J Clin 2004;54:8–29.
2. Heintz AP. Surgery in advanced ovarian carcinoma: is there proof to show the benefit? Eur J Surg Oncol 1988;14:91–9.
3. McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL, Davidson M. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996;334:1–6.
4. Takei Y, Suzuki M, Ohwada M, Saga Y, Kohno T, Machida S, Sato I. A feasibility study of paclitaxel and carboplatin therapy in Japanese patients with epithelial ovarian cancer. Oncol Rep 2003;10: 951–5.
5. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.
6. Roszkowski P, Wronkowski Z, Szamborski J, Romejko M. Evaluation of selected prognostic factors in ovarian cancer. Eur J Gynaecol Oncol 1993;14 (Suppl):140–5.
7. Cooper BC, Ritchie JM, Broghammer CL, Coffin J, Sorosky JI, Buller RE, Hendrix MJ, Sood AK. Preoperative serum vascular endothelial growth factor levels: significance in ovarian cancer. Clin Cancer Res 2002;8: 3193–7.

8. Davidson B, Goldberg I, Gotlieb WH, Kopolovic J, Ben-Baruch G, Nesland JM, Reich R. The prognostic value of metalloproteinases and angiogenic factors in ovarian carcinoma. Mol Cell Endocrinol 2002; 187(1/2):39–45.
9. Watanabe Y, Nakai H, Ueda H, Nozaki K, Hoshiai H, Noda K. Platelet-derived endothelial cell growth factor predicts of progression and recurrence in primary epithelial ovarian cancer. Cancer Lett 2003;200: 173–6.
10. Sowter HM, Corps AN, Smith SK. Hepatocyte growth factor (HGF) in ovarian epithelial tumour fluids stimulates the migration of ovarian carcinoma cells. Int J Cancer 1999;83:476–80.
11. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 1993;90:10705–9.
12. Sawano A, Takahashi T, Yamaguchi S, Aonuma T, Shibuya M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor (PlGF), which is related to vascular endothelial growth factor (VEGF). Cell Growth Differ 1996;7:213–21.
13. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene 1995;10:135–47.
14. Hasumi Y, Mizukami H, Urabe M, Kohno T, Takeuchi K, Kume A, Momoeda M, Yoshikawa H, Tsuruo T, Shibuya M, Taketani Y, Ozawa K. Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. Cancer Res 2002;62:2019–23.
15. Fisher KJ, Jooss K, Alston J, Yang Y, Haecker SE, High K, Pathak R, Raper SE, Wilson JM. Recombinant adeno-associated virus for muscle directed gene therapy. Nat Med 1997;3:306–12.
16. Kessler PD, Podsakoff GM, Chen X, McQuiston SA, Colosi PC, Matelis LA, Kurtzman GJ, Byrne BJ. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. Proc Natl Acad Sci USA 1996;93:14082–7.
17. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol 1996;70:8098–108.
18. Imai S, Kiyozuka Y, Maeda H, Noda T, Hosick HL. Establishment and characterization of a human ovarian serous cystadenocarcinoma cell line that produces the tumor markers CA-125 and tissue polypeptide antigen. Oncology 1990;47:177–84.
19. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–74.
20. Urabe M, Hasumi Y, Ogasawara Y, Matsushita T, Kamoshita N, Nomoto A, Colosi P, Kurtzman GJ, Tobita K, Ozawa K. A novel dicistronic AAV vector using a short IRES segment derived from hepatitis C virus genome. Gene 1997;200(1/2):157–62.
21. Yoshimura I, Mizuguchi Y, Miyajima A, Asano T, Tadakuma T, Hayakawa M. Suppression of lung metastasis of renal cell carcinoma by the intramuscular gene transfer of a soluble form of vascular endothelial growth factor receptor I. J Urol 2004;171(6, Part 1):2467–70.
22. Wigler M, Pellicer A, Silverstein S, Axel R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 1978;14:725–31.
23. Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther 1998;5:938–45.
24. Mochizuki S, Mizukami H, Kume A, Muramatsu S, Takeuchi K, Matsushita T, Okada T, Kobayashi E, Hoshika A, Ozawa K. Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes. Gene Ther Mol Biol 2004;8:9–18.
25. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. J Virol 1999;73:3994–4003.
26. Hermens WT, ter Brake O, Dijkhuizen PA, Sonnemans MA, Grimm D, Kleinschmidt JA, Verhaagen J. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther 1999;10:1885–91.
27. Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 1999;6:973–85.
28. Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat Med 2000;6:1335–40.
29. Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, Ferrara N. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. J Biol Chem 1996;271:5638–46.
30. Tanaka K, Yamaguchi S, Sawano A, Shibuya M. Characterization of the extracellular domain in vascular endothelial growth factor receptor-1 (Flt-1 tyrosine kinase). Jpn J Cancer Res 1997;88:867–76.
31. Takayama K, Ueno H, Nakanishi Y, Sakamoto T, Inoue K, Shimizu K, Oohashi H, Hara N. Suppression of tumor angiogenesis and growth by gene transfer of a soluble form of vascular endothelial growth factor receptor into a remote organ. Cancer Res 2000;60:2169–77.
32. Hoshida T, Sunamura M, Duda DG, Egawa S, Miyazaki S, Shineha R, Hamada H, Ohtani H, Satomi S, Matsuno S. Gene therapy for pancreatic cancer using an adenovirus vector encoding soluble flt-1 vascular endothelial growth factor receptor. Pancreas 2002;25:111–21.
33. Chiorini JA, Kim F, Yang L, Kotin RM. Cloning and characterization of adeno-associated virus type 5. J Virol 1999;73:1309–19.
34. Chiorini JA, Yang L, Liu Y, Safer B, Kotin RM. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J Virol 1997;71:6823–33.
35. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM. Clades of adeno-associated viruses are widely disseminated in human tissues. J Virol 2004;78:6381–8.
36. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA 2002;99:11854–9.
37. Mori S, Wang L, Takeuchi T, Kanda T. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology 2004;330:375–83.
38. Muramatsu S, Mizukami H, Young NS, Brown KE. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology 1996;221:208–17.
39. Rutledge EA, Halbert CL, Russell DW. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J Virol 1998;72:309–19.
40. Cobleigh MA, Langmuir VK, Sledge GW, Miller KD, Haney L, Novotny WF, Reimann JD, Vassel A. A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. Semin Oncol 2003;30(5, Suppl 16):117–24.
41. Garofalo A, Naumova E, Manenti L, Ghilardi C, Ghisleni G, Caniatti M, Colombo T, Cherrington JM, Scanziani E, Nicoletti MI, Giavazzi R. The combination of the tyrosine kinase receptor inhibitor SU6668 with paclitaxel affects ascites formation and tumor spread in ovarian carcinoma xenografts growing orthotopically. Clin Cancer Res 2003;9:3476–85.
42. Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 2003;21:60–5.
43. Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, Bohlen P, Hicklin DJ. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res 1999;59:5209–18.
44. Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rosel J, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res 2000;60:2178–89.
45. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 2003;349:427–34.
46. Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, Kalluri R. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem 2003;278:12605–8.
47. Niethammer AG, Xiang R, Becker JC, Wodrich H, Pertl U, Karsten G, Eliceiri BP, Reisfeld RA. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat Med 2002;8:1369–75.
48. Zeimet AG, Marth C. Why did *p53* gene therapy fail in ovarian cancer? Lancet Oncol 2003;4:415–22.
49. Saga Y, Mizukami H, Takei Y, Ozawa K, Suzuki M. Suppression of cell migration in ovarian cancer cells mediated by PTEN overexpression. Int J Oncol 2003;23:1109–13.
50. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995;146:1029–39.

JOURNAL OF VIROLOGY, Dec. 2006, p. 11899–11910 Vol. 80, No. 24
0022-538X/06/$08.00+0 doi:10.1128/JVI.00890-06

Induction of Robust Immune Responses against Human Immunodeficiency Virus Is Supported by the Inherent Tropism of Adeno-Associated Virus Type 5 for Dendritic Cells[▽]

Ke-Qin Xin,[1] Hiroaki Mizukami,[2] Masashi Urabe,[2] Yoshihiko Toda,[1] Kaori Shinoda,[1] Atsushi Yoshida,[1] Kenji Oomura,[1] Yoshitsugu Kojima,[1] Motohide Ichino,[3] Dennis Klinman,[4] Keiya Ozawa,[2] and Kenji Okuda[1]*

Departments of Molecular Biodefense Research[1] and Immunology,[3] Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan[2]; and Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland 20892[4]

Received 2 May 2006/Accepted 11 September 2006

The ability of adeno-associated virus serotype 1 to 8 (AAV1 to AAV8) vectors expressing the human immunodeficiency virus type 1 (HIV-1) Env gp160 (AAV-HIV) to induce an immune response was evaluated in BALB/c mice. The AAV5 vector showed a higher tropism for both mouse and human dendritic cells (DCs) than did the AAV2 vector, whereas other AAV serotype vectors transduced DCs only poorly. AAV1, AAV5, AAV7, and AAV8 were more highly expressed in muscle cells than AAV2. An immunogenicity study of AAV serotypes indicates that AAV1, AAV5, AAV7, and AAV8 vectors expressing the Env gp160 gene induced higher HIV-specific humoral and cell-mediated immune responses than the AAV2 vector did, with the AAV5 vector producing the best responses. Furthermore, mice injected with DCs that had been transduced ex vivo with an AAV5 vector expressing the gp160 gene elicited higher HIV-specific cell-mediated immune responses than did DCs transduced with AAV1 and AAV2 vectors. We also found that AAV vectors produced by HEK293 cells and insect cells elicit similar levels of antigen-specific immune responses. These results demonstrate that the immunogenicity of AAV vectors depends on their tropism for both antigen-presenting cells (such as DCs) and non-antigen-presenting cells (such as muscular cells) and that AAV5 is a better vector than other AAV serotypes. These results may aid in the development of AAV-based vaccine and gene therapy.

Recombinant adeno-associated viruses (AAVs) have been widely used as gene delivery vectors in animal models (17, 18), and these have entered human clinical trials (34). AAVs have been found in many animal species, including nonhuman primates, canines, fowls, and humans. An increasing number of AAV serotypes have been reported. AAV2, AAV3, and AAV5 are found in humans, while AAV4, AAV7, and AAV8 are found in nonhuman primates (21, 45, 50). The reservoir for AAV1 is unclear because these viruses have not been primarily isolated from tissues; however, reactive antibodies (Abs) against AAV1 have been found to exist in both humans and nonhuman primates (9). AAV6 appears to be a recombinant between AAV1 and AAV2 (60). Most of the current studies involving AAV are based on AAV2 since it was the first available infectious clone (51). The use of AAV2 as a vector to introduce exogenous genes encoding immunogenic proteins for the purpose of vaccination has been explored in several studies conducted by us (65, 66) and other researchers (17, 30, 46, 47). An ex vivo experiment demonstrated that the AAV2 vector can transduce dendritic cells (DCs) and that these cells then present the AAV-encoded antigen to T cells (46, 65). Other AAV serotypes may have advantages as vaccine vectors because AAV serotypes differ in their tissue and/or cell tropism (23, 25, 30, 47). For example, AAV1 and AAV7 are more efficient than AAV2 for the transduction of skeletal muscle (21, 60), while AAV3 is superior for the transduction of megakaryocytes (27). AAV5 and AAV6 infect apical airway cells more efficiently (24, 68). AAV2, AAV4, and AAV5 infect cells of the central nervous system; however, differences with regard to the distribution and target cell types exist among these three serotypes (68).

AAV is a small, single-stranded DNA virus that lacks an envelope. This virus requires a helper virus to facilitate efficient replication. The genome of wild-type AAV is known to integrate into the human genome at a specific site on chromosome 19q (36). However, in nondividing cells, AAV vector genomes mostly adopt the form of concatameric circular episomes that comprise active transcriptional units (16, 54). AAV is currently the only nonpathogenic viral vector that has been shown to mediate long-term gene expression without causing toxicity in vivo. Using this vector system, exogenous genes have been efficiently transferred into a number of tissues, including brain (18), muscle (29), lung (19), gut (17), liver (55), and eye (37). A human clinical trial of AAV2 has been conducted (34, 41).

The objectives of the present study were (i) to compare the immunogenicities of AAV serotypes (1 to 8) expressing human

* Corresponding author. Mailing address: Department of Molecular Biodefense Research, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Phone: 81(45)787-2602. Fax: 81 (45)787-2851. E-mail: kokuda@med.yokohama-cu.ac.jp.

▽ Published ahead of print on 27 September 2006.

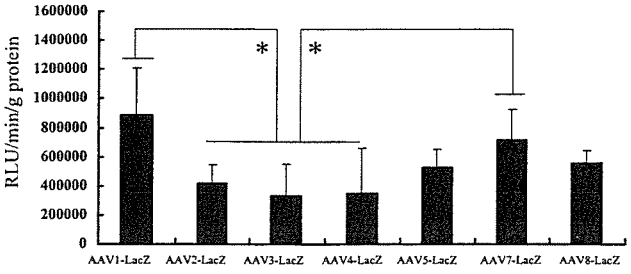
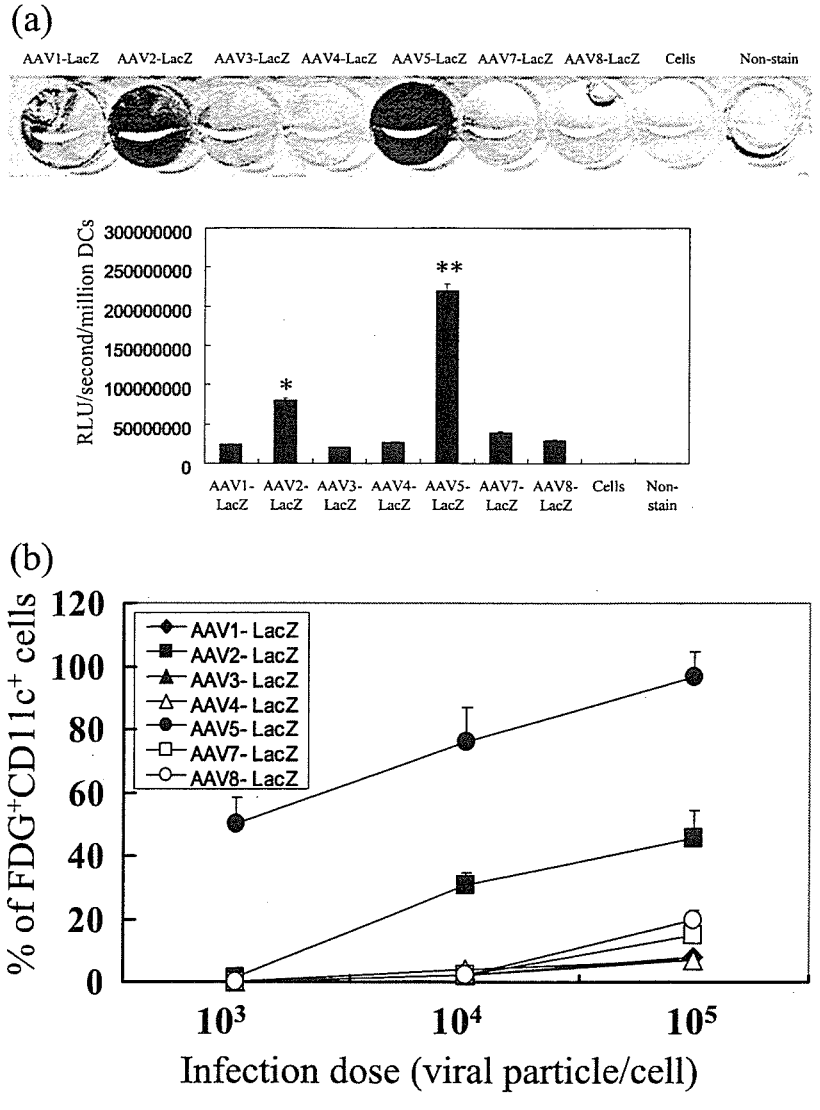


FIG. 1. Transduction of mouse muscle by AAV serotype vectors. AAV LacZ-expressing vectors (10^{10} vp) were injected intramuscularly into five mice/group. β-Galactosidase activity in the muscle was examined 2 weeks later using the Beta-Glo Assay System. An asterisk indicates a significant difference between the two groups ($P < 0.05$). RLU, relative light units.

immunodeficiency virus (HIV) Env gp160 in BALB/c mice, (ii) to compare the immunogenicities of AAV serotype vectors produced by HEK293 cells and insect cells, and (iii) to evaluate the mechanisms involved in the observed responses.

MATERIALS AND METHODS

AAV production. AAV vectors were generated as described previously (65, 66). In brief, a *lacZ* gene or a fragment containing HIV Env gp160 and Rev coding genes, which were derived from the HIV IIIB strain, was subcloned into a shuttle vector containing the cytomegalovirus (CMV) promoter, poly(A), and the AAV2 inverted terminal repeat (the AAV5 inverted terminal repeat was used for construction of the AAV5 vector; the AAV2 inverted terminal repeat was used for other AAV serotype vectors). We included a Rev coding gene in the construct because expression of HIV Env gp160 is dependent on Rev protein. This approach resulted in increased HIV Env gp160 protein expression in vitro and enhanced immune responses against HIV Env gp160 in vivo (33). The recombinant shuttle vector was packaged by triple transfection of HEK293 cells with an adenovirus helper plasmid, a chimeric packaging construct in which the AAV2 Rep gene was fused to the cap gene derived from either AAV serotype, and a shuttle vector plasmid to produce pseudotypes AAV2-based AAV1-LacZ, AAV2-LacZ, AAV3-LacZ, AAV4-LacZ, AAV5-LacZ, AAV7-LacZ, and AAV8-LacZ or AAV1-HIV, AAV2-HIV, AAV3-HIV, AAV4-HIV, AAV5-



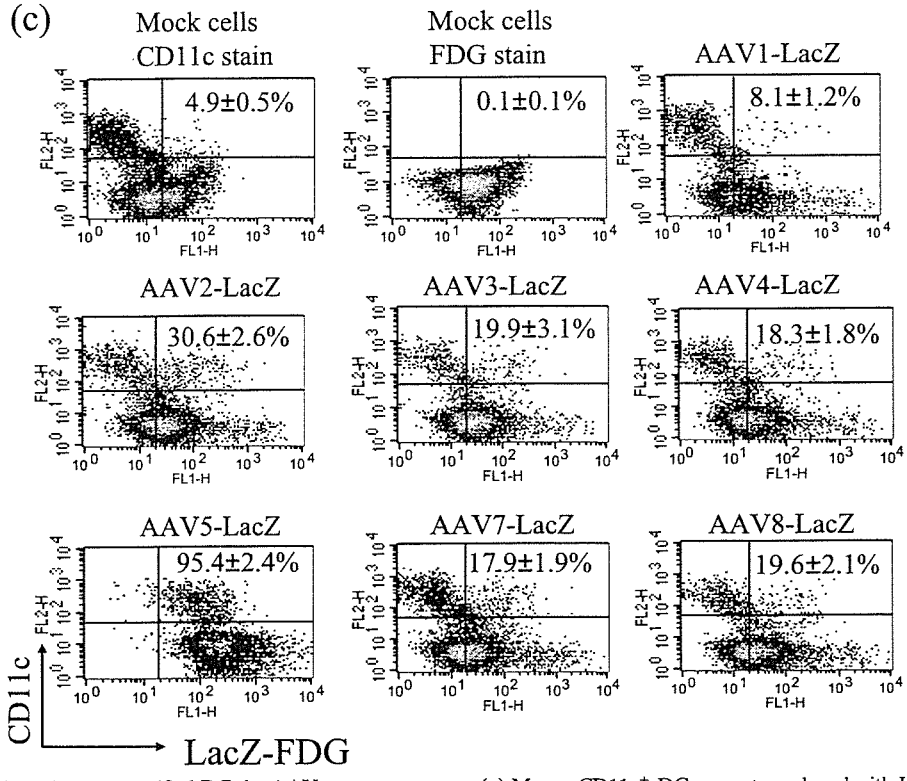


FIG. 2. Transduction of mouse purified DCs by AAV serotype vectors. (a) Mouse CD11c$^+$ DCs were transduced with LacZ-expressing AAV vectors at 10^4 vp/cell (triplicate for each sample). Two days after infection, the cells were stained with X-Gal (upper panel), and the β-galactosidase activity was measured using the Beta-Glo Assay System (bottom panel). The data presented were averaged from three separate experiments. The asterisk indicates a significant difference when AAV2-LacZ-transduced cells were compared to AAV1-LacZ-, AAV3-LacZ-, AAV4-LacZ-, AAV7-LacZ-, and AAV8-LacZ-transduced cells and mock-transduced cells; the double asterisk indicates a significant difference when AAV5-LacZ-transduced cells were compared to AAV2-LacZ-transduced cells. RLU, relative light units. (b) Mouse CD11c$^+$ DCs were transduced with AAV-LacZ vectors at 10^3 to 10^5 vp/cell (triplicate for each sample). Two days after transduction, the cells were stained with X-Gal, and the percentages of LacZ-expressing DCs (FDG$^+$ CD11c$^+$ DCs) were determined. The data presented were averaged from three separate experiments. (c) Enriched immature DCs were transduced with 10^5 vp/cell of various AAV-LacZ vectors (triplicate for each sample). Two days after transduction, the cells were treated with PE-conjugated anti-mouse CD11c Ab and FDG followed by flow cytometric analysis. The data provided represent the fraction of FDG/CD11c dual-positive cells as a percentage of the total population of CD11c$^+$ cells. The data presented were averaged from three independent experiments.

HIV, AAV7-HIV, and AAV8-HIV vectors. The AAV vectors were purified by the standard cesium chloride sedimentation method (65, 66). The titer was determined by quantitative DNA dot blot hybridization.

The insect cell-produced AAV vectors (BacAAVs) were generated as described previously (58). The fragment containing the CMV promoter, HIV Env gp160 and Rev coding genes, and poly(A) was excised from the AAV shuttle vector by using NotI; the resulting fragment was inserted into the corresponding site of a baculovirus transfer plasmid between the serotype 2 or 5 inverted terminal repeats. Recombinant baculoviruses were generated by using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). Recombinant baculoviruses containing the HIV Env gp160 and Rev coding genes; an AAV Rep of serotypes 1, 2, and 5; and an AAV1, AAV2, or AAV5 capsid were used to infect insect cells in order to produce BacAAV1-HIV, BacAAV2-HIV, and BacAAV5-HIV vectors, respectively. The AAV vectors produced were purified by two rounds of ultracentrifugation with a standard cesium chloride density gradient (65, 66). The titer was determined by quantitative DNA dot blot hybridization.

In vivo expression of β-galactosidase. The AAV-LacZ vector (10^{10} viral particles [vp]/mouse) was injected into mouse muscle. Two weeks after the administration, the mouse was sacrificed and the β-galactosidase activity in the muscle was monitored periodically from 1 week through 6 months after administration by using the Beta-Glo Assay System (Promega, Madison, WI).

Mouse DC preparation. DCs were isolated from BALB/c mouse bone marrow, as described previously (7, 62). In brief, the bone marrow was obtained from the tibia and femur of BALB/c mice. The DCs at a density of 5×10^5 cells/ml were cultured in RPMI 1640 medium containing 10% fetal calf serum, 1 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Kirin Beer Corp., Tokyo, Japan), and recombinant interleukin-4 (IL-4) for 6 days.

Transduction of AAV vectors to mouse cells. Immature mouse DCs were stained with phycoerythrin (PE)-conjugated anti-mouse CD11c antibody (clone N418; eBioscience, Boston, MA). The CD11c$^+$ DCs were sorted using a MoFlo Cell Sorter (Takara Bio Corp., Tokyo, Japan). The cells were transduced with LacZ-expressing serotype AAV vectors at 37°C at 10^3 to 10^5 vp/cell for 2 days. The transduced cells were washed with phosphate-buffered saline (PBS) and stained with 40 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in staining buffer [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS] at 37°C for 2 h. The β-galactosidase activity was detected by using the Beta-Glo Assay System (Promega). To count LacZ-expressing cells, the sorted CD11c$^+$ DCs were treated with 1 μM of fluorescein digalactoside (FDG; Molecular Probes, Eugene, OR) followed by flow cytometric analysis (the data are shown in Fig. 2b). The enriched, unsorted DCs were transduced with LacZ-expressing serotype AAV vectors at 37°C at 10^5 vp/cell for 2 days. The cells were stained with anti-mouse CD11c antibody and treated with FDG followed by flow cytometric analysis (the data are shown in Fig. 2c).

The mouse DCs used in this study were derived from GM-CSF- and IL-4-treated bone marrow cells. To explore the efficiencies of transduction of AAV serotype vectors to hematopoietic cells, unpurified mouse splenocytes and bone marrow cells were transduced with LacZ-expressing serotype AAV vectors at 10^5 vp/cell at 37°C for 2 days. The bone marrow cells were treated with 1 μM of FDG, and splenocytes were treated with 1 μM of FDG and PE-conjugated

anti-mouse CD3e (clone145-2C11), PE-conjugated anti-mouse CD11b (clone M1/70), or PE-conjugated anti-mouse CD19 (clone MB19-1) (all antibodies were from eBioscience) followed by flow cytometric analysis for staining T cells, macrophages, and B cells, respectively.

Human DC preparation. To explore the transduction efficiencies of AAV serotype vectors to human cells, human peripheral blood mononuclear cells (PBMCs) and an enriched population of PBMC-derived immature DCs were prepared as previously described (67). Briefly, human PBMCs were isolated from the blood of a healthy person using Lymphoparu I (Immune Biological Laboratory, Gunma, Japan) according to the manufacturer's instructions. Fresh human PBMCs at 3×10^6 cells/ml in RPMI 1640 medium were dispensed into individual wells of 12-well plates (1 ml/well), which had been previously coated with autologous plasma for 30 min at 37°C. The PBMC cultures were allowed to incubate at 37°C for 1 h. After gentle washing with serum-free RPMI 1640 medium, the adherent cells were cultured in Iscove's modification of Dulbecco modified Eagle medium (2 ml/well) containing human GM-CSF (500 ng/ml) and IL-4 (200 μg/ml) (both from Pierce Biotechnology, Rockford, IL) for 5 days.

Transduction of AAV vectors to human cells. The human PBMCs and an enriched population of PBMC-derived immature DCs were transduced with LacZ-expressing serotype AAV vectors at 10^5 vp/cell at 37°C for 2 days. The AAV-transduced DCs were treated with 1 μM of FDG, and AAV-transduced PBMCs were treated with FDG and PE-conjugated anti-human CD11c (clone 3.9), PE-conjugated anti-human CD3 (clone HIT3a), PE-conjugated anti-human CD11b (clone ICRF44), or PE-conjugated anti-human CD19 (J4-166) (all antibodies were from eBioscience) followed by flow cytometric analysis for staining DCs, T cells, macrophages, and B cells, respectively.

Western blot analysis. To confirm the expression of HIV Env gp160, HEK293 cells were transduced with the AAV vectors encoding the HIV gene in a six-well plate. Two days posttransduction, the cells were washed in PBS and lysed with 0.1 M Tris-HCl (pH 7.8) and 0.125% Nonidet P-40. The cell lysates were mixed with an equal volume of 2× sodium dodecyl sulfate buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol) and boiled for 10 min. Then the cell lysates were loaded on an 8% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The HIV gp160 and β-actin proteins were detected using a mouse anti-HIV gp120 monoclonal Ab (MAb) (hybridoma 902; AIDS Research and Reference Reagent Program, National Institutes of Health, Maryland) and an anti-human β-actin MAb (Sigma), respectively. An affinity-purified horseradish peroxidase-labeled anti-mouse immunoglobulin (Ig; ICN Pharmaceuticals, Inc., Solon, OH) was used as the secondary Ab. Protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Animals and immunization. Eight-week-old BALB/c female mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in the animal center located at Yokohama City University and maintained on a 12-h day-night cycle. The mice were intramuscularly (i.m.) immunized three times with 10^{10} particles of the AAV vector at 2-week intervals.

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay was performed as described elsewhere (63, 65). To summarize, 96-well microtiter plates were coated with 1 μg/ml of HIV_{SF2} gp120 protein (donated by the AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They were then treated with 100 μl of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified horseradish peroxidase-labeled anti-mouse antibody (Sigma). The mean antibody titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 standard deviations.

IFN-γ ELISPOT assay. Two weeks after the final immunization, a gamma interferon (IFN-γ) ELISPOT assay was performed as described previously (62). In brief, MultiScreen-IP plates (Millipore, Bedford, MA) were coated with 50 μl of 10-μg/ml purified rat anti-mouse IFN-γ antibody (XMG1.2; PharMingen, San Diego, CA) in PBS overnight at 4°C. The plate was then blocked with PBS containing 5% BSA and 0.025% Tween 20 for 2 h at room temperature. Lymphocytes (1×10^5 to 10×10^5) isolated from the spleen were added to each well in triplicate. The spleen cells were stimulated with or without 10 μg/ml of the HIV V3 peptide (RGPGRAFVTI) for 24 h at 37°C. After incubation, the cells were removed and incubated with 0.5 μg/ml of biotinylated anti-mouse IFN-γ antibody (PharMingen) for 2 h at 37°C, followed by the addition of 100 μl/well of 0.2% alkaline phosphatase-streptavidin (Vector Laboratories, Burlingame, CA) in PBS containing 0.05% Tween 20 and 5% BSA for 1.5 h. Finally, the plate was treated with 50 μl/well of 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium membrane phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at room temperature for 20 min, and the reaction was terminated by holding the plate under running distilled water. The number of spots was counted using a computer-assisted video image analyzer. The results were expressed as spot-forming cells (SFC) per million cells.

Tetramer assay. The tetramer assay was performed as described previously (62–65). The H-$2D^d$/p18 tetramer (RGPGRAFVTI) labeled with PE was prepared by ProImmune Limited (Oxford, United Kingdom). In brief, mouse splenocytes were incubated with 4% healthy mouse serum in PBS for 15 min at 4°C. The cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD8α (Ly-2; PharMingen) at 0.5 μg/10^6 cells for 30 min at 4°C. After two washes in staining buffer (3% fetal calf serum, 0.1% NaN_3 in PBS), the cells were incubated with the tetramer reagent for 30 min at 4°C and analyzed by flow cytometry.

In vivo CTL assay. The assay was performed as previously described (52). Briefly, cytotoxic T-lymphocyte (CTL) target cells were derived from naïve splenocytes and then pulsed with 10 μg/ml of the HIV V3 peptide (RGPGRAFVTI) or with no peptide at 37°C for 1 h. Peptide-pulsed cells were labeled with a high concentration of carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 μM; Molecular Probes, Eugene, Oreg.), or unpulsed cells were labeled with a low concentration of CFSE (0.5 μM). Cells were then washed and enumerated. For the in vivo CTL assay, 5×10^6 pulsed and 5×10^6 unpulsed cells were combined into a final 200 μl of PBS and intravenously injected into vaccinated mice. Mice were killed 24 h after in vivo target cell incubation. The mouse splenocytes were analyzed by flow cytometry. The percent in vivo killing of CFSE-labeled target cells was defined as the relative loss of such cells after in vivo incubation and thus was taken as the measure of CTL. It was calculated using the formula % killing = [1 − (unpulsed cells/peptide-pulsed cells from unimmunized control)/(unpulsed cells/peptide-pulsed cells from immunized group)] × 100.

Surface expression of HIV gp120 and ex vivo transduction. To investigate whether the AAV-HIV-transduced DCs could present the HIV peptide to T cells, mouse bone marrow-derived DCs were transduced with AAV-HIV vectors at 10^5 vp/cell at 37°C for 2 days. To explore whether HIV gp160 is expressed on the surface of DCs, the AAV-HIV-transduced DCs were stained with HIV gp120 MAb (hybridoma 902) followed by FITC-conjugated rabbit anti-mouse IgG and PE-conjugated anti-mouse CD11c antibody (clone N418; eBioscience). The cells were analyzed by flow cytometry. Enriched mouse DCs were transduced with the AAV-HIV vectors for 2 h and then washed with PBS, and 10^5 cells were intravenously administered to recipient BALB/c mice. One week after the administration, HIV-specific responses were detected by a tetramer assay.

Data analysis. All the values are expressed as means ± standard errors. Statistical analysis of the experimental data and controls was conducted using one-way factorial analysis of variance. Statistical significance was defined as a value of $P < 0.05$.

RESULTS

Different expression levels of AAV serotype vectors in mouse muscle. Muscular cells are one of the major targets transduced when viral vectors are delivered via the i.m. route. In this study, the relative efficiency with which various AAV serotype vectors were transduced and expressed in vivo was explored. Mice were injected i.m. with AAV serotype vectors carrying the *lacZ* gene. β-Galactosidase activity was monitored periodically from 1 week through 6 months after administration. Results show that the AAV1 and AAV7 vectors elicited significantly higher β-galactosidase activity than the AAV2, AAV3, and AAV4 vectors, with AAV5 and AAV8 yielding intermediate levels of expression at 2 weeks after administration (Fig. 1). We found that AAV expression was maximal 2 to 4 weeks after AAV vector administration, although β-galactosidase activity remained elevated for up to 6 months (data not shown).

Strong tropism of AAV5 for mouse DCs. The ability of each AAV serotype to transduce DCs was then examined. Enriched $CD11c^+$ DCs were purified and incubated for 2 days with 10^4 vp/cell of each AAV serotype. Some cells were stained with X-Gal, and others were examined for β-galactosidase expression. As shown in Fig. 2a, very strong X-Gal staining was

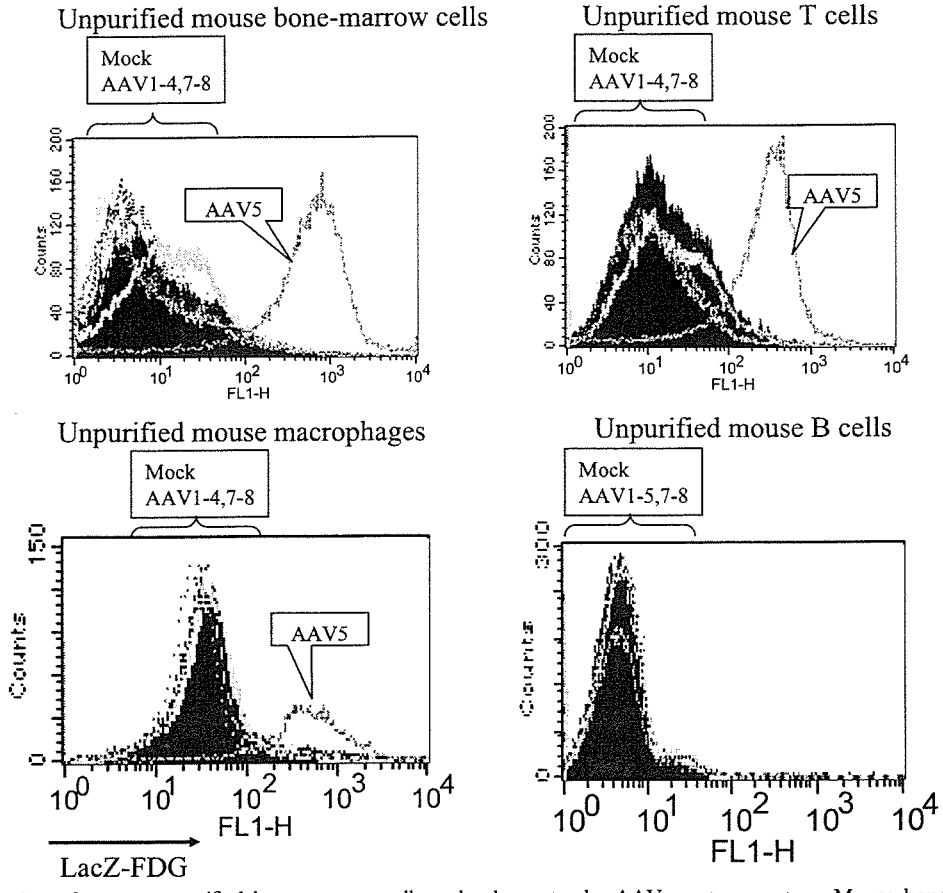


FIG. 3. Transduction of mouse unpurified bone marrow cells and splenocytes by AAV serotype vectors. Mouse bone marrow cells and splenocytes were transduced with 10^5 vp/cell of AAV-LacZ vectors. Two days after transduction, the cells were treated with FDG and the splenocytes were stained with anti-mouse CD3 (T cells), anti-mouse CD11b (macrophages), or anti-mouse CD19 (B cells) antibody followed by flow cytometric analysis. Panels show results from one of three independent experiments.

observed in cells treated with AAV5 vector, weaker staining in those treated with AAV2, and none in cells exposed to the other AAV serotypes. Similar results were obtained when purified $CD11c^+$ DCs were incubated with 10^3 to 10^5 vp/cell, and X-Gal expression was monitored by flow cytometry. As the dose of vector increased, up to 96.5% of DCs were transduced by the AAV5 vector and 45.7% by the AAV2 vector, but <10% for all other vectors (Fig. 2b). Similar results were observed using enriched bone marrow-derived immature DCs (Fig. 2c). Of note, the AAV5 vector strongly transduced both bone marrow-derived $CD11c^+$ cells and $CD11c^-$ cells (Fig. 2c). Further study demonstrated that AAV5 transfected most unpurified bone marrow cells, whereas AAV2 was much less effective and other AAV serotypes largely ineffective (Fig. 3). AAV5 was also effective at transducing T cells (>85% of $CD3^+$ cells) and macrophages (>30% of $CD11b^+$ cells), but less than 10% of T cells and macrophages were transduced by other AAV serotypes (Fig. 3). However, there was no significant difference in the abilities of various vectors to transduce B cells (<15%).

Strong tropism of AAV5 for human DCs. To explore the transduction efficiency of AAV5 for human target cells, DCs were prepared from human PBMCs. At an infection dose of 10^5 vp/cell, 26.8% of enriched human $CD11^+$ DCs were transduced by AAV5, but less than 5% of DCs were transduced by other serotypes (Fig. 4). The cellular tropism of AAV5 was also examined by flow cytometry. AAV5 infected 56.4% of $CD3^+$ T cells (Fig. 4) and 23.5% of $CD11b^+$ macrophages (Fig. 4). However, other serotype vectors were poorly transduced into both T cells and macrophages (<5%, Fig. 4). No significant difference in transduction of $CD19^+$ B cells was observed among AAV serotype vectors (<10% of B cells). In general, human DCs, macrophages, and T cells were transduced less effectively by AAV5 than were murine cells, perhaps reflecting differences in AAV5 receptor expression between these two species.

Similar immunogenicities of AAV vectors produced by HEK293 cells and insect cells. Difficulty in producing high-titered AAV stocks has limited the clinical utility of this class of vectors. To resolve the problem, AAV vectors have been produced utilizing baculovirus in insect cells (42, 56, 58) and herpes simplex virus (8, 14). In this study, HEK293 cells were infected with 10^5 vp/cell of AAV-HIV vectors and the expression of AAV-HIV vectors was confirmed by Western blotting (Fig. 5a). HIV gene expression by AAV1 and AAV2 vectors was slightly stronger than that by other serotype vectors. The

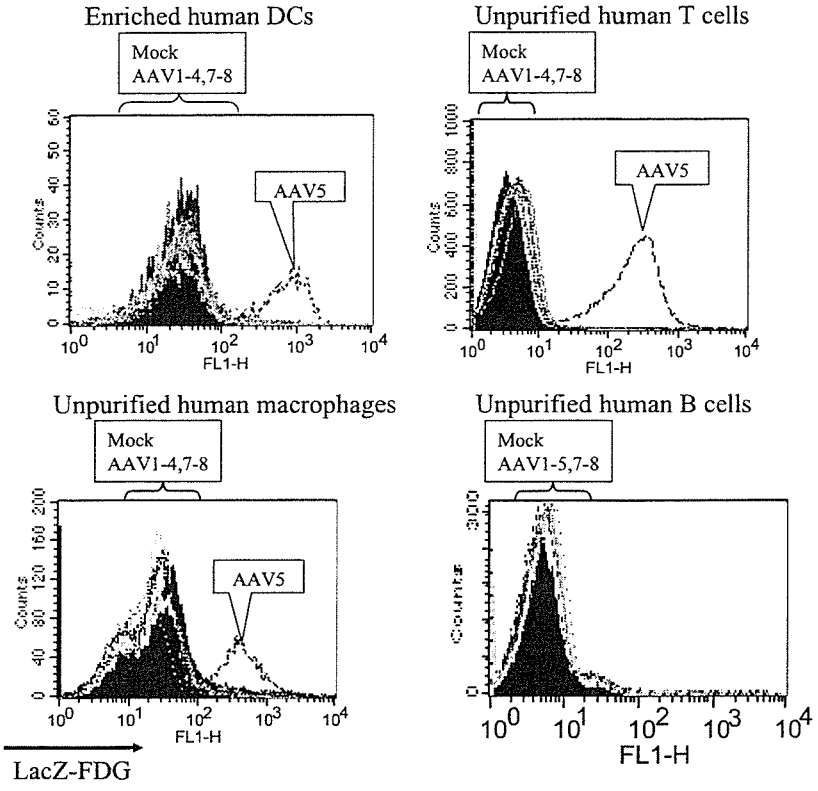


FIG. 4. Transduction of enriched human DCs and unpurified PBMCs by AAV serotype vectors. Human enriched DCs or PBMCs were transduced with 10^5 vp/cell of AAV-LacZ vectors. Two days after transduction, the DCs were treated with FDG and anti-human CD11c Ab, and PBMCs were treated with FDG and anti-human CD3 (T cells), anti-human CD11b (macrophages), or anti-human CD19 (B cells) antibody followed by flow cytometric analysis. Panels show results from one of three independent experiments.

abilities to produce three AAV serotypes (AAV1, AAV2, and AAV5) in insect cells and in HEK293 cells were compared. Expression levels of insect cell-produced AAV vectors similar to those of HEK293-produced corresponding AAV vectors were observed. Again, as seen in Fig. 6, similar cell-mediated immune responses were elicited by the corresponding AAV vectors. However, AAV vectors derived from insect cells were slightly more effective at inducing humoral immune responses than vectors derived from HEK293 cells (Fig. 5b). This may be due to vector contamination by baculovirus components, since baculovirus itself can have an adjuvant effect on vaccine-induced immune responses (1, 22).

AAV-HIV immunization stimulates a humoral immune response. To explore the immunogenicity of the AAV vectors, BALB/c mice were immunized three times with 10^{10} vp of various AAV-HIV vectors. The resultant HIV-specific serum Ab response was examined 1 month after the last boost. The strongest HIV-specific serum IgG response was generated in mice immunized with the AAV5-HIV vector; the poorest humoral responses were observed in mice immunized with AAV3-HIV or AAV4-HIV (Fig. 5b). AAV1-HIV, AAV7-HIV, and AAV8-HIV induced intermediate humoral responses (Fig. 5b).

AAV-HIV immunization stimulates a cellular immune response. The induction of HIV-specific IFN-γ-secreting cells was then examined in the immunized mice. As observed for humoral immunity, animals immunized with the AAV5-HIV vector generated the strongest cellular immune response (1,500 SFC/million cells). The AAV1, AAV2, AAV7, and AAV8 HIV vectors also stimulated >300 IFN-γ-secreting SFC/million spleen cells, whereas the AAV3- and AAV4-HIV vectors elicited significantly weaker cellular responses ($P < 0.05$, Fig. 6a). The AAV5-HIV produced by either HEK293 or insect cells induced significantly higher cellular immune responses than the AAV1-HIV produced by corresponding cells.

A tetramer binding assay was used to identify major histocompatibility complex class I-restricted HIV-specific T cells (3). Mice immunized with the AAV1-, AAV5-, and AAV7-HIV vectors induced strong HIV-specific $CD8^+$ T-cell responses (>2%, Fig. 6b), and AAV2-HIV was intermediate (1.2%), while AAV3- and AAV4-HIV elicited only 0.4% HIV-specific $CD8^+$ T cells.

An in vivo CTL assay was used to detect vaccine-elicited cell-mediated immune responses. Whereas the AAV3-, AAV4-, and AAV8-HIV vectors elicited approximately 40% CTL activity, the AAV1-, AAV5-, and AAV7-HIV vectors were significantly more effective (generally >50% activity), with the AAV2-HIV vector being intermediate (Fig. 6c).

DCs present HIV peptide to T cells in vivo. To examine whether DCs play a role in AAV-HIV-induced immune responses, DCs were enriched from the bone marrow of naïve mice. Enriched DCs were then treated with AAV5-LacZ, AAV1-HIV, AAV2-HIV, or AAV5-HIV vectors in vitro (10^5

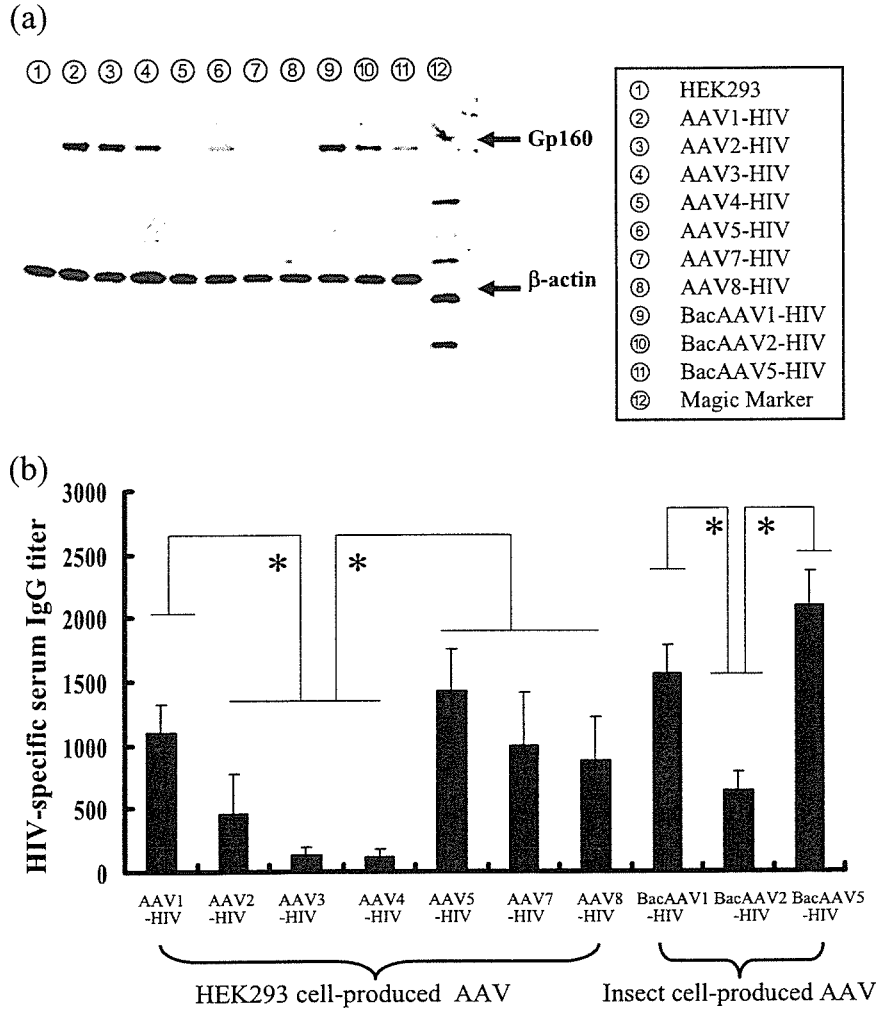


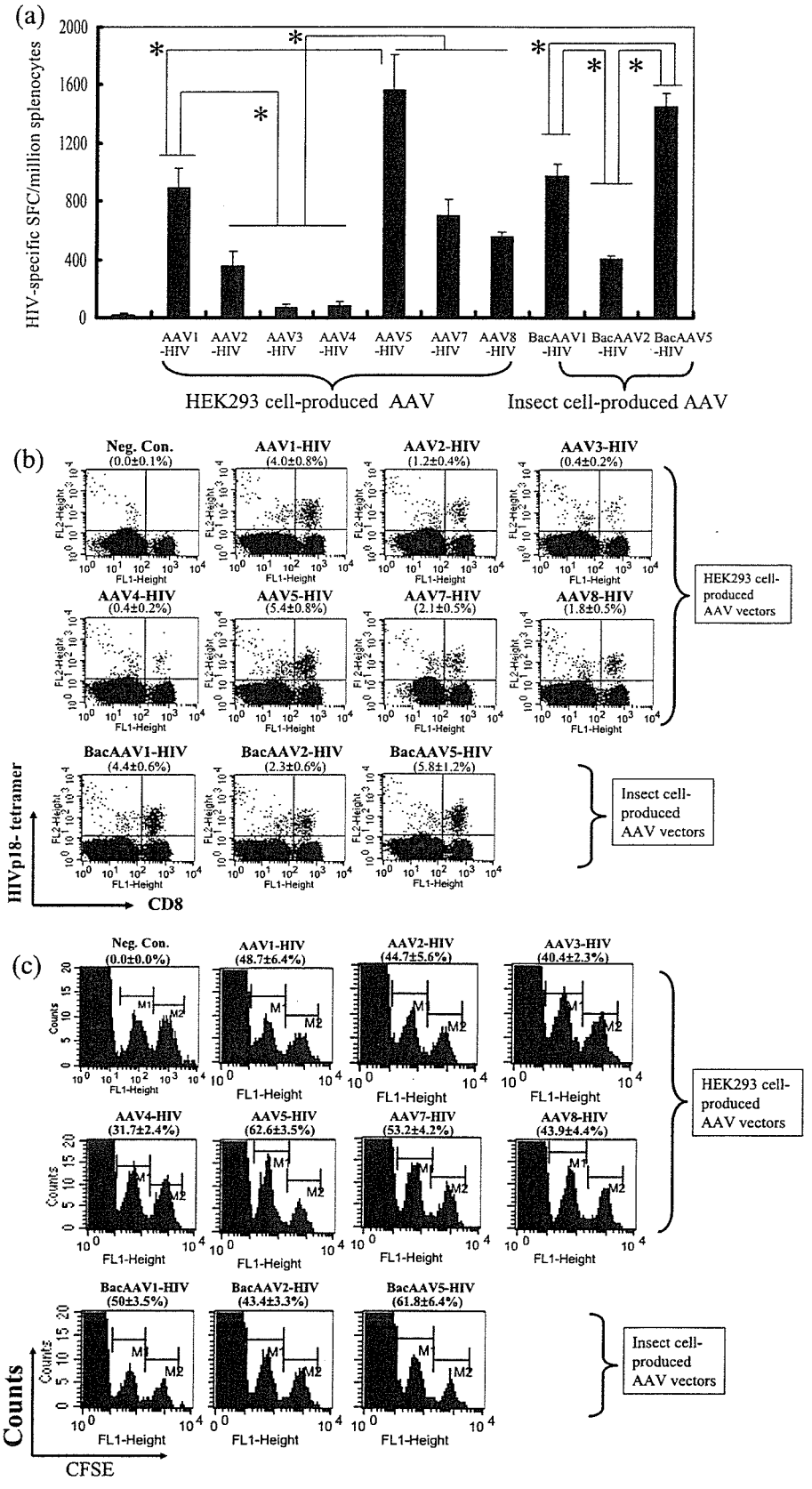
FIG. 5. Expression of HIV gp160 and HIV-specific serum IgG titer by AAV serotype vectors. (a) HEK293 cells were transduced with AAV vectors carrying the HIV Env gp160 gene. Two days after infection, cell lysates were analyzed by Western blotting using anti-HIV Env MAb and anti-human β-actin MAb. (b) BALB/c mice (five mice/group) were immunized with AAV-HIV vectors on days 0, 14, and 28. HIV-specific serum IgG was measured 1 month after the final immunization. The BacAAV vectors were generated in insect cells, whereas other vectors were produced in HEK293 cells. The asterisks indicate significant differences between the two groups ($P < 0.05$).

vp/cell). The expression of HIV gp160 protein on the surface of mouse DCs was confirmed by anti-mouse HIV gp120 monoclonal antibody and anti-mouse CD11c antibody staining followed by flow cytometric analysis. HIV gp160 protein was detected in 13.5%, 19.4%, and 65.2% of $CD11c^+$ DCs which were transduced with AAV1-HIV, AAV2-HIV, and AAV5-HIV vectors, respectively (Fig. 7a). On the other hand, enriched mouse DCs were transduced with AAV-HIV vectors and injected into naïve recipients. Seven days later, HIV-specific tetramer binding $CD8^+$ cells were quantified using the tetramer assay. Recipients of the AAV2-HIV (0.6%)- and AAV5-HIV (2.1%)-transduced cells had significantly higher HIV peptide-binding $CD8^+$ T cells than the AAV-LacZ-treated control (0.0%) (Fig. 7b). In contrast, a weak immune response was observed in recipients of AAV1-HIV vector-transduced cells (0.1%).

DISCUSSION

The present study demonstrates that AAV5 is a superior vector for achieving humoral and cellular immune responses, and the immunogenicity of AAV vectors depends on their tropism for both antigen-presenting cells (APCs) (such as DCs) and non-antigen-presenting cells (such as muscular cells).

Consistent with previous studies, AAV-HIV vectors of different serotypes had distinct effects on the induction of HIV-specific humoral and cell-mediated immune responses (10, 17, 20, 28, 31, 38, 40, 53, 65, 66). However, most of these studies examined the durability of transgene expression after i.m. administration of the AAV vector but did not typically monitor cell-mediated immune responses against the encoded antigen (19, 32, 61). In contrast to studies in which the AAV vector encoded a self protein (as in gene therapy) or in which a


(a)
HIV-specific SFC/million splenocytes
2000
1600
1200
800
400
0
AAV1 -HIV
AAV2 -HIV
AAV3 -HIV
AAV4 -HIV
AAV5 -HIV
AAV7 -HIV
AAV8 -HIV
BacAAV1 -HIV
BacAAV2 -HIV
BacAAV5 -HIV
HEK293 cell-produced AAV
Insect cell-produced AAV
(b)
Neg. Con. (0.0±0.1%)
AAV1-HIV (4.0±0.8%)
AAV2-HIV (1.2±0.4%)
AAV3-HIV (0.4±0.2%)
AAV4-HIV (0.4±0.2%)
AAV5-HIV (5.4±0.8%)
AAV7-HIV (2.1±0.5%)
AAV8-HIV (1.8±0.5%)
BacAAV1-HIV (4.4±0.6%)
BacAAV2-HIV (2.3±0.6%)
BacAAV5-HIV (5.8±1.2%)
FL2-Height
FL1-Height
HEK293 cell-produced AAV vectors
Insect cell-produced AAV vectors
HIVp18- tetramer
CD8
(c)
Neg. Con. (0.0±0.0%)
AAV1-HIV (48.7±6.4%)
AAV2-HIV (44.7±5.6%)
AAV3-HIV (40.4±2.3%)
AAV4-HIV (31.7±2.4%)
AAV5-HIV (62.6±3.5%)
AAV7-HIV (53.2±4.2%)
AAV8-HIV (43.9±4.4%)
BacAAV1-HIV (50±3.5%)
BacAAV2-HIV (43.4±3.3%)
BacAAV5-HIV (61.8±6.4%)
Counts
FL1-Height
M1
M2
HEK293 cell-produced AAV vectors
Insect cell-produced AAV vectors
Counts
CFSE

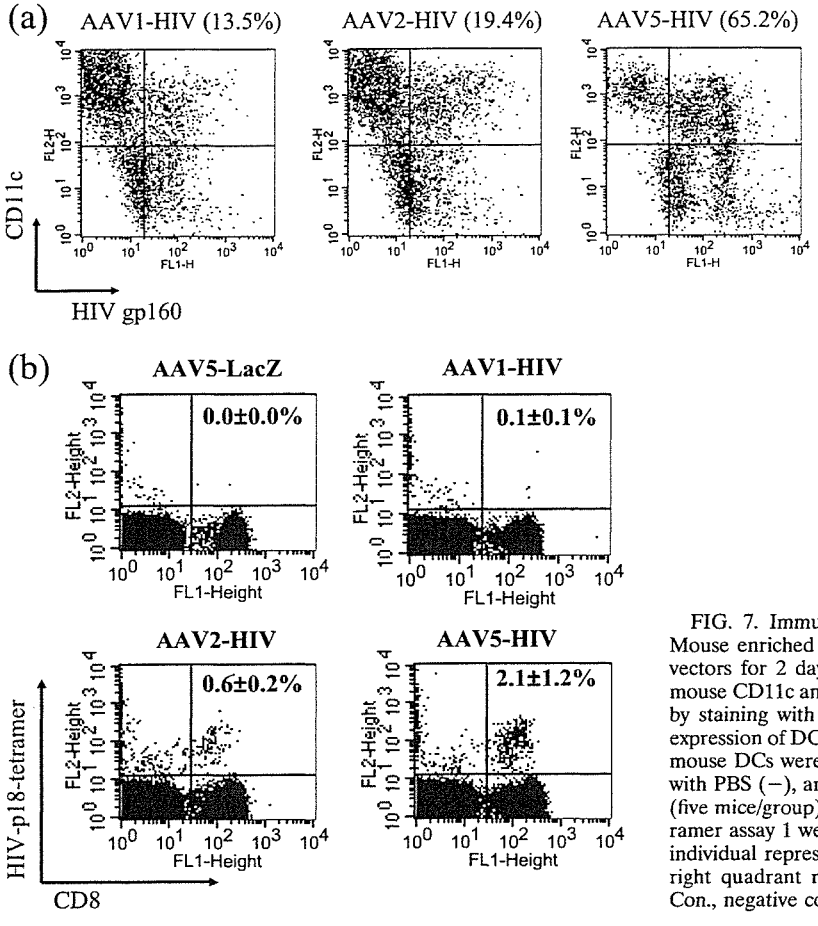


FIG. 7. Immune responses induced by ex vivo DC transduction. Mouse enriched DCs were transduced with 10^5 vp/cell of AAV-HIV vectors for 2 days. The cells were stained with PE-conjugated anti-mouse CD11c antibody plus mouse anti-HIV gp120 antibody followed by staining with FITC-conjugated anti-mouse IgG. The HIV gp160 expression of DC surface was analyzed by flow cytometry (a). Enriched mouse DCs were transduced with AAV-HIV vectors for 2 h, washed with PBS (−), and injected intravenously into recipient BALB/c mice (five mice/group). HIV-specific responses were detected using the tetramer assay 1 week after administration. Dot plots show results from individual representative animals, while the data shown in the upper right quadrant represent the averages of five mice/group (b). Neg. Con., negative control.

neoantigen was used to evaluate long-term expression (such as β-galactosidase), the immune response elicited by our HIV Env gp160-encoding vector was quite strong (10, 17, 20, 28, 31, 38, 40, 53, 65, 66).

Most currently used AAV vectors for vaccine or gene therapy are based on the AAV2 serotype (10, 17, 20, 28, 31, 38, 40, 53, 65, 66). The current work compared the immunogenicity of AAV2-HIV to that of other AAV serotype-based vectors and found that the AAV5 vector induced the strongest cellular and humoral immune responses. This finding may help in the development of novel AAV vector-based vaccines, particularly because natural immunity to AAV5 is rare (30). Of note, while we used a prime/boost regimen involving repeated administration of the same AAV serotype vector, it may be even more effective to prime with one serotype and boost with another (since neutralizing Abs raised against the first vector are unlikely to cross-react with a different serotype) (48).

Following vaccination with the AAV vector, antigen-specific immune responses can be induced through at least two pathways. In one of the pathways, the vaccine vector directly infects APCs, such as DCs, where the encoded antigen is expressed. The antigen is then processed and presented by APCs to T cells (Fig. 7) (65). The percentage of HIV gp160$^+$ CD11c$^+$ cells in Fig. 7a was lower than the percentage of FDG$^+$ CD11c$^+$ cells in Fig. 2c. This may be due to the presentation of HIV gp160 peptides by DCs present in culture. In the other pathway, the vaccine vector infects non-APCs, such as muscle cells. APCs take up the antigen expressed by the infected cells and present peptides from the exogenous antigen to T cells by major histocompatibility complex class I-restricted cross-presentation. It has been known that adenovirus vectors may use both pathways; however, AAV2 relies on the latter pathway due to a low efficiency of transfection of DCs (53). Current findings indicate that AAV1,

FIG. 6. HIV-specific cell-mediated immune responses induced by AAV vector vaccination. BALB/c mice (five mice/group) were immunized with AAV-HIV vectors on days 0, 14, and 28. Five mice per group for the IFN-γ ELISPOT and tetramer assay and five mice per group for the in vivo CTL assay were used. The HIV-specific IFN-γ ELISPOT assay was performed (a), the percentage of HIV-specific tetramer binding CD8$^+$ cells was determined (b), and the in vivo CTL assay was performed (c) 2 weeks after the final immunization. BacAAV vectors were generated in insect cells, whereas other AAV vectors were generated in HEK293 cells. Asterisks indicate significant differences between the two groups ($P < 0.05$). Panels b and c show results from individual representative animals, while the data represent the averages of five mice/group.

AAV7, and AAV8 vectors preferentially infect muscle cells, whereas the AAV5 vector optimally infected DCs (Fig. 1 to 4 and 7). Following i.m. administration, all four vectors induced higher HIV-specific immunogenicity than the AAV2 vector (Fig. 5 and 6).

DCs are primarily responsible for stimulating resting naïve T lymphocytes and initiating a CTL response (5). Immature DCs residing in the peripheral tissues capture foreign antigens, mature, and then migrate to secondary lymphoid organs, where the processed antigen is presented. To determine whether DCs might contribute to the immune responses induced by the AAV-HIV vector, enriched DCs were transduced with the AAV5-HIV vector and transferred into naïve mice. As seen in Fig. 7b, recipients generated HIV-specific tetramer binding $CD8^+$ T cells. These results demonstrate that DCs can present vector-encoded antigen to naïve T cells efficiently. In this context, the levels of immune response induced by the AAV1-HIV, AAV2-HIV, and AAV5-HIV vectors correlated with their tropism for DCs (70).

A number of studies show that recombinant AAV vectors can be used to transduce DCs (2, 13, 32, 35, 39, 46, 49, 53, 70). Current results document that AAV5 can transfect mouse and human DCs much more efficiently than other AAV serotype vectors (Fig. 2 to 4). In addition, AAV5 was efficiently transduced into T cells and macrophages (Fig. 3 and 4), which may be useful for gene therapy of diseases involving hematopoietic cells (4, 26, 43, 57, 59, 69).

Eight AAV serotypes (AAV1 to AAV8) have been described, with each serotype having unique binding and cell tropism characteristics (6, 11, 12, 21, 44, 50, 60). A primary coreceptor for AAV5 is sialic acid, while the platelet-derived growth factor receptor is involved in the binding and cell entry of AAV5 (15). Platelet-derived growth factor receptor is mainly expressed on fibroblasts, smooth muscle cells, glial cells, and chondrocytes but not on hematopoietic cells. Surprisingly, we found that AAV5 was efficiently transduced into hematopoietic cells (Fig. 3 and 4), suggesting that another receptor(s) may be used by AAV5 for cell entry.

The AAV2 vector can be produced in insect cells by using baculovirus expression vectors in suspension culture, a strategy that is amenable to easy scale-up (58). In this study, AAV1, AAV2, and AAV5 vectors expressing the HIV gp160 gene were prepared using either insect cells or HEK293 cells. Similar immune responses were obtained using vectors produced by either method (Fig. 5b and 6), indicating that insect cell-produced AAV vectors should be considered for the large-scale preparation of AAV vectors.

In summary, this is the first report that systematically compares DC tropism and immunogenicity of AAV serotype vectors. Results show that AAV5 vectors can efficiently transduce DCs and produce stronger antigen-specific immune responses than other AAV vectors. Thus, AAV5-based vectors deserve further consideration for clinical vaccine development and immune therapy.

ACKNOWLEDGMENTS

We are grateful to M. Kawano for her technical assistance and A. De La Fuente for her secretarial assistance. We also extend our appreciation to J. M. Wilson and G. P. Gao for providing helper plasmids of AAV7 and AAV8.

This work was partially supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan and the Japanese National Institute of Biomedical Innovation (no. 05-1).

REFERENCES

1. **Abe, T., H. Hemmi, H. Miyamoto, K. Moriishi, S. Tamura, H. Takaku, S. Akira, and Y. Matsuura.** 2005. Involvement of the Toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. J. Virol. **79**:2847–2858.
2. **Aldrich, W. A., C. Ren, A. F. White, S. Z. Zhou, S. Kumar, C. B. Jenkins, D. R. Shaw, T. V. Strong, P. L. Triozzi, and S. Ponnazhagan.** 2006. Enhanced transduction of mouse bone marrow-derived dendritic cells by repetitive infection with self-complementary adeno-associated virus 6 combined with immunostimulatory ligands. Gene Ther. **13**:29–39.
3. **Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274**:94–96.
4. **Babcock, A. M., D. Standing, K. Bullshields, E. Schwartz, C. M. Paden, and D. J. Poulsen.** 2005. In vivo inhibition of hippocampal Ca^{2+}/calmodulin-dependent protein kinase II by RNA interference. Mol. Ther. **11**:899–905.
5. **Banchereau, J., and R. M. Steinman.** 1998. Dendritic cells and the control of immunity. Nature **392**:245–252.
6. **Bantel-Schaal, U., H. Delius, R. Schmidt, and H. zur Hausen.** 1999. Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. J. Virol. **73**:939–947.
7. **Behboudi, S., D. Chao, P. Klenerman, and J. Austyn.** 2000. The effects of DNA containing CpG motif on dendritic cells. Immunology **99**:361–366.
8. **Booth, M. J., A. Mistry, X. Li, A. Thrasher, and R. S. Coffin.** 2004. Transfection-free and scalable recombinant AAV vector production using HSV/AAV hybrids. Gene Ther. **11**:829–837.
9. **Boucher, D. W., W. P. Parks, and J. L. Melnick.** 1970. A sensitive neutralization test for the adeno-associated satellite viruses. J. Immunol. **104**:555–559.
10. **Brockstedt, D. G., G. M. Podsakoff, L. Fong, G. Kurtzman, W. Mueller-Ruchholtz, and E. G. Engleman.** 1999. Induction of immunity to antigens expressed by recombinant adeno-associated virus depends on the route of administration. Clin. Immunol. **92**:67–75.
11. **Chiorini, J. A., F. Kim, L. Yang, and R. M. Kotin.** 1999. Cloning and characterization of adeno-associated virus type 5. J. Virol. **73**:1309–1319.
12. **Chiorini, J. A., L. Yang, Y. Liu, B. Safer, and R. M. Kotin.** 1997. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J. Virol. **71**:6823–6833.
13. **Chiriva-Internati, M., Y. Liu, E. Salati, W. Zhou, Z. Wang, F. Grizzi, J. J. Roman, S. H. Lim, and P. L. Hermonat.** 2002. Efficient generation of cytotoxic T lymphocytes against cervical cancer cells by adeno-associated virus/human papillomavirus type 16 E7 antigen gene transduction into dendritic cells. Eur. J. Immunol. **32**:30–38.
14. **Conway, J. E., C. M. Rhys, I. Zolotukhin, S. Zolotukhin, N. Muzyczka, G. S. Hayward, and B. J. Byrne.** 1999. High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. Gene Ther. **6**:986–993.
15. **Di Pasquale, G., B. L. Davidson, C. S. Stein, I. Martins, D. Scudiero, A. Monks, and J. A. Chiorini.** 2003. Identification of PDGFR as a receptor for AAV-5 transduction. Nat. Med. **9**:1306–1312.
16. **Duan, D., P. Sharma, J. Yang, Y. Yue, L. Dudus, Y. Zhang, K. J. Fisher, and J. F. Engelhardt.** 1998. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. J. Virol. **72**:8568–8577.
17. **During, M. J., R. Xu, D. Young, M. G. Kaplitt, R. S. Sherwin, and P. Leone.** 1998. Peroral gene therapy of lactose intolerance using an adeno-associated virus vector. Nat. Med. **4**:1131–1135.
18. **Fan, D. S., M. Ogawa, K. I. Fujimoto, K. Ikeguchi, Y. Ogasawara, M. Urabe, M. Nishizawa, I. Nakano, M. Yoshida, I. Nagatsu, H. Ichinose, T. Nagatsu, G. J. Kurtzman, and K. Ozawa.** 1998. Behavioral recovery in 6-hydroxydopamine-lesioned rats by cotransduction of striatum with tyrosine hydroxylase and aromatic L-amino acid decarboxylase genes using two separate adeno-associated virus vectors. Hum. Gene Ther. **9**:2527–2535.
19. **Flotte, T. R., S. A. Afione, C. Conrad, S. A. McGrath, R. Solow, H. Oka, P. L. Zeitlin, W. B. Guggino, and B. J. Carter.** 1993. Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. Proc. Natl. Acad. Sci. USA **90**:10613–10617.
20. **Gallez-Hawkins, G., X. Li, A. E. Franck, L. Thao, S. F. Lacey, D. J. Diamond, and J. A. Zaia.** 2004. DNA and low titer, helper-free, recombinant AAV prime-boost vaccination for cytomegalovirus induces an immune response to CMV-pp65 and CMV-IE1 in transgenic HLA A*0201 mice. Vaccine **23**:819–826.
21. **Gao, G. P., M. R. Alvira, L. Wang, R. Calcedo, J. Johnston, and J. M. Wilson.** 2002. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. USA **99**:11854–11859.
22. **Gronowski, A. M., D. M. Hilbert, K. C. Sheehan, G. Garotta, and R. D. Schreiber.** 1999. Baculovirus stimulates antiviral effects in mammalian cells. J. Virol. **73**:9944–9951.

23. **Hacker, U. T., L. Wingenfeld, D. M. Kofler, N. K. Schuhmann, S. Lutz, T. Herold, S. B. King, F. M. Gerner, L. Perabo, J. Rabinowitz, D. M. McCarty, R. J. Samulski, M. Hallek, and H. Buning.** 2005. Adeno-associated virus serotypes 1 to 5 mediated tumor cell directed gene transfer and improvement of transduction efficiency. J. Gene Med. **7**:1429–1438.
24. **Halbert, C. L., J. M. Allen, and A. D. Miller.** 2001. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. J. Virol. **75**:6615–6624.
25. **Halbert, C. L., E. A. Rutledge, J. M. Allen, D. W. Russell, and A. D. Miller.** 2000. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J. Virol. **74**:1524–1532.
26. **Han, W., M. Wind-Rotolo, R. L. Kirkman, and C. D. Morrow.** 2004. Inhibition of human immunodeficiency virus type 1 replication by siRNA targeted to the highly conserved primer binding site. Virology **330**:221–232.
27. **Handa, A., S. Muramatsu, J. Qiu, H. Mizukami, and K. E. Brown.** 2000. Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. J. Gen. Virol. **81**:2077–2084.
28. **Hara, H., A. Monsonego, K. Yuasa, K. Adachi, X. Xiao, S. Takeda, K. Takahashi, H. L. Weiner, and T. Tabira.** 2004. Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. J. Alzheimers Dis. **6**:483–488.
29. **Herzog, R. W., J. N. Hagstrom, S. H. Kung, S. J. Tai, J. M. Wilson, K. J. Fisher, and K. A. High.** 1997. Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proc. Natl. Acad. Sci. USA **94**:5804–5809.
30. **Hildinger, M., A. Auricchio, G. Gao, L. Wang, N. Chirmule, and J. M. Wilson.** 2001. Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. J. Virol. **75**:6199–6203.
31. **Johnson, P. R., B. C. Schnepp, M. J. Connell, D. Rohne, S. Robinson, G. R. Krivulka, C. I. Lord, R. Zinn, D. C. Montefiori, N. L. Letvin, and K. R. Clark.** 2005. Novel adeno-associated virus vector vaccine restricts replication of simian immunodeficiency virus in macaques. J. Virol. **79**:955–965.
32. **Jooss, K., Y. Yang, K. J. Fisher, and J. M. Wilson.** 1998. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. J. Virol. **72**:4212–4223.
33. **Jounai, N., K. Okuda, Y. Kojima, Y. Toda, K. Hamajima, K. Ohba, D. Klinman, and K. Q. Xin.** 2003. Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. J. Gene Med. **5**:609–617.
34. **Kay, M. A., C. S. Manno, M. V. Ragni, P. J. Larson, L. B. Couto, A. McClelland, B. Glader, A. J. Chew, S. J. Tai, R. W. Herzog, V. Arruda, F. Johnson, C. Scallan, E. Skarsgard, A. W. Flake, and K. A. High.** 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. **24**:257–261.
35. **Koppold, B., G. Sauer, H. Buning, M. Hallek, R. Kreienberg, H. Deissler, and C. Kurzeder.** 2005. Efficient gene transfer of CD40 ligand into ovarian carcinoma cells with a recombinant adeno-associated virus vector. Int. J. Oncol. **26**:95–101.
36. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. USA **87**:2211–2215.
37. **Lewin, A. S., K. A. Drenser, W. W. Hauswirth, S. Nishikawa, D. Yasumura, J. G. Flannery, and M. M. LaVail.** 1998. Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. Nat. Med. **4**:967–971.
38. **Liu, D. W., J. L. Chang, Y. P. Tsao, C. W. Huang, S. W. Kuo, and S. L. Chen.** 2005. Co-vaccination with adeno-associated virus vectors encoding human papillomavirus 16 L1 proteins and adenovirus encoding murine GM-CSF can elicit strong and prolonged neutralizing antibody. Int. J. Cancer **113**:93–100.
39. **Liu, Y., A. D. Santin, M. Mane, M. Chiriva-Internati, G. P. Parham, A. Ravaggi, and P. L. Hermonat.** 2000. Transduction and utility of the granulocyte-macrophage colony-stimulating factor gene into monocytes and dendritic cells by adeno-associated virus. J. Interferon Cytokine Res. **20**:21–30.
40. **Manning, W. C., X. Paliard, S. Zhou, M. P. Bland, A. Y. Lee, K. Hong, C. M. Walker, J. A. Escobedo, and V. Dwarki.** 1997. Genetic immunization with adeno-associated virus vectors expressing herpes simplex virus type 2 glycoproteins B and D. J. Virol. **71**:7960–7962.
41. **Manno, C. S., A. J. Chew, S. Hutchison, P. J. Larson, R. W. Herzog, V. R. Arruda, S. J. Tai, M. V. Ragni, A. Thompson, M. Ozelo, L. B. Couto, D. G. Leonard, F. A. Johnson, A. McClelland, C. Scallan, E. Skarsgard, A. W. Flake, M. A. Kay, K. A. High, and B. Glader.** 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood **101**:2963–2972.
42. **Meghrous, J., M. G. Aucoin, D. Jacob, P. S. Chahal, N. Arcand, and A. A. Kamen.** 2005. Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: from shake flasks to a 20-L bioreactor. Biotechnol. Prog. **21**:154–160.
43. **Moore, M. D., M. J. McGarvey, R. A. Russell, B. R. Cullen, and M. O. McClure.** 2005. Stable inhibition of hepatitis B virus proteins by small interfering RNA expressed from viral vectors. J. Gene Med. **7**:918–925.
44. **Muramatsu, S., H. Mizukami, N. S. Young, and K. E. Brown.** 1996. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology **221**:208–217.
45. **Padron, E., V. Bowman, N. Kaludov, L. Govindasamy, H. Levy, P. Nick, R. McKenna, N. Muzyczka, J. A. Chiorini, T. S. Baker, and M. Agbandje-McKenna.** 2005. Structure of adeno-associated virus type 4. J. Virol. **79**:5047–5058.
46. **Ponnazhagan, S., G. Mahendra, D. T. Curiel, and D. R. Shaw.** 2001. Adeno-associated virus type 2-mediated transduction of human monocyte-derived dendritic cells: implications for ex vivo immunotherapy. J. Virol. **75**:9493–9501.
47. **Rabinowitz, J. E., F. Rolling, C. Li, H. Conrath, W. Xiao, X. Xiao, and R. J. Samulski.** 2002. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J. Virol. **76**:791–801.
48. **Rabinowitz, J. E., and R. J. Samulski.** 2000. Building a better vector: the manipulation of AAV virions. Virology **278**:301–308.
49. **Ribas, A.** 2005. Genetically modified dendritic cells for cancer immunotherapy. Curr. Gene Ther. **5**:619–628.
50. **Rutledge, E. A., C. L. Halbert, and D. W. Russell.** 1998. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J. Virol. **72**:309–319.
51. **Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka.** 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. Proc. Natl. Acad. Sci. USA **79**:2077–2081.
52. **Santosuosso, M., X. Zhang, S. McCormick, J. Wang, M. Hitt, and Z. Xing.** 2005. Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. J. Immunol. **174**:7986–7994.
53. **Sarukhan, A., S. Camugli, B. Gjata, H. von Boehmer, O. Danos, and K. Jooss.** 2001. Successful interference with cellular immune responses to immunogenic proteins encoded by recombinant viral vectors. J. Virol. **75**:269–277.
54. **Schnepp, B. C., K. R. Clark, D. L. Klemanski, C. A. Pacak, and P. R. Johnson.** 2003. Genetic fate of recombinant adeno-associated virus vector genomes in muscle. J. Virol. **77**:3495–3504.
55. **Snyder, R. O., C. H. Miao, G. A. Patijn, S. K. Spratt, O. Danos, D. Nagy, A. M. Gown, B. Winther, L. Meuse, L. K. Cohen, A. R. Thompson, and M. A. Kay.** 1997. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat. Genet. **16**:270–276.
56. **Sollerbrant, K., J. Elmen, C. Wahlestedt, J. Acker, H. Leblois-Prehaud, M. Latta-Mahieu, P. Yeh, and M. Perricaudet.** 2001. A novel method using baculovirus-mediated gene transfer for production of recombinant adeno-associated virus vectors. J. Gen. Virol. **82**:2051–2060.
57. **Tomar, R. S., H. Matta, and P. M. Chaudhary.** 2003. Use of adeno-associated viral vector for delivery of small interfering RNA. Oncogene **22**:5712–5715.
58. **Urabe, M., C. Ding, and R. M. Kotin.** 2002. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum. Gene Ther. **13**:1935–1943.
59. **Xia, H., Q. Mao, S. L. Eliason, S. Q. Harper, I. H. Martins, H. T. Orr, H. L. Paulson, L. Yang, R. M. Kotin, and B. L. Davidson.** 2004. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat. Med. **10**:816–820.
60. **Xiao, W., N. Chirmule, S. C. Berta, B. McCullough, G. Gao, and J. M. Wilson.** 1999. Gene therapy vectors based on adeno-associated virus type 1. J. Virol. **73**:3994–4003.
61. **Xiao, X., J. Li, and R. J. Samulski.** 1996. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J. Virol. **70**:8098–8108.
62. **Xin, K. Q., Y. Hoshino, Y. Toda, S. Igimi, Y. Kojima, N. Jounai, K. Ohba, A. Kushiro, M. Kiwaki, K. Hamajima, D. Klinman, and K. Okuda.** 2003. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood **102**:223–228.
63. **Xin, K. Q., N. Jounai, K. Someya, K. Honma, H. Mizuguchi, S. Naganawa, K. Kitamura, T. Hayakawa, S. Saha, F. Takeshita, K. Okuda, M. Honda, and D. M. Klinman.** 2005. Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. Gene Ther. **12**:1769–1777.
64. **Xin, K. Q., Y. Kumamoto, N. Jounai, Y. Kojima, K. Hamajima, and K. Okuda.** 2002. Recombinant vaccinia virus (WR strain) may not be suitable for ex vivo stimulation. Vaccine **21**:5–6.
65. **Xin, K. Q., T. Ooki, H. Mizukami, K. Hamajima, K. Okudela, K. Hashimoto, Y. Kojima, N. Jounai, Y. Kumamoto, S. Sasaki, D. Klinman, K. Ozawa, and K. Okuda.** 2002. Oral administration of recombinant adeno-associated virus elicits human immunodeficiency virus-specific immune responses. Hum. Gene Ther. **13**:1571–1581.

66. **Xin, K. Q., M. Urabe, J. Yang, K. Nomiyama, H. Mizukami, K. Hamajima, H. Nomiyama, T. Saito, M. Imai, J. Monahan, K. Okuda, and K. Ozawa.** 2001. A novel recombinant adeno-associated virus vaccine induces a long-term humoral immune response to human immunodeficiency virus. Hum. Gene Ther. **12**:1047–1061.
67. **Yoshida, A., R. Tanaka, T. Murakami, Y. Takahashi, Y. Koyanagi, M. Nakamura, M. Ito, N. Yamamoto, and Y. Tanaka.** 2003. Induction of protective immune responses against R5 human immunodeficiency virus type 1 (HIV-1) infection in hu-PBL-SCID mice by intrasplenic immunization with HIV-1-pulsed dendritic cells: possible involvement of a novel factor of human $CD4^+$ T-cell origin. J. Virol. **77**:8719–8728.
68. **Zabner, J., M. Seiler, R. Walters, R. M. Kotin, W. Fulgeras, B. L. Davidson, and J. A. Chiorini.** 2000. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J. Virol. **74**:3852–3858.
69. **Zhang, W., R. Singam, G. Hellermann, X. Kong, H. S. Juan, R. F. Lockey, S. J. Wu, K. Porter, and S. S. Mohapatra.** 2004. Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. Genet. Vaccines Ther. **2**:8.
70. **Zhang, Y., N. Chirmule, G. Gao, and J. Wilson.** 2000. CD40 ligand-dependent activation of cytotoxic T lymphocytes by adeno-associated virus vectors in vivo: role of immature dendritic cells. J. Virol. **74**:8003–8010.