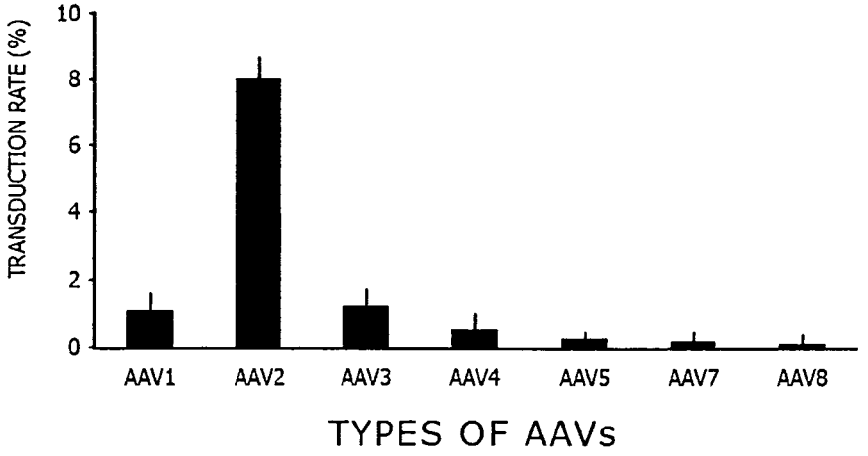

Fig. 3. Transduction rates of different serotypes of adeno-associated viruses at the highest concentration (multiplicities of infection, 100) at 5 days after transduction. For each viral serotype, four randomly selected fields from each of the two wells were observed. Each observation field under the microscope was considered an individual sample. The sample size, therefore, was eight for each virus at multiplicities of infection of 100. AAV2 had the highest transduction rate. By contrast, other adeno-associated viruses had only less than 50 percent transduction rates compared with the transduction rate of AAV2.

promising for future in vivo gene therapy for healing flexor tendons. Persistently higher levels of gene expression and production of the transgene indicate that the effects of gene transfer are sufficiently long to encompass the critical period of tendon healing.

It is known that different serotypes of adeno-associated virus vary in their preferences for target cells.[22–26] AAV2 has been best characterized so far and has had a broad range of infectivity.[22–24] AAV1 vectors can efficiently transduce skeletal muscle and retina[22,23]; AAV5 vectors transduce central nervous system and lung.[24] Because AAV6 is thought to be a recombinant between AAV1 and AAV2, it was not included in our test. AAV7 and AAV8 are newly discovered serotypes isolated from heart DNA of a rhesus monkey.[26] AAV7 transduces skeletal muscle, and AAV8 has a higher transduction rate in liver than other serotypes. As part of this study, transduction rates of seven adeno-associated virus serotypes were compared to identify the most efficient at transducing tenocytes. At all concentrations of viral particles tested, AAV2 was the vector most efficient at introducing genes to tenocytes. Even at high concentrations, other virus serotypes showed no or minimal transduction.

The efficiency of gene transfer by AAV2 in tenocytes was significantly higher than that by means of the plasmid vector. Transduction substantially increased expression of the transferred *bFGF* gene and its production of bFGF. Nevertheless, the transduction rate of AAV2 is approximately 10 percent, which is not high compared with that in highly permissive cells.[22–26] The optimal rates of transduction and production of growth factors for tendon healing are not yet clear. Low levels of gene expression or production of certain growth factors are considered innate biological reasons underlying the poor healing capacity of the intrasynovial tendons. In contrast, overexpression of some growth factors leads to excessive production of collagen and formation of adhesions, thus impeding tendon gliding. Future in vivo studies should help to identify optimal efficiency of gene transfer to tenocytes by vectors and appropriate regulation in selective growth factor production, thus strengthening the healing process and not stimulating adhesion formation. AAV2 vectors have delivered genes to some types of chondrocytes and endothelial cells, and the efficiency of gene transfer was similar to that of the tenocytes in this study.[27,28]

CONCLUSIONS

The tendons, particularly those in the intrasynovial area, contain low levels of growth factor and lack sufficient cellularity, limiting their intrinsic healing capacity.[4,5,29] This is the basic reason that adhesions or ruptures occur after surgery and outcomes are less than perfect.[30–36] We conclude from this study that AAV2 can efficiently transduce intrasynovial tenocytes, but other AAV serotypes

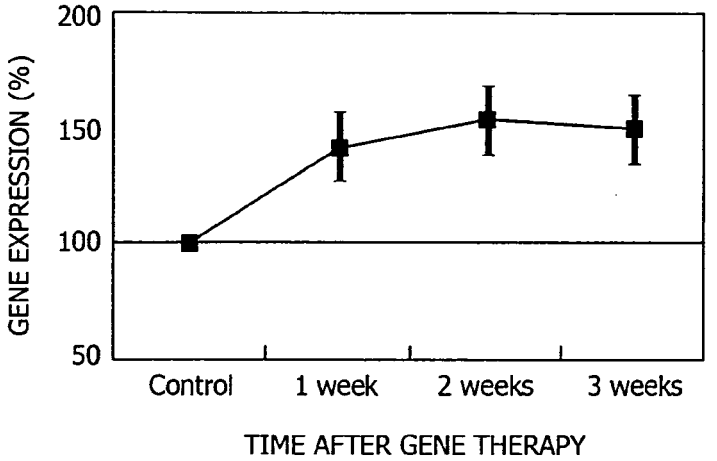

Fig. 4. Expression of the *bFGF* gene at 1, 2, and 3 weeks after gene transfer. Values of gene expression were given as mean percentage changes and SD of gene expression in the experimental (five samples) compared with those in the nontreatment control (five samples) at each period after gene therapy.

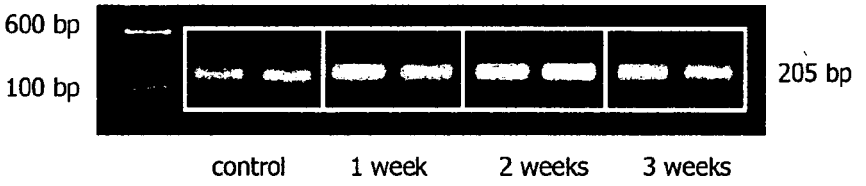

Fig. 5. Expression of the *bFGF* gene at 1, 2, and 3 weeks after gene transfer. Gel electrophoresis shows the location and density of the bands of the *bFGF* gene of tenocytes receiving exogenous bFGF and those in the control.

have much lower transduction efficiency. Transfer of the bFGF gene significantly increases expression of the bFGF gene over 3 tested weeks. Our study lays the groundwork for future investigations of gene therapy in tendons using adeno-associated virus vectors and will help design more appropriate and efficient gene transfer protocols for injured tendons. Delivery of exogenous growth factor genes by means of the AAV2 vector is a novel and promising method of promoting tendon healing. Our findings warrant future in vivo studies to test the effectiveness of this new gene therapy approach and its constructs. With certain extensions of the techniques outlined above, we believe that AAV2 can be used to deliver growth factor genes critical to traumatized tendons, strengthening tendon healing in vivo.

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ACKNOWLEDGMENTS

We thank Drs. J. M. Wilson and G. P. Gao, University of Pennsylvania, Philadelphia, for the helper plasmids for AAV7 and AAV8.

DISCLOSURE

None of the authors has any financial interest in the products, devices, or drugs mentioned in this article.

REFERENCES

1. Chow, J. A., Thomes, L. J., Dovelle, S., Milnor, W. H., Seyfer, A. E., and Smith, A. C. A combined regimen of controlled motion following flexor tendon repair in "no man's land." *Plast. Reconstr. Surg.* 79: 447, 1987.
2. Savage, R., and Risitano, G. Flexor tendon repair using a "six strand" method of repair and early active mobilization. *J. Hand Surg. (Br.)* 14: 396, 1989.
3. Tang, J. B., Gu, Y. T., Rice, K., Chen, F., and Pan, C. Z. Evaluation of four methods of flexor tendon repair for postoperative active mobilization. *Plast. Reconstr. Surg.* 107: 742, 2001.
4. Mass, D. P., and Tuel, R. Human flexor tendon participation in the in vitro repair process. *J. Hand Surg. (Am.)* 14: 64, 1989.
5. Chan, B. P., Fu, S., Qin, L., Lee, K., Rolf, C. G., and Chan, K. Effects of basic fibroblast growth factor (bFGF) on early

stages of tendon healing: A rat patellar tendon model. *Acta Orthop. Scand.* 71: 513, 2000.
6. Lou, J., Manske, P. R., Aoki, M., and Joyce, M. E. Adenovirus-mediated gene transfer into tendon and tendon sheath. *J. Orthop. Res.* 14: 513, 1996.
7. Lou, J., Kubota, H., Hotokezaka, S., Ludwig, F. J., and Manske, P. R. In vivo gene transfer and overexpression of focal adhesion kinase (pp125 FAK) mediated by recombinant adenovirus-induced tendon adhesion formation and epitenon cell change. *J. Orthop. Res.* 15: 911, 1997.
8. Lou, J., Tu, Y., Burns, M., Silva, M. J., and Manske, P. R. BMP-12 gene transfer augmentation of lacerated tendon repair. *J. Orthop. Res.* 19: 1199, 2001.
9. Wang, X. T., Liu, P. Y., and Tang, J. B. Tendon healing in vitro: Genetic modification of tenocytes with exogenous PDGF gene and promotion of collagen gene expression. *J. Hand Surg. (Am.)* 29: 884, 2004.
10. Brenner, M. Gene transfer by adenovectors. *Blood* 94: 3965, 1999.
11. Chandler, L. A., and Sosnowski, B. A. Gene therapy for cutaneous wound repair. *Wounds* 16: 23, 2004.
12. McMahon, J. M., Wells, K. E., Bamfo, J. E., Cartwright, M. A., and Wells, D. J. Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. *Gene Ther.* 5: 1283, 1998.
13. Nidome, T., and Huang, L. Gene therapy progress and prospects: Nonviral vectors. *Gene Ther.* 9: 1647, 2002.
14. Schwarz, E. M. The adeno-associated virus vector for orthopedic gene therapy. *Clin. Orthop.* 379S: 31, 2000.
15. Lai, C. M., Lai, Y. K., and Rakoczy, P. E. Adenovirus and adeno-associated virus vectors. *DNA Cell Biol.* 21: 895, 2002.
16. Yokoo, N., Saito, T., Uesugi, M., et al. Repair of articular cartilage defect by autologous transplantation of basic fibroblast growth factor gene-transduced chondrocytes with adeno-associated virus vector. *Arthritis Rheum.* 52: 164, 2005.
17. Wang, X. T., Liu, P. Y., and Tang, J. B. PDGF gene therapy enhances expression of VEGF and bFGF genes and activates NF-kB gene in signal pathways in ischemic flaps. *Plast. Reconstr. Surg.* 117: 129, 2006.
18. Chan, B. P., Chan, K. M., Maffulli, N., Webb, S., and Lee, K. H. Effect of basic fibroblast growth factor: An in vitro study of tendon healing. *Clin. Orthop.* 342: 239, 1997.
19. Abrahamsson, S. O. Similar effects of recombinant human insulin-like growth factor-I and II on cellular activities in flexor tendons of young rabbits: Experimental studies in vitro. *J. Orthop. Res.* 15: 256, 1997.
20. Tang, J. B., Xu, Y., Ding, F., and Wang, X. T. Tendon healing in vitro: Promotion of collagen gene expression by bFGF with NF-kB gene activation. *J. Hand Surg. (Am.)* 28: 215, 2003.
21. Klein, M. B., Yalamanchi, N., Pham, H., Longaker, M. T., and Chang, J. Flexor tendon healing in vitro: Effects of TGF-β on tendon cell collagen production. *J. Hand Surg. (Am.)* 27: 615, 2002.
22. Xiao, W., Chirmule, N., Berta, S. C., McCullough, B., Gao, G., and Wilson, J. M. Gene therapy vectors based on adeno-associated virus type 1. *J. Virol.* 73: 3994, 1999.
23. Auricchio, A., Kobinger, G., Anand, V., et al. Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: The retina as a model. *Hum. Mol. Genet.* 10: 3075, 2001.
24. Davidson, B. L., Stein, C. S., Heth, J. A., et al. Recombinant adeno-associated virus type 2, 4, and 5 vectors: Transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 97: 3428, 2000.
25. Louboutin, J. P., Wang, L., and Wilson, J. M. Gene transfer into skeletal muscle using novel AAV serotypes. *J. Gene Med.* 7: 442, 2005.
26. Gao, G. P., Alvira, M. R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J. M. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 99: 11854, 2002.
27. Vinther, M. U., Duch, M. R., O'Keefe R. J., Schwarz, E. M., and Pedersen, F. S. In vivo gene delivery to articular chondrocytes mediated by an adeno-associated virus vector. *J. Orthop. Res.* 22: 726, 2004.
28. Pajusola, K., Gruchala, M., Joch, H., Luscher, T. F., Yla-Herttuala, S., and Bueler, H. Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. *J. Virol.* 76: 11530, 2002.
29. Tang, J. B., Xu, Y., and Wang, X. T. Tendon healing in vitro: Activation of NIK, IIKα, IKKβ and NF-κB genes in signal pathway and proliferation of tenocytes. *Plast. Reconstr. Surg.* 113: 1703, 2004.
30. Small, J. O., Brennen, M. D., and Colville, J. Early active mobilization following flexor tendon repair in zone 2. *J. Hand Surg. (Br.)* 14: 383, 1989.
31. Cullen, K. W., Tolhurst, P., Lang, D., and Page, R. E. Flexor tendon repair in zone 2 followed by controlled active mobilization. *J. Hand Surg. (Br.)* 14: 392, 1989.
32. Elliot, D., Moiemen, N. S., Flemming, A. F. S., Harris, S. B., and Foster, A. J. The rupture rate of acute flexor tendon repairs mobilized by the controlled active motion regimen. *J. Hand Surg. (Br.)* 19: 607, 1994.
33. Kitsis, C. K., Wade, P. J. F., Krikler, S. J., Parsons, N. K., and Nicholls, L. K. Controlled active motion following primary flexor tendon repair: A prospective study over 9 years. *J. Hand Surg. (Br).* 23: 344, 1998.
34. Harris, S. B., Harris, D., Foster, A. J., and Elliot, D. The aetiology of acute rupture of flexor tendon repairs in zones 1 and 2 of the fingers during early mobilization. *J. Hand Surg. (Br.)* 24: 275, 1999.
35. Boyer, M. I. Flexor tendon biology. *Hand Clin.* 21: 159, 2005.
36. Tang, J. B. Clinical outcomes associated with flexor tendon repair. *Hand Clin.* 21: 199, 2005.

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Antibody-dependent enhancement of adeno-associated virus infection of human monocytic cell lines

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Received 14 November 2007; returned to author for revision 4 January 2008; accepted 25 January 2008

Abstract

In host animals, adeno-associated virus (AAV) is detectable mainly in the lymphoid tissue, which appears to be a target in natural infection. We used the human monocytic cell lines THP-1 and U937 to study the effect of mouse anti-AAV2 antiserum on infection with an AAV2 vector having the luciferase gene (AAV2/Luc). AAV2/Luc was found to infect THP-1 and U937 cells much less efficiently than HeLa cells, as monitored with the induced enzyme activity. Pre-incubation of AAV2/Luc with anti-AAV2 antiserum at a sub-neutralizing concentration enhanced by 2-to-10 fold infection of THP-1 and U937 with AAV2/Luc, but not of HeLa. Similarly, anti-AAV10 serum at a low level enhanced infection of THP-1 with AAV10/Luc. Sera of two cynomolgus monkeys, which had been probably infected with an AAV2-like virus, enhanced infection of THP-1 with AAV2/Luc. The enhancement was reduced with blocking the IgG-receptors Fcγ-RI and Fcγ-RII, which were displayed on the surface of THP-1 and U937 but not HeLa cells, with anti-Fcγ-RI antibody or anti-Fcγ-RII antibody. The data indicate that infection of Fcγ receptor-bearing cells with AAV is enhanced by anti-AAV IgG antibodies at a sub-neutralizing concentration that play a role in linking AAV particles and Fcγ receptors.

Keywords: AAV; Anti-AAV antibody; ADE; Fcγ-receptor

Introduction

Adeno-associated virus (AAV) is a nonenveloped icosahedral particle (a diameter of 25 nm) containing a single-stranded linear DNA (4.7 kb). To date several AAV serotypes and over 100 AAV variants have been recorded (Wu et al., 2006). Among them AAV serotype type 2 (AAV2) has been studied most extensively and used for gene therapy trials. Efficient propagation of AAV2 in cultured cells requires coinfection with a helper virus (f y ic). Without a helper virus the AAV DNA is integrated into cell DNA at a specific site and maintained as a provirus ( ). When a latently infected cell is super-infected with adenovirus the integrated AAV is induced to replicate (Cheung et al., 1980). A low level induction has been observed upon Fas-mediated apoptosis of the latently infected cells (Mori et al., 2002).

The tissue tropism of AAV in natural infection is not fully elucidated. AAV10, AAV11, and AAVcy.7 have been detected mainly in the lymphoid tissue and ileum of the naturally infected cynomolgus monkeys (08). AAV2 vectors injected to monkeys are detectable in the lymphoid tissue ( ) and the leukocytes ( ). These studies suggest that the leukocytes are one of the major targets for AAV.

Infection of the leukocytes with dengue virus (DENV) is enhanced by either sub-neutralizing level of serotype-specific anti-DENV antibody or cross-reacti DENV antibody ( ). The antibody, which binds to the virion at its variable region and to the cell-surface Fcγ receptor (Fcγ-R) at its Fc-region, mediates attachment of the virion to the surface

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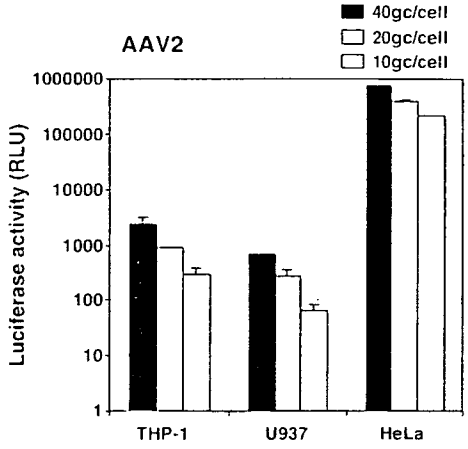

Fig. 1. Infection of THP-1, U937, and HeLa cells with AAV2/Luc. AAV2/Luc was inoculated to the cells at three multiplicities of infection (gc/cell). Two days later, the cells were lysed and luciferase activities of the lysates were measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

of monocytes and macrophages (Clyde et al., 2006). This antibody-dependent enhancement (ADE) of DENV is believed to be associated with dengue hemorrhagic fever. Similar ADE has been reported in infection of Fcγ-R-positive cells with West Nile virus (Peiris and Porterfield, 1979; Peiris et al., 1981), human parvovirus B19 (Munakata et al., 2006), and Aleutian mink disease parvovirus (Kanno et al., 1993).

In this study we examined infection of THP-1 and U937, human monocytic cell lines that were expressing Fcγ-R, with recombinant AAV vectors that had been pre-incubated with diluted anti-AAV antiserum and found that a sub-neutralizing level of the antiserum enhanced infection of these cells with AAV vectors.

Results

Antibody-dependent-enhancement of infection of THP-1 and U937 cells with AAV2 and AAV10

Infection of THP-1 and U937, human monocytic cell lines, with AAV2/Luc, a recombinant AAV having the luciferase gene, was much less efficient than that of HeLa. Serially diluted AAV2/Luc was inoculated to the cells and 2 days later the cells were lysed and the luciferase activities of the lysates were measured (Fig. 1). While the luciferase activity of the HeLa culture inoculated with AAV2/Luc at a multiplicity of infection (MOI) of 10 genome copies (gc) was 200,000 U, those of the THP-1 and U937 cultures inoculated at an MOI of 40 gc were 2500 U and 1000 U, respectively.

Infection of THP-1 and U937 cells with AAV2/Luc was enhanced by pre-incubation of AAV2/Luc with a sub-neutralizing concentration of anti-AAV2 antiserum. AAV2/Luc was incubated with mouse anti-AAV2 antiserum or mouse normal serum at 37 °C for 30 min and then inoculated to 3×10^5 of THP-1 cells (at MOIs of 10, 20, and 40 gc), U937 cells (at MOIs of 50, 100, and 200 gc), and HeLa cells (at MOIs of 1, 2, and 4 gc). Two days later the luciferase activity of the cellular lysate was measured. The antiserum diluted at 1:100 induced a major decrease of the luciferase activities of the three cell lines, indicating that AAV2/Luc was neutralized with the antiserum (Figs. 2, A, B, and C). Although the antiserum diluted at 1:1000 induced a slight decrease of the luciferase activity of HeLa (Fig. 2C), it induced increase of the luciferase activities of THP-1 and of U937 (Figs. 2, A and B). The antiserum diluted at 1:10,000 and the normal mouse serum did not affect the luciferase activities of the three cell lines (Fig. 2). Thus, the antiserum diluted at 1:1000 enhanced infection of THP-1 and U937 with AAV2/Luc, indicating that typical ADE occurred in infection to these cells with AAV2.

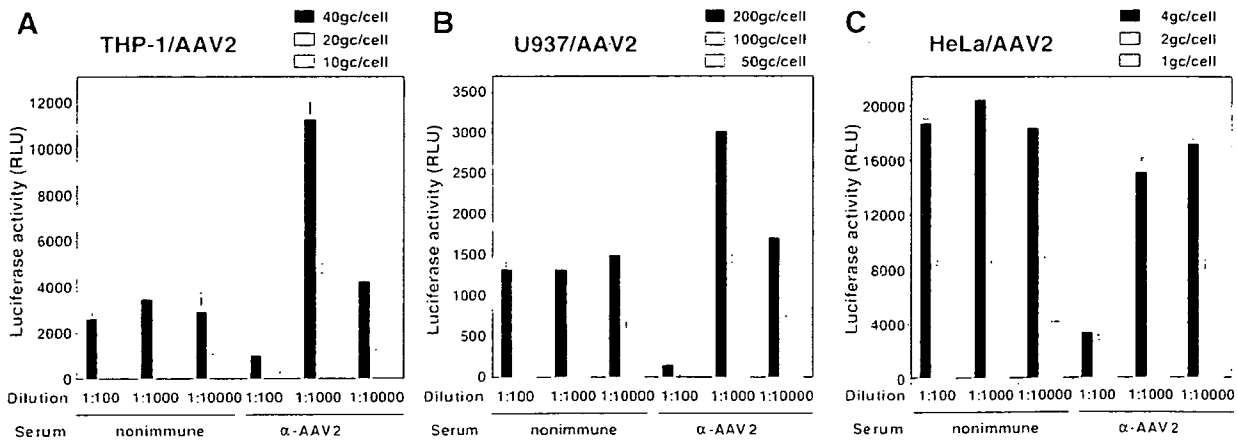

Fig. 2. Effect of anti-AAV2 antiserum on the infection of THP-1, U937, and HeLa cells with AAV2/Luc. AAV2/Luc was mixed with the diluted nonimmune serum or anti-AAV2 antiserum and incubated at 37 °C for 30 min. The mixtures were inoculated to THP-1 (A), U937 (B), or HeLa (C) cells at various multiplicities of infection (gc/cell) indicated. Two days later, the luciferase activity of the cellular lysate was measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

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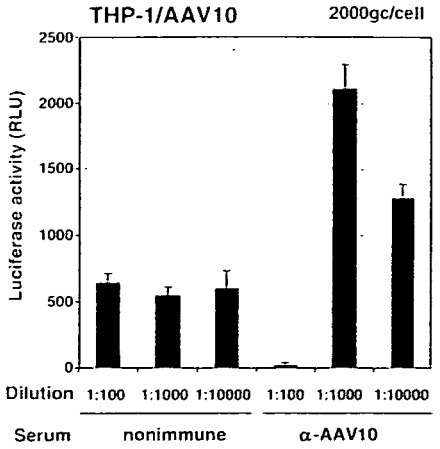

Fig. 3. Effect of anti-AAV10 antiserum on the infection of THP-1 cells with AAV10/Luc. AAV10/Luc was mixed with the diluted nonimmune serum or anti-AAV10 antiserum and incubated at 37 °C for 30 min. The mixtures were inoculated to THP-1 cells at the multiplicity of infection (gc/cell) indicated. Two days later, the luciferase activity of the cellular lysate was measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

Similarly, infection of THP-1 cells with AAV10 was enhanced by a sub-neutralizing concentration of anti-AAV10 serum. AAV10/Luc pre-incubated with mouse anti-AAV10 serum was inoculated to THP-1 (3×10^5 cells) at an MOI of 2000 gc and the luciferase activity was measured as the experiments with AAV2/Luc. The antiserum reduced the infectivity of AAV10/Luc at a dilution of 1:100 but increased the infectivity at dilutions of 1:1000 and 1:10,000 (Fig. 3).

Anti-AAV2 and anti-AAV10 sera did not increase the infectivity of AAV10/Luc and AAV2/Luc, respectively (Fig. 4), indicating clearly that the antibody capable of binding to AAV particles mediates the enhancement of infection of THP-1 cells with AAV.

Two monkey sera enhanced infection of THP-1 cells with AAV2/Luc. Sera were obtained from two healthy male cynomolgus monkeys of 5 years of age. The pre-incubation of AAV2/Luc with the monkey-A serum that had been undiluted or diluted at 1:10 reduced the infectivity of AAV2/Luc to HeLa and to THP-1 (Figs. 5, A and B), indicating that the serum contained antibody capable of neutralizing AAV2. Pre-incubation of AAV2/Luc with the monkey-A serum that had been diluted at 1:100 and 1:1000 did not affect the infectivity to HeLa but it increased the infectivity to THP-1 (Figs. 5, A and B). Similarly, pre-incubation of AAV2/Luc with the undiluted monkey-B serum, which did not neutralize AAV2/Luc (Fig. 5A), enhanced infection of THP-1 (Fig. 5B). The data clearly indicate that monkey-B serum diluted at 1:10 and 1:100 did not affect the infectivity of AAV2/Luc (Fig. 5B). The data strongly suggest that the two monkeys had been probably infected with AAV types antigenically related to AAV2 and the sub-neutralizing levels of the antibodies in the sera enhanced infection of THP-1 with AAV2/Luc.

Involvement of the cell-surface Fcγ-receptors (Fcγ-R) in the ADE of AAV infection

Both Fcγ-RI and Fcγ-RII were present on the surface of THP-1 and U937 cells. THP-1, U937, and HeLa cells were incubated with anti-CD64 mouse monoclonal antibody (mAb) recognizing Fcγ-RI or anti-CD32 mouse mAb recognizing Fcγ-RII at 4 °C for 1 h. Then the mAb on the surface was detected with labeled-goat anti-mouse IgG antibody by using a FACS. Both mAbs clearly bound to the surface of THP-1 and U937 cells but did not bind to the surface of HeLa cells (Fig. 6).

Pretreatment of THP-1 and U937 with anti-CD64 mAb or anti-CD32 mAb interfered with ADE in the infection of these cells with AAV2/Luc. THP-1 and U937 cells were incubated with anti-CD64 mAb (5 μg/ml) and/or anti-CD32 (5 μg/ml) mAb at 4 °C for 1 h. The cells were inoculated with AAV2/Luc that had been pre-incubated with the anti-AAV2 antiserum diluted at 1:1000 and the luciferase activities of the lysates were measured at 2 days after the inoculation. The level of the enhancement of infection of both cell lines with AAV2/Luc was reduced by pretreatment with mAbs (Fig. 7), indicating that

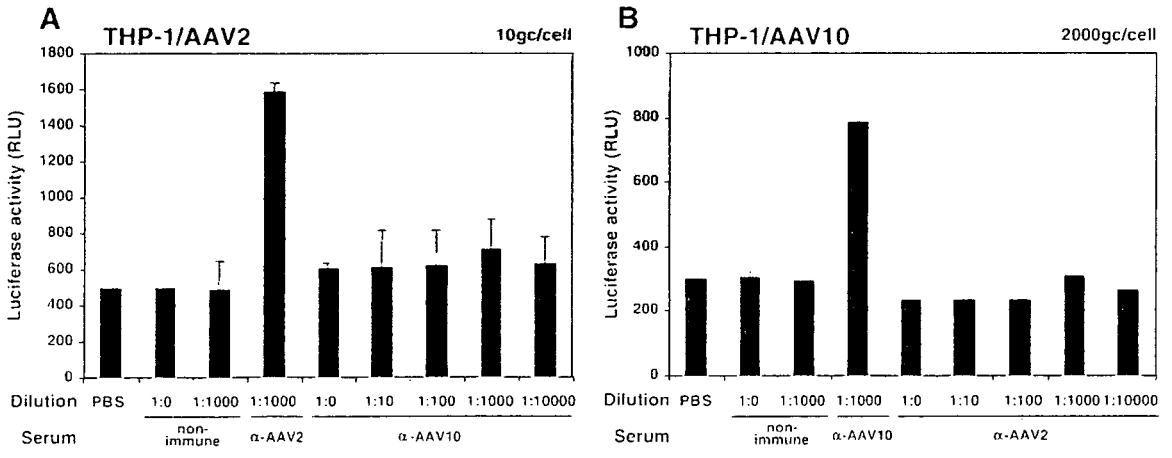

Fig. 4. Requirement of type-specific antibody for ADE of infection of THP-1 cells with AAV2/Luc and AAV10/Luc. AAV2/Luc (A) and AAV10/Luc (B) were pre-incubated with the undiluted or diluted anti-AAV2 or anti-AAV10 antiserum and inoculated to THP-1 cells. Two days later, the luciferase activity of the cellular lysate was measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

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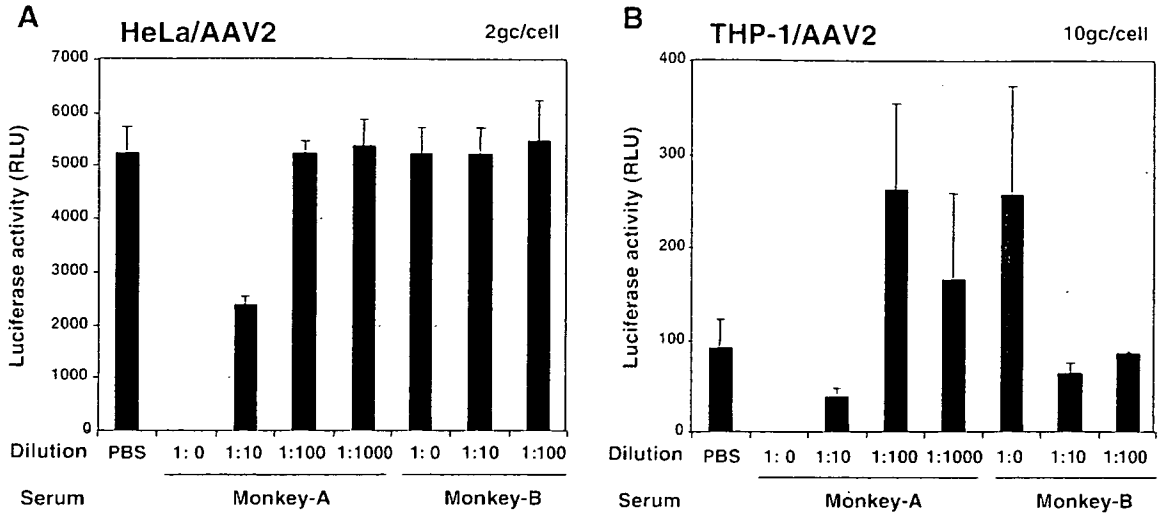

Fig. 5. Effect of the two monkey sera on the infection of THP-1 and HeLa cells with AAV2/Luc. AAV2/Luc was pre-incubated with the undiluted or the diluted cynomolgus monkey sera and inoculated to HeLa (A) or THP-1 (B) cells. Two days later, the luciferase activity of the cellular lysate was measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

both Fcγ-RI and Fcγ-RII are involved in the enhancement. Anti-CD64 mAb was more effective than anti-CD32 mAb for the reduction and the mixture of anti-CD64 and anti-CD32 mAbs completely abolished the enhancement.

Discussion

We demonstrated that infection of THP-1 and U937, human monocytic cell lines, with AAV2/Luc was enhanced by the sub-neutralizing concentration of anti-AAV2 antibody. Similarly infection of THP-1 with AAV10/Luc was enhanced by the sub-neutralizing concentration of anti-AAV10 antibody. Cell-surface Fcγ-RI and Fcγ-RII were required for the enhancement, indicating that the antibodies play a role in linking the AAV particle and the Fcγ-R on the cell surface. A variety of leukocytes are positive for Fcγ-RI (monocytes/macrophages, dendritic cells, and neutrophils) and Fcγ-RII (monocytes/macrophages, dendritic cells, neutrophils, B lymphocytes, and mast cells)(Cohen-Solal et al., 2004). It is possible that infection of these cells with AAV is enhanced with a low level of anti-AAV antibodies in vivo.

The naturally infected AAV does not induce the strong immune response of host animals. Although a great majority of humans are infected with AAV2 during childhood, the sera of

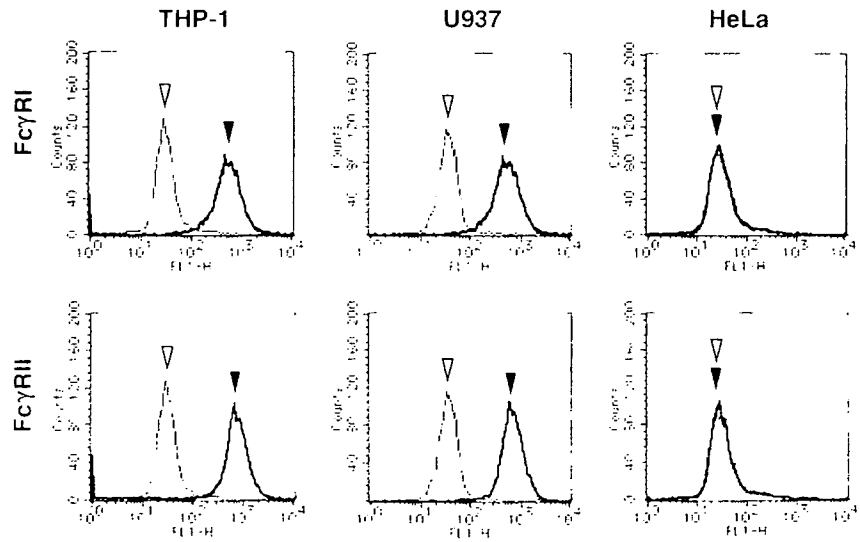

Fig. 6. Presence of Fcγ-RI and Fcγ-RII on THP-1 and U937 cells. THP-1, U937 and HeLa cells were incubated with anti-Fcγ-RI or anti-Fcγ-RII monoclonal antibodies followed by staining with the Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody. The fluorescence on the cells was measured by using a flow cytometer. The bold line (indicated with filled arrowheads) shows the distribution of the resultant fluorescence. The thin line (indicated with open arrowheads) shows the distribution of the fluorescence of the cells incubated with only the secondary antibody.

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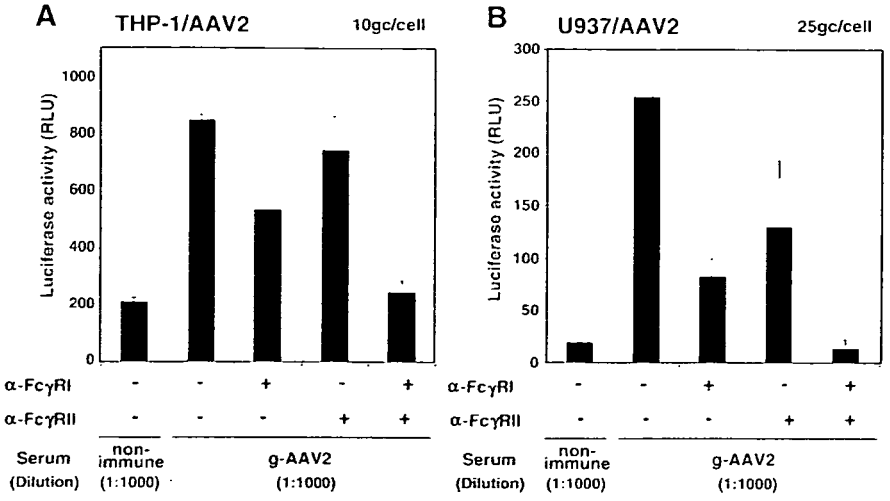

Fig. 7. Inhibition of ADE of AAV2 infection with anti-Fcγ-RI and anti-Fcγ-RII antibodies. THP-1 (A) and U937 (B) cells were pre-incubated with anti-Fcγ-RI and/or anti-Fcγ-RII mAbs. Then, AA2/Luc that had been incubated with anti-AAV2 antiserum was inoculated to the cells. Two days later, the luciferase activity of the cellular lysate was measured. Each bar represents the average of three independent experiments with the standard deviation indicated by an error bar. RLU: relative light units.

humans aged over 30 are positive for anti-AAV2 IgG and IgM antibodies simultaneously, indicating anti-AAV2 antibody induced by the primary infection does not protect the hosts from the secondary infection (Erles et al., 1999). Among human sera positive for anti-AAV antibody, only 30% of the sera are neutralizing (Chirmule et al., 1999). The level of anti-AAV antibody in the serum of the host persistently infected with AAV could be appropriate for the enhancement of AAV infection. Indeed the undiluted monkey-B sera, whose level of neutralizing antibody was below the detectable level, enhanced infection of THP-1 with AAV2/Luc (Fig. 5).

ADE is probably important for AAV to be maintained in host animals. Because the chance for the AAV-infected cell to be super-infected with the helper virus must be very rare in vivo, AAV may survive with a low level autonomous propagation induced by the Fas-mediated apoptosis of the host cell (Mori et al., 2002). ADE may support infection of a new host cell, mainly a leukocyte, with AAV.

Previous studies have shown that the capability of an antibody to contribute to ADE is independent of neutralizing activity. The monoclonal neutralizing antibodies against West Nile virus and against Dengue virus (Pierson et al., 2007; Y... 8) and non-neutralizing antibodies against human respiratory syncytial virus (... 6) enhance infection of FcR bearing cells with these viruses, respectively. It is necessary to examine a panel of anti-AAV monoclonal antibodies for their capability of contributing to ADE and for their binding characteristics to AAV particles in future studies.

ADE may be useful for ex vivo immunotherapy, which uses antigen-presenting cells transduced with AAV vectors in vitro (...). Because the antigen-presenting cells, such as monocytes and dendritic cells, are positive for Fcγ-RI and Fcγ-RII, pre-incubation of the AAV vectors with anti-AAV antibodies at a sub-neutralizing level, would increase the efficiency of transduction.

This is the first report showing the ADE of infection of Fcγ-R-bearing cells with the dependovirus. ADE has been observed in infection of Fcγ-R-bearing cells with the autonomous parvoviruses, such as human parvovirus B19 (Munakata et al., 2006) and Aleutian mink disease parvovirus (Kanno et al., 1993). The Fcγ-R-mediated ADE of infection may be common to those belonging to *Parvoviridae*.

Experimental Procedures

Cells

THP-1 and U937, human monocytic cell lines, were purchased from American Type Culture Collection (Manassas, VA). THP-1 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μM 2-β-mercaptoethanol at 37 °C. U937 was cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C. HeLa cells that had been adapted to the suspension condition were cultured in suspension minimal essential medium (S-MEM) supplemented with 10% FBS with gentle shaking at 37 °C.

AAV vectors

A vector plasmid pAAVLuc was packaged into the AAV2 and AAV10 capsids to produce AAV2/Luc and AAV10/Luc, respectively, in HEK293 cells as described previously (...). The pAAVLuc is composed of the 5′-inverted terminal repeats (ITR) of AAV2, simian virus 40 early enhancer promoter, firefly luciferase gene, and 3′-ITR. AAV2/Luc and AAV10/Luc were purified by heparin affinity column chromatography (...) and CsCl equilibrium centrifugation (...), respectively. Genome copy numbers of the vector stocks were measured by real-time PCR with TaqMan probes for the firefly luciferase gene (Perkin-Elmer Biosystems, Foster City, CA).

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Antisera

Anti-AAV2 antiserum was obtained by an intramuscular injection of purified AAV2 vector having β-galactosidase gene (10^9 genome copies per mouse) to eight-week-old female BALB/c mice. The serum was collected at 6 weeks after the injection. Mouse anti-AAV10 serum was produced previously by immunizing mice with VP2, one of three capsid proteins (Mori et al., 2004). Mouse anti-CD64 and anti-CD32 monoclonal antibodies (BD Bioscience PharMingen, San Diego, CA) were used in the detection and blocking of Fcγ-RI and Fcγ-RII, respectively.

Monkey sera

Sera were obtained from two cynomolgus monkeys kept in Tsukuba Primate Research Center, National Institute of Biomedical Innovation. The monkeys were sedated during collection of blood by administration of Ketamine (10 mg/kg). Animal studies were performed in accordance with the guidelines for animal experiments in the National Institute of Infectious Diseases.

Assay for AAV infection

AAV vectors were suspended in phosphate-buffered saline (PBS) containing 5% FBS. 15 μl of vector suspension was mixed with an equal volume of the test serum that had been heat-inactivated (56 °C for 30 min) and diluted with PBS, and incubated at 37 °C for 30 min. The mixture was inoculated to 3×10^5 cells in a microtube. After incubation at 4 °C for 1 h with occasional rocking, the cells were washed with culture medium twice and resuspended in 780 μl of culture medium. The cell-suspension was seeded in 3 wells (250 μl/well) of a 48-well culture plate and incubated at 37 °C for 2 days. The cells were harvested and lysed. Luciferase activity of the lysate was measured by using Luciferase Assay System (Promega, Madison,WI) and Mithras LB940 Multilabelreader (Berthold Technologies, Bad Wildbad, Germany).

Flow cytometry

THP-1, U937, and HeLa cells were incubated with 2.5 μg/ml of anti-CD64 or anti-CD32 monoclonal antibody in reaction buffer (PBS containing 2% FBS) for 1 h at 4 °C. The cells were washed with the buffer twice and incubated in the buffer containing 2.5 μg/ml of Alexa Fluor 488-conjugated goat anti-mouse IgG serum (Molecular Probes, Eugene, OR). The cells were washed twice with the buffer and then fixed with 2% paraformaldehyde in PBS. Fluorescence was measured using a flow cytometer (BD FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

Blocking of FcγR on THP-1 and U937 cells

THP-1 and U937 cells were incubated with the culture medium containing 5 μg/ml of anti-CD64 (BD Bioscience PharMingen) and/or anti-CD32 (BD Bioscience PharMingen) at 4 °C for 1 h. The cells were washed twice with the culture medium and used in the assay for AAV infection.

Acknowledgments

We thank Dr. Kunito Yoshiike for critical reading of the manuscript. This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare for the Third-Term Comprehensive 10-year Strategy for Cancer Control.

References

Auricchio, A., Hildinger, M., O'Connor, E., Gao, G.P., Wilson, J.M., 2001. Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. Hum. Gene Ther. 12, 71–76.

Buller, R.M., Janik, J.E., Sebring, E.D., Rose, J.A., 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J. Virol. 40, 241–247.

Carter, B.J., Laughlin, C.A., de la Maza, L.M., Myers, M., 1979. Adeno-associated virus autointerference. Virology 92, 449–462.

Cheung, A.K., Hoggan, M.D., Hauswirth, W.W., Berns, K.I., 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. 33, 739–748.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., Wilson, J., 1999. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. 6, 1574–1583.

Clyde, K., Kyle, J.L., Harris, E., 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J. Virol. 80, 11418–11431.

Cohen-Solal, J.F., Cassard, L., Fridman, W.H., Sautes-Fridman, C., 2004. Fcγ receptors. Immunol. Lett. 92, 199–205.

Conrad, C.K., Allen, S.S., Afione, S.A., Reynolds, T.C., Beck, S.E., Fee-Maki, M., Barrazza-Ortiz, X., Adams, R., Askin, F.B., Carter, B.J., Guggino, W.B., Flotte, T.R., 1996. Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. Gene Ther. 3, 658–668.

Davidoff, A.M., Gray, J.T., Ng, C.Y., Zhang, Y., Zhou, J., Spence, Y., Bakar, Y., Nathwani, A.C., 2005. Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. Molec. Ther. 11, 875–888.

Erles, K., Sebokova, P., Schlehofer, J.R., 1999. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). J. Med. Virol. 59, 406–411.

Favre, D., Provost, N., Blouin, V., Blancho, G., Cherel, Y., Salvetti, A., Moullier, P., 2001. Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. Molec. Ther. 4, 559–566.

Flotte, T.R., Berns, K.I., 2005. Adeno-associated virus: a ubiquitous commensal of mammals. Hum. Gene Ther. 16, 401–407.

Gimenez, H.B., Chisholm, S., Dornan, J., Cash, P., 1996. Neutralizing and enhancing activities of human respiratory syncytial virus-specific antibodies. Clin. Diagn. Lab. Immunol. 3, 280–286.

Handa, H., Shiroki, K., Shimojo, H., 1977. Establishment and characterization of KB cell lines latently infected with adeno-associated virus type 1. Virology 82, 84–92.

Kanno, H., Wolfinbarger, J.B., Bloom, M.E., 1999. Aleutian mink disease virus infection of mink macrophages: role of the macrophage cell line U937 demonstration of antibody-dependent enhancement of infection. Virology 917–7024.

Lai, L., Davison, B.B., Veazey, R.S., Fisher, K., Baskin, G.B., 2002. A preliminary evaluation of recombinant adeno-associated virus biodistribution in rhesus monkeys after intrahepatic inoculation in utero. Hum. Gene Ther. 13, 2027–2039.

McPherson, R.A., Rosenthal, L.J., Rose, J.A., 1985. Human cytomegalovirus completely helps adeno-associated virus replication. Virology 147, 217–222.

Please cite this article as: Mori, S., et al., Antibody-dependent enhancement of adeno-associated virus infection of human monocytic cell lines. Virology (2008). doi:

Mori, S., Murakami, M., Takeuchi, T., Kozuka, T., Kanda, T., 2002. Rescue of AAV by antibody-induced Fas-mediated apoptosis from viral DNA integrated in HeLa chromosome. Virology 15, 90–98.

Mori, S., Wang, L., Takeuchi, T., Kanda, T., 2004. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology 330, 375–383.

Mori, S., Takeuchi, T., Enomoto, Y., Kondo, K., Sato, K., Ono, F., Iwata, N., Sata, T., Kanda, T., 2006. Biodistribution of a low dose of intravenously administered AAV-2, 10, and 11 vectors to cynomolgus monkeys. Jpn. J. Infect. Dis. 59, 285–293.

Mori, S., Takeuchi, T., Enomoto, Y., Kondo, K., Sato, K., Ono, F., Sata, T., Kanda, T., 2008. Tissue distribution of cynomolgus adeno-associated viruses AAV10, AAV11, and AAVcy.7 in naturally infected monkeys. Arch. Virol. 153, 375–380.

Munakata, Y., Kato, I., Saito, T., Kodera, T., Ishii, K.K., Sasaki, T., 2006. Human parvovirus B19 infection of monocytic cell line U937 and antibody-dependent enhancement. Virology 345, 251–257.

Nathwani, A.C., Davidoff, A.M., Hanawa, H., Hu, Y., Hoffer, F.A., Nikanorov, A., Slaughter, C., Ng, C.Y., Zhou, J., Lozier, J.N., Mandrell, T.D., Vanin, E.F., Nienhuis, A.W., 2002. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood 100, 1662–1669.

Peiris, J.S., Porterfield, J.S., 1979. Antibody-mediated enhancement of flavivirus replication in macrophage-like cell lines. Nature 282, 509–511.

Peiris, J.S., Gordon, S., Unkeless, J.C., Porterfield, J.S., 1981. Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. Nature 289, 189–191.

Pierson, T.C., Xu, Q., Nelson, S., Oliphant, T., Nybakken, G.E., Fremont, D.H., Diamond, M.S., 2007. The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. Cell Host Microbe. 1, 135–145.

Ponnazhagan, S., Mahendra, G., Curiel, D.T., Shaw, D.R., 2001. Adeno-associated virus type 2-mediated transduction of human monocyte-derived dendritic cells: implications for ex vivo immunotherapy. J. Virol. 75, 9493–9501.

Richardson, W.D., Westphal, H., 1981. A cascade of adenovirus early functions is required for expression of adeno-associated virus. Cell 27, 133–141.

Veron, P., Allo, V., Riviere, C., Bernard, J., Douar, A.M., Masurier, C., 2007. Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2. J. Virol. 81, 5385–5394.

Wu, Z., Asokan, A., Samulski, R.J., 2006. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Molec. Ther. 14, 316–327.

Yamanaka, A., Kosugi, S., Konishi, E., 2008. Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J. Virol. 82, 927–937.

Please cite this article as: Mori, S., et al., Antibody-dependent enhancement of adeno-associated virus infection of human monocytic cell lines, Virology (2008), doi:

Arch Virol (2008) 153: 375–380
DOI 10.1007/s00705-007-1097-8
Printed in The Netherlands

Archives of Virology

Brief Report

Tissue distribution of cynomolgus adeno-associated viruses AAV10, AAV11, and AAVcy.7 in naturally infected monkeys

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Received 20 August 2007; Accepted 29 October 2007; Published online 10 December 2007

Summary

Adeno-associated virus (AAV) is used in gene-therapy studies, but its tissue distribution is unknown in natural infection. We examined cynomolgus AAVs (previously isolated AAV10 and AAV11 and novel AAVcy.7) for their tissue distribution in 14 cynomolgi by type-specific PCR. We found AAV10, AAV11, and AAVcy.7 in 6, 10, and 14 monkeys, respectively, and two or three types in 11 monkeys, showing that these AAVs are widespread in the monkeys. We detected AAV at a higher level mainly in the lymphatic tissues and ileum, which suggests that AAV may invade the host through Peyer's patches in the ileum and infect immune cells.

*

Adeno-associated virus (AAV), a member of the genus *Dependovirus* of the family *Parvoviridae*, is a nonenveloped icosahedral particle with a genome of 4.7 kb single-stranded linear DNA. Pathogenicity of AAV has not been reported. The AAV genome has the rep and cap genes that encode the nonstructural Rep proteins and the capsid proteins (VP1, VP2 and VP3), respectively. The initial studies of AAV type 2 (AAV2) have shown that, upon infection of cultured human cells, the viral genome is integrated into chromosome 19 and is maintained as a latent provirus unless the cells are co-infected with a helper virus, such as adenovirus [8].

Recently Gao et al. [3, 4] have obtained various AAV DNAs from human and nonhuman primate tissue samples by PCR amplifying the signature region, which has a highly type-specific nucleotide sequence, with consensus primers that hybridize to the conserved region in the capsid gene. AAV DNA was detected in the heart, lung, liver, spleen, duodenum, kidney, and lymph node of rhesus monkeys. By using a similar PCR strategy, Mori et al. [6] newly detected AAV10 and AAV11 in DNA samples from cynomolgus monkeys, and Chen et al. [1] detected AAV DNA in the tonsil, lung, and spleen of children. These findings indicate that many AAVs, including unidentified ones, are widely disseminated throughout multiple tissues of humans and nonhuman primates [4]. To date we have only a limited knowledge of the AAV life cycle, such as the portal of entry, the role of helper viruses in

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primary infection, sites of replication and latency, and molecular forms of viral DNA.

Recombinant AAV has been studied for its potential use as a gene-transfer vector in gene therapy. It has been indicated that pseudotyped vectors, which are the AAV2 vector genome packaged in the capsids of AAVs other than AAV2, transduced various tissues with different efficiency. For example, AAV1-pseudotype transduced mouse muscle efficiently by the injection of the vector into the muscle, and AAV8-pseudotype transduced mouse liver efficiently by the injection of the vector into the portal vein [2]. These findings suggest that each AAV type has its own tissue preference. However, the tissue tropism of AAV in the natural host has not been studied extensively. In this study, we examined three cynomolgus monkey AAVs for their tissue distribution in the naturally infected host.

Fourteen cynomolgus monkeys (4 to 5 years of age and weighing 3 to 5 kg), numbered from #1 to #14, were obtained from Tsukuba Primate Research Center, National Institute of Biomedical Innovation. All the animal studies were conducted in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases. Twelve monkeys (#1 to #4 and #6 to #13) had been intravenously injected with a AAV2 vector containing a beta-galactosidase gene or EGFP-tubulin fusion gene in the previous study [7]. The entire coding regions of AAV10 (GenBank accession number: AY631965) and AAV11 (AY631966) had been isolated from the ileum of monkey #4 and the liver of monkey #5, respectively, as described previously [6]. Animal tissues were harvested at necropsy and stored at −80 °C until use. DNA was extracted from approximately 25 mg of each frozen tissue by using a QIAamp DNA extraction kit (Qiagen GmbH, Hilden, Germany). The quality and quantity of DNA in the samples were verified by amplifying a part of the cellular G3PDH gene as described previously [7].

We found a novel AAV sequence in monkey #12. The DNA samples extracted from the spleen and ileum were examined by PCR with the consensus primers CP1 and CP5 (Fig. 1A), whose nucleotide sequences are highly conserved in the cap genes of

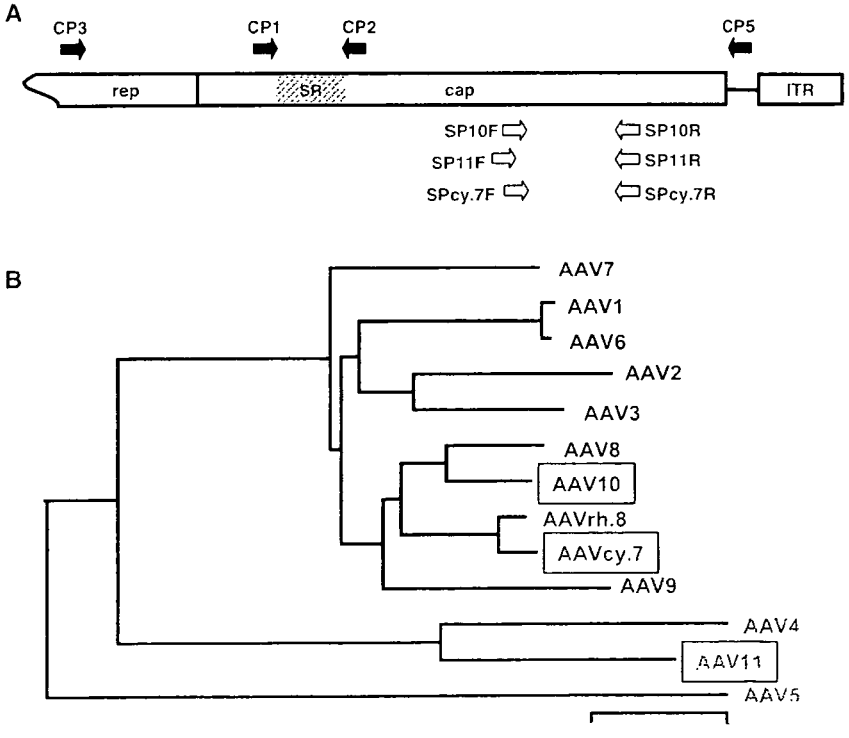

Fig. 1. Schematic representation of the AAV cap gene and phylogenetic tree of AAVs. (**A**) The signature region of AAV capsid gene and the PCR primers are indicated. (**B**) Phylogenetic tree based on the amino acid sequences of the capsid proteins. AAV types analysed in this study are indicated by boxes

the eleven AAV types, from AAV1 to AAV11 (6). Although AAV DNA was not amplified with the spleen sample, a DNA fragment of approximately 1.6 kilobase pairs (bp) was obtained from the ileum sample. The nucleotide sequence of the amplified fragment did not match those of any reported AAVs.

The entire coding region of the cap gene of the novel AAV was obtained by two sets of PCR using the DNA sample extracted from the ileum of monkey #12 and the consensus primers [6] (Fig. 1A). The 5′ region was amplified by using CP3 and CP2, and the 3′ region was amplified by using CP1 and CP5. The amino acid (aa) sequence deduced from the nucleotide sequence did not match those of any known AAVs. Therefore, we named the novel AAV AAVcy.7. The aa sequence of AAVcy.7 was similar to that of AAVrh.8 (97% homology), which had been obtained from a rhesus monkey [4]. The phylogenetic tree based on aa sequences of capsid protein is presented in Fig. 1B.

We attempted to detect the presence of AAV10, AAV11, and AAVcy.7 in the DNA samples of the various tissues of the monkeys by the PCR-amplification with primers specific for one of these types (Fig. 1A). The nucleotide sequences of the type-specific primers are as follows: forward primer for AAV10 (SP10F), 5′-GGGCCTGCAAACATGT CGGC; reverse primer for AAV10 (SP10R), 5′-CTGTTGACATTTCCCACAAT; SP11F, 5′-CTG AATCAAGGCAATGCAGC; SP11R, 5′-GCAGTC ACGTTGCCGGTTAT; SPcy.7F, 5′-GGGCCCAGC ACTATGGCCAA; SPcy.7R, 5′-TTGTGAACAAG TCCAGTCTG. These primer sets have been designed to amplify fragments of 395, 446, and 395 bp from AAV10, AAV11, and AAVcy.7 DNA, respectively. These primer sets amplified the target AAV DNA in the test samples containing AAV2, AAV4, AAV5, AAV8, AAV10, AAV11, or AAVcy.7 without non-specific amplification (data not shown). The level of the amplified DNA was estimated by the comparison of DNA amplified from the standard samples, which were a mixture of the cellular DNA extracted from African green monkey COS-1 cells (10^5 cells) and the plasmid DNA containing the AAV cap gene in the range from 10 to 10^5 copies, after ethidium bromide staining of the agarose gel. Approximately 10 copies of the cap gene in the standard sample were the minimum level of detection.

Nucleotide sequencing of the randomly selected PCR products showed that the detection and typing by the PCR with the type-specific primers was reliable. Seventeen PCR products obtained with the AAV10-specific primers and DNA samples of monkeys #3 and #5 were sequenced after cloning using pGEM-T vector system (Promega, Madison, WI). All of the DNA fragments were 395 nucleotides long. The aa sequences were deduced from the nucleotide sequences. The aa sequences of 4 clones were the same as with that of the registered AAV10. Ten clones had one aa replacement, R at aa 552 was replaced with K (AAV10/R552K), and 3 clones had one additional aa replacement (AAV10/R552K/S558I, AAV10/R552K/E577G, AAV10/R552K/D584N). Seven PCR products obtained with the AAV11-specific primers and DNA samples from monkeys #2 and #5 were sequenced. All of the DNA fragments were 446 nucleotides long. The aa sequence of one clone was same with that of the registered AAV11. One clone had one aa replacement (AAV11/Y490D), and 4 clones obtained from monkey #2 had two aa replacements (AAV11/V544I/N547T). Twenty PCR products obtained with the AAVcy.7-specific primers and DNA samples of monkeys #12 and #13 were sequenced. All of the DNA fragments were 395 nucleotides long. The aa sequences of 15 clones obtained from the cerebrum, spinal cord, ileum, and mesenteric lymph node of monkey #12 were identical to that of AAVcy.7. Five clones obtained from monkeys #13 had one aa replacement (AAVcy.7/A579S).

Table 1 shows the presence of AAV10, AAV11, and AAVcy.7 in the various tissues with the levels estimated by comparison to the standard samples. The blanks indicate that the samples were negative for the DNA amplification with the three sets of type-specific primers. AAV10, AAV11, and AAVcy.7 were detected in 6, 10, and 14 monkeys, respectively. Five monkeys (#1, #2, #5, #12, and #13) were infected with AAV10, AAV11, and AAVcy.7. Five monkeys (#3, #6, #7, #8, and #9) were infected with AAV11 and AAVcy.7. One monkey (#4) was infected with AAV11 and AAVcy.7. The data indicate that these AAVs are widespread in cynomolgus

Table 1. AAV10, 11, and cy.7 genomes in cynomolgus monkeys

	#1♀			#2♀			#3♀			#4♂			#5♀			#6♂			#7♀		
tissues	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7
cerebrum		+	+			+															
cerebellum																					
spinal cord	ND	ND	ND				ND	ND	ND	ND	ND	ND		+	+						
bone marrow										++		+	+	+++							
skin										ND	ND	ND									
eye			+											+	+						
muscle			+							ND	ND	ND									
bronchus																					
lung														++							
heart			+					+		+					+			++			
liver		+								+++				+++							+
gallbladder														+			+				
pancreas																					
spleen	+	++	+	+	+					++		+++	++	+++++						+	++
esophagus														+							
stomach										+		++		+							
jejunum				ND	ND	ND				++++			ND	ND	ND						++
ileum	+	+	+			+++				+++		+++		++++	+						
colon							ND	ND	ND					+							
kidney										+				+++							
adrenal gland		+								+			++	+++							
bladder										ND	ND	ND									
tonsil			+			+++			+++					+				+			
thymus													ND	ND	ND				ND	ND	ND
parotid gland																					
submandibular gland																					
thyroid gland														+							
axillary lymph node			+			+				++			+	+							
hilar lymph node	ND	ND	ND					+						+							+
mesenteric lymph node										ND	ND	ND	+	+++	+			+			++
iliac lymph node	ND	ND	ND			+	ND	ND	ND	ND	ND	ND		+							
inguinal lymph node	+		+							+			+	++				+			
testis/ovary																					
epididymis/uterus	ND	ND	ND															++			

	#8♂			#9♀			#10♀			#11♀			#12♂			#13♂			#14♀		
tissues	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7	10	11	cy.7
cerebrum															+						
cerebellum																					
spinal cord															+						
bone marrow																					
skin																					
eye																					
muscle																					
bronchus																					
lung																					
heart					++							+									
liver																					
gallbladder						+															
pancreas													+								
spleen									+			+									+
esophagus			++																		
stomach			++									+									
jejunum									+												
ileum															+			+			
colon						++															
kidney																					
adrenal gland																					
bladder																					
tonsil														+							
thymus			++																		
parotid gland			+																		
submandibular gland																					
thyroid gland			+																		
axillary lymph node																+	+	+			+
hilar lymph node							ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
mesenteric lymph node															+						
iliac lymph node		+																	ND	ND	ND
inguinal lymph node						+								+							
testis/ovary																					
epididymis/uterus																					

10 AAV10, *11* AAV11, *cy.7* AAVcy.7.
+ 10–10^2, ++ 10^2–10^3, +++ 10^3–10^4, ++++ 10^4–10^5, +++++ 10^5 < genome copies/500 ng DNA.
ND Not done.

Table 2. Frequency of AAV genome detection in the tissues of cynomolgus monkeys

Tissues	AAV10			AAV11			AAVcy.7		
	Number of monkeys		Frequency	Number of monkeys		Frequency	Number of monkeys		Frequency
	A	B	A/B (%)	A	B	A/B (%)	A	B	A/B (%)
Cerebrum	0	6	0	1	10	10	3	14	21
Cerebellum	0	6	0	0	10	0	0	14	0
Spinal cord	0	4	0	1	8	13	2	11	18
Bone marrow	2	6	33	1	10	10	1	14	7
Skin	0	5	0	0	10	0	0	13	0
Eye	0	6	0	1	10	10	2	14	14
Muscle	0	5	0	0	10	0	1	13	8
Bronchus	0	6	0	0	10	0	0	14	0
Lung	0	6	0	1	10	10	0	14	0
Heart	1	6	17	2	10	20	4	14	29
Liver	1	6	17	2	10	20	1	14	7
Gallbladder	0	6	0	2	10	20	1	14	7
Pancreas	1	6	17	0	10	0	0	14	0
Spleen	4	6	67	4	10	40	6	14	43
Esophagus	0	6	0	1	10	10	1	14	7
Stomach	1	6	17	1	10	10	3	14	21
Jejunum	1	4	25	0	8	0	2	12	17
Ileum	2	6	33	2	10	20	6	14	43
Colon	0	6	0	1	9	11	1	13	8
Kidney	1	6	17	1	10	10	0	14	0
Adrenal gland	2	6	33	2	10	20	0	14	0
Bladder	0	5	0	0	10	0	0	13	0
Tonsil	0	6	0	2	10	20	4	14	29
Thymus	0	5	0	0	8	0	1	12	8
Parotid gland	0	6	0	0	10	0	1	14	7
Submandibular gland	0	6	0	0	10	0	0	14	0
Thyroid gland	0	6	0	1	10	10	1	14	7
Axillary lymph node	3	6	50	2	10	20	4	14	29
Hilar lymph node	0	3	0	2	7	29	1	8	13
Mesenteric lymph node	1	5	20	1	10	10	4	13	31
Iliac lymph node	0	4	0	2	8	25	1	10	10
Inguinal lymph node	3	6	50	2	10	20	3	14	21
Testis	0	3	0	0	4	0	1	5	20
Ovary	0	3	0	0	6	0	0	9	0
Epididymis	0	3	0	0	4	0	1	5	20
Uterus	0	2	0	0	5	0	0	7	0

A The number of the monkeys whose indicated tissue was positive for AAV DNA.
B The number of the monkeys with AAV-positive tissue(s) and whose indicated tissue was available for the test.

monkeys and that monkeys are commonly infected with multiple types of AAV.

Table 2 shows the frequency of the AAV DNA detection in the various tissues of monkeys having one or more tissues positive for the AAV DNA. Regardless of the types, these AAVs were frequently detected in the lymphoid tissues, such as the spleen and lymph nodes, as previously reported [3, 4], and in the ileum. The AAV10, AAV11, and AAVcy.7 were detected in the ileum of 33%, 20%, and 43% of the monkeys infected with AAV10, AAV11 and AAVcy.7, respectively, suggesting that AAV may invade the host through Peyer's patches in the ileum and infect immune cells, such as lymphocytes and macrophages, as many other pathogens do [5]. AAV10 and AAV11 were detected in

the kidney and adrenal gland less frequently, whereas AAVcy.7 was not detected in those tissues. Although AAV10 was not detected in the central nervous system (CNS), such as cerebrum and spinal cord, AAV11 and AAVcy.7 were detected in the CNS of two (#1 and #5) and four monkeys (#1, #2, #5, and #12), respectively. These results provide useful information for designing pseudotyped AAV vectors targeting the kidney or CNS.

Acknowledgements

We thank Dr. Kunito Yoshiike for critical reading of the manuscript. This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare for the Third-Term Comprehensive 10-year Strategy for Cancer Control.

References

1. Chen CL, Jensen RL, Schnepp BC, Connell MJ, Shell R, Sferra TJ, Bartlett JS, Clark KR, Johnson PR (2005) Molecular characterization of adeno-associated viruses infecting children. J Virol 79: 14781–14792
2. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA 99: 11854–11859
3. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM (2003) Adeno-associated viruses undergo substantial evolution in primates during natural infections. Proc Natl Acad Sci USA 100: 6081–6086
4. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM (2004) Clades of Adeno-associated viruses are widely disseminated in human tissues. J Virol 78: 6381–6388
5. Jones B, Pascopella L, Falkow S (1995) Entry of microbes into the host: using M cells to break the mucosal barrier. Curr Opin Immunol 7: 474–478
6. Mori S, Wang L, Takeuchi T, Kanda T (2004) Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology 330: 375–383
7. Mori S, Takeuchi T, Enomoto Y, Kondo K, Sato K, Ono F, Iwata N, Sata T, Kanda T (2006) Biodistribution of a low dose of intravenously administered AAV-2, 10, and 11 vectors to cynomolgus monkeys. Jpn J Infect Dis 59: 285–293
8. Muzyczka N, Berns I (2001) Parvoviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds) Fields virology, vol. 2. Lippincott Williams & Wilkins, Philadelphia, pp 2327–2359

Chapter 2

Targeted Insertion of Transgene into a Specific Site on Chromosome 19 by Using Adeno-Associated Virus Integration Machinery

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Site-specific integration of the therapeutic transgene is favorable for gene therapy applications since it minimizes the risk of insertional mutagenesis and thereby prevents target cells from developing tumors. Adeno-associated virus (AAV), a member of parvovirus, is unique in that it integrates its genome into a specific site termed the AAVS1 locus (19q13.4) in the human genome. A non-structural replication initiator protein of AAV, Rep78 or Rep68, binds the inverted terminal repeat (ITR) sequence at either end of the AAV genome via tandem repeats of the GAGC/GCTC motif. A homologous sequence exists at the AAVS1 site. The Rep protein recognizes it and drives the integration of the AAV genome into AAVS1. The ITR is a *cis* element sufficient for AAVS1-specific integration. The incorporation of the ITR sequence into plasmid DNA is thus discussed in terms of Rep-mediated site-specific integration and of AAVS1 as a hazard-free target for transgene integration. Therefore, the use of the AAV integration machinery should allow us to develop a safer gene delivery system.

Keywords: Site-specific transgene integration; adeno-associated virus; Rep protein; AAVS1 locus.

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1. Introduction

Stem cells such as hematopoietic stem cells, embryonic stem (ES) cells and mesenchymal stem cells (MSCs) are attractive targets for gene therapy since they replicate themselves and differentiate into various cell lineages. To manipulate genes in these cells, it is especially important to utilize a system to introduce therapeutic DNA with a minimal risk of insertional mutagenesis. Insertion of the gene of interest into a defined site in the human chromosome is desirable. However, current strategies that achieve the integration of transgene into host chromosomal DNA insert it randomly, which is an insertional mutagenic/oncogenic hazard as shown with retroviral vectors. It is undoubtedly a milestone in gene therapy that 10 patients with X-linked severe combined immune deficiency, a lethal inherited disease characterized by an early block in T and natural killer lymphocyte differentiation due to mutations of the gene encoding the γc cytokine receptor subunit, were treated by infusion of autologous $CD34^+$ hematopoietic stem cells transduced with a murine retrovirus vector encoding the common γ chain (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002). Unfortunately T-cell leukemia developed in four patients approximately three years after gene therapy (Hacein-Bey-Abina *et al.*, 2003; Baum, 2007). The vector sequence was integrated into the upstream region or the first intron of the LMO2 gene, which is known to be a T-cell proto-oncogene. The activation of the LMO2 gene by chromosomal translocation has been reported in patients with T-cell acute leukemia (Nam and Rabbitts, 2006). The integrated vector promoter, the long terminal repeat (LTR) enhanced the activity of the LMO2 promoter and resulted in aberrant LMO2 expression and premalignant cell proliferation (Hacein-Bey-Abina *et al.*, 2003). To prevent such an adverse event, it is absolutely necessary to employ a strategy that introduces foreign DNA specifically into a predefined safe region of chromosomal DNA.

Prokaryotic site-specific recombinases such as Cre (Sauer and Henderson, 1988) or Flt (Andrews *et al.*, 1985) are widely used to introduce DNA into a site that their respective recognition sequence in eukaryotic cells as well as prokaryotic cells. However, the recognition sequence must be inserted into target chromosomal DNA in advance. Phage phiC31 integrase recognizing phage *attP* and *attB* sites has been shown to mediate site-specific DNA integration in human genome at native "pseudo" *attP* sites (Thyagarajan *et al.*, 2001).

AAV integrates its genome into a particular site in human chromosome 19, termed AAVS1 (19q13.4) (Kotin *et al.*, 1992; Samulski *et al.*, 1991), through the activity of a specific replicase/integrase protein, Rep. Taking advantage

of the AAV integration machinery, systems for AAVS1-specific integration of therapeutic DNA have been developed (Balague *et al.*, 1997; Surosky *et al.*, 1997). The systems are particularly valuable for *ex vivo* gene therapy applications for stem cells. Here the authors will review the current trend of the development of Rep-mediated AAVS1-targeted integration as well as basic biology of the site-specific integration of AAV.

2. Overview of AAV

The adeno-associated virus (AAV) belongs to the family *Parvoviridae* and is classified into the genus *Dependovirus*. A number of AAV serotypes have been reported so far. The AAV serotype 2 was sequenced more than 20 years ago and has been most extensively studied (for general review, see: Muzyczka, 2001; Smith, 2002). The type 2 is referred here as AAV. The AAV depends for its replication and propagation on a helper virus such as adenovirus, herpes virus, papilloma virus and vaccinia virus. When the AAV infects cells alone, it enters a latent infection phase and integrates into the human genome preferentially into the AAVS1 locus on chromosome 19 (19q14.2) (Kotin *et al.*, 1992; Samulski *et al.*, 1991). The integrated genome can be activated and rescued by subsequent superinfection by a helper virus (Fig. 1). There is no disease reported associated with AAV infection. More than 80% of adults are seropositive for antibodies against the AAV. The AAV is used as a gene-transfer vector particularly for long-term gene expression in livers, neurons and muscles. However, AAV vectors that are devoid of the *rep* gene fail to integrate into AAVS1 (see below for details).

The wild-type AAV has been reported to integrate into the AAVS1 site in immortalized cell lines at a frequency of 68% (Kotin *et al.*, 1990) or 94% (Kearns *et al.*, 1996). The insertion of AAV DNA into the AAVS1 site was also reported in human testis tissue (Mehrle *et al.*, 2004). The AAV vector genome has also been shown to persist extrachromosomally in cells (Afione *et al.*, 1996; Duan *et al.*, 1998; Nakai *et al.*, 2001). A recent study reported that the AAV genome in humans appears to persist as episomal forms, not as integrated forms; an attempt to detect a junction DNA sequence between the AAV genome and host chromosome by PCR from clinical tonsil-adenoid samples obtained from children was made. The junction sequence, however, could not be amplified, suggesting that the AAV genome existed in the respiratory tissues as an episomal form (Schnepp *et al.*, 2005). Thus, the mode of persistence of the AAV genome may be different among cell types.

The AAV genome is a single-stranded DNA of 4.8 kb in size. Either end of the genome shows a unique T-shaped hairpin configuration, which is

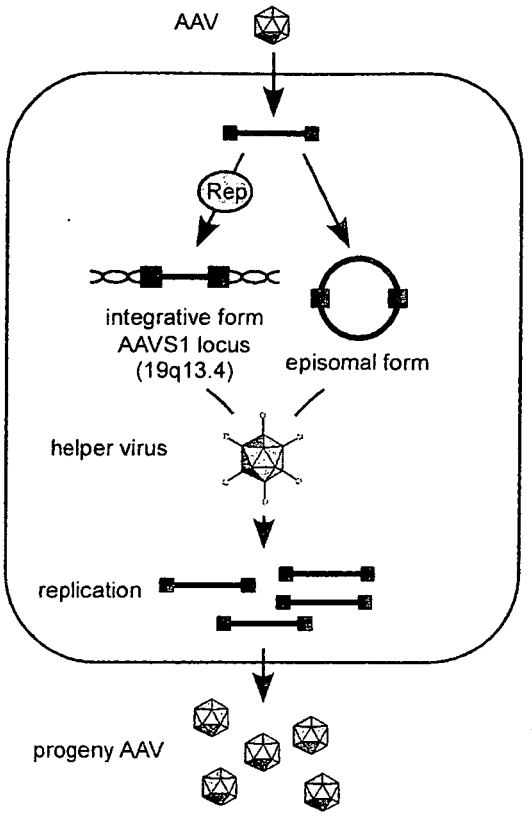

Fig. 1. Life cycle of AAV. Following infection, the AAV genome persists as an integrative or episomal form. The AAV genome preferentially integrates into the AAVS1 site (19q13.4) in the presence of the Rep protein. When AAV and a helper virus (e.g., adenovirus) coinfect cells or a helper virus superinfects the cells harboring the AAV genome, burst replication of AAV occurs.

called an inverted terminal repeat (ITR). The ITR serves as an origin of AAV genome replication. Between the ITRs are two open reading frames corresponding to *rep* and *cap* [Fig. 2(A)]. The *rep* gene encodes four overlapping nonstructural proteins, Rep78, Rep68, Rep52 and Rep40. The *cap* gene codes for structural proteins, VP1, VP2 and VP3. On the genome are three promoters, p5, p19, and p40, designated according to their map positions. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep 68, respectively, while Rep52 and Rep40 are translated from p19-unspliced and -spliced transcripts. Rep78 or Rep68 is a replication initiator of the AAV genome, which possesses site-specific, strand-specific endonuclease activity, ATP-dependent helicase activity (Im and Muzyczka, 1990), and ligase activity (Smith and Kotin, 2000). The Rep78 and Rep68 proteins bind