Our results indicate that, among these promoters, CAG promoter was the most efficient in transducing the cochlear cells.

Dose-response relationship for AAV1-CAG vector

Within the dose-effect groups with AAV1-CAG, a significant effect of dosage on the number of EGFP-expressing cochlear cells was found by one-way ANOVA ($P < 0.001$). CAG promoter driven EGFP expression in inner hair cells was detected at a dose as low as 3×10^7 genome copies, with expression increasing in a dose-dependent manner.

Discussion

The various factors affecting the transcription of a transgene includes the promoter containing 5'flanking region, 3'UTR, enhancer, suppressor, insulator sequences and the type and activity of transcription factors available in the transfected cells. This study showed that all 6 promoters, including viral, mammalian cells promoters, were capable of driving EGFP expression in HEK 293 cells, suggesting that the HEK 293 cell possesses all essential transcription factors for recognizing the diverse promoter sequences applied in this study. The CAG promoter appeared to be more efficient than other promoters based on the following observations: (1) more cells transduced in an equal dose comparison; (2) greater spreads of EGFP-expressing cell groups; and (3) CAG promoter-driven expression in more cell types, especially the inner hair cells, which were rarely found with other promoters except for CMV promoter. These results extend our knowledge about the promoter-related characteristics of AAV1-mediated gene transfer in the cochlea.

Interestingly, our data demonstrate that, in comparison with NSE, EF-1α and RSV promoters, CAG, CMV and hair cells specific promoter-Myo can drive EGFP expression in the inner hair cells. Even though certain promoters, such as EF-1α and RSV promoters, showed robust activity in many tissues, their activities are limited in the cochlear cells. Nowadays, it still has not been known why NSE, EF-1α and RSV promoters are inactive in the inner hair cells. One likely explanation is that inner hair cells may not express some transcription factors, which were necessary for these promoters to be actived. On the other hand, the inner hair cells may have all necessary components for the full transcriptional activity of the CAG, CMV or Myo promoters. Another possibility is transcriptional shutdown or promoter silencing in the inner hair cells. These phenomena have been observed previously in different organs such as the liver, lungs, and muscles (Hartikka *et al.*, 1996; Chen *et al.*, 2001; Gill *et al.*, 2001). Besides the known mechanisms for promoter silencing, such as DNA methylation, it has been demonstrated that the inclusion of EBN1 and OriP sequences into vector constructs may delay the process of promoter silencing (Al-Dosari *et al.*, 2006). Those elements also have been known to play an important role during viral infection in retention, replication, nuclear localization, binding to the nuclear matrix of the target cell, and tran-scriptional up-regulation (Cui *et al.*, 2001). Nevertheless, the mechanism of promoter inactivation remains to be poorly understood. The development of the vectors, which was capable of expressing high levels of transgene products, remains as an occasional finding. A thorough investigation on the mechanisms underlying episomal gene expression would be important for successful development of gene therapy.

Dose-dependent response for AAV gene transfer in other tissue has been previously reported (Klein *et al.*, 2002), and the wide range of doses has established minimal doses for transgene expression (10^7 particles). There is a shift toward lower potency for bicistronic vectors. The present study determined the dose dependency and minimum effective doses for AAV1 gene transfer using CAG promoter for the cochlear cells.

The direct measurement of transgene expression level in individual cells of cochlea *in vivo* is an important evaluation for cochlear gene therapy. Nevertheless, quantifying the number of cells transduced remains an important additional issue and a combination of protein expression and cell transduction efficiency may permit us to estimate the amount of gene product per cell under appropriate conditions. The gene transduction of the cochlear inner hair cells requires efficient promoter systems, which ensure potent and stable expression of exogenous genes. In this study, we demonstrated that the CAG and CMV promoters showed stable and efficient activity in the cochlear cells, the Myo promoter was specific for gene transfer in the inner hair cells. The stable and efficient promoter activities might be necessary for cochlear gene therapy strategies. From the data obtained by this study, it might be able to extend our knowledge both in the basic and clinical gene research fields.

Acknowledgement

This study was supported in part by Research Grants from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Vehicle Racing Commemorative Foundation.

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Prevention of diabetic retinopathy by intraocular soluble *flt-1* gene transfer in a spontaneously diabetic rat model

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Received August 11, 2006; Accepted October 2, 2006

Abstract. The number of patients suffering from diabetes mellitus is constantly rising worldwide, and diabetic retinopathy (DR) has become the most frequent cause of postnatal blindness. Vascular endothelial growth factor (VEGF) is known to play a central role during DR development. Thus, inhibiting the effects of VEGF may hamper the disease progression, and gene transfer of the soluble VEGF receptor *sflt-1* is an attractive approach for this purpose. However, the lack of suitable animal models hindered the evaluation of this strategy. Recently, the spontaneously diabetic non-obese Torii (SDT) rat was established and is considered as one of the ideal models for human DR. In this study, we evaluated the efficacy of gene therapy in SDT rats by using adeno-associated viral vectors (AAV-*sflt-1*) injected into the subretinal space. Thirty weeks later, the progression of DR was assessed by fluorescein angiography using three parameters; the presence of an avascular area, extensive hyperfluorescein and arterial narrowing. These changes were significantly less evident in the 'treated' eyes than in the control. No adverse effects were observed throughout the study. These results indicate that local *sflt-1* gene transfer inhibits DR progression in SDT rats and offers powerful therapeutic potential for the management of human DR.

Introduction

Diabetic retinopathy (DR) is one of the major complications of diabetes mellitus (DM), and the most frequent cause of postnatal blindness (1,2). The number of patients suffering from DM is steadily increasing worldwide (3), and the prevention of DR has become a matter of great importance. Unfortunately, the number of patients who are losing their vision due to DR is increasing despite the technological advancements, especially laser photocoagulation and vitreous surgery. Therefore, the development of a novel therapeutic approach to prevent DR progression has a vital significance.

Proliferative diabetic retinopathy (PDR) is an advanced form of DR characterized by neovascularization, vitreous hemorrhage and tractional retinal detachment. Although a number of biochemical changes, including increased polyol pathway activity (4,5), activation of protein kinase C (6-8) and accumulation of advanced glycation end-products (9,10) were reported in the development of PDR, vascular endothelial growth factor (VEGF), a potent endothelial cell-specific mitogen, plays a critical role in the angiogenesis of PDR (11-13). The actions of VEGF are mediated by the fms-like receptors, Flt-1 and Flk-1/KDR, which are expressed on vascular endothelial cells, and result in endothelial cell proliferation, migration, and increased vasopermeability with tyrosine kinase activity (14-17). Expression of VEGF is upregulated by hypoxia, and increased vitreous VEGF levels were observed in patients with PDR (12,18,19). Moreover, overexpression of VEGF by photo-receptors in transgenic mice promoted retinal neovascular-ization (20), whereas antagonists for VEGF suppressed neovascularization in the retina and iris (13,21,22). A soluble form of the VEGF receptor Flt-1 (sFlt-1) is the only known endogenous specific inhibitor for VEGF, and has drawn considerable attention for its potential clinical application in the inhibition of angiogenesis (23-28). It lacks the immuno-globulin-like domain, the transmembrane spanning region and the intracellular tyrosine-kinase domain. The anti-angiogenic activity of sFlt-1 results from the inhibition of VEGF by two mechanisms; the sequestration of VEGF and the formation of inactive heterodimers with membrane spanning isoforms of the VEGF receptors Flt-1 and KDR (26,29). Studies have shown that the administration of viral vectors encoding *sflt-1* inhibited retinal neovascularization in animal models (30,31). However, the actual merits of sFlt-1 in clinically relevant DR models have not been evaluated.

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Key words: diabetic retinopathy, gene therapy, *sflt-1*, spontaneously diabetic non-obese Torii rat, adeno-associated viral vector

Recently, a spontaneously diabetic, non-obese Torii (SDT) rat strain was established from the Sprague-Dawley lineage (32). The animals develop DM at ~20 weeks of age and later manifest DR, which is characterized by tractional retinal detachment, retinal hemorrhage, extensive venous dilatation, extensive hyperfluorescence and a non-perfusion area beyond 55 weeks of age (33). These findings are similar to those found in DR patients; therefore, the SDT rat is one of the best-suited models for studying human DR.

Adeno-associated viral (AAV) vectors are becoming popular in the field of gene therapy because of their safety and long-term effectiveness (34,35). A number of studies have demonstrated the efficacy of ocular gene therapy using AAV vectors (30,36), and vectors derived from serotype 5 (rAAV5) showed the highest utility for retinal gene transfer among serotypes tested (37-39). For this reason, we set out to test the utility of gene therapy on preventing DR in SDT rats using an rAAV5 vector encoding human *sflt-1* (rAAV5-*sflt-1*).

Materials and methods

rAAV vector construction and production. The *sflt-1* cDNA was amplified by PCR from the cDNA library of human umbilical vein endothelial cells (HUVEC). The *in vitro* effect of *sflt-1* expression was confirmed according to a method previously reported (40,41). Briefly, a plasmid was transfected into 293 cells with the Ca-phosphate method, the medium from 293 cells was added at specified dilutions to a 96-well plate containing HUVEC, and the cell density was assessed. An rAAV5 vector encoding *sflt-1* cDNA driven by the human cytomegalovirus (CMV) promoter (rAAV5-CMV-*sflt-1*) was constructed (Fig. 1). rAAV vectors were produced with an adenovirus-free system, and were purified by ultra-centrifugation through an Iodixanol (Axis-Shield PoC AS, Oslo, Norway) gradient followed by dialysis (42,43). The titers of the vector stocks were determined by quantitative dot-blot analysis using a BAS-1500 image analyzer (Fuji Film, Tokyo, Japan).

Animals. All animal experiments were performed in accordance with the standards in the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23) and the institutional guidelines. Male SDT rats, provided by the Association for the Spontaneously Diabetic Torii Rat, were used in this study. Standard rodent diet and water were provided *ad libitum*. Casual blood glucose levels were measured by the glucose-oxydase method every four weeks using Glutest A (Sanwa Chemical, Tokyo, Japan). Glycosylated hemoglobin (HbA1c) was measured with a latex agglutination test (SRL, Tokyo, Japan), and plasma sFlt-1 levels were determined using a commercially available ELISA kit (Bender MedSystems, San Bruno, CA) at the end of the study.

Subretinal injection of vector solution. Rats at the age of 27 weeks were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mg/kg), and 0.4% oxybuprocaine chloride eye drops were used for additional analgesia. All surgical procedures were performed under a surgical microscope. The tip of a 10-mm 39-gauge nylon needle

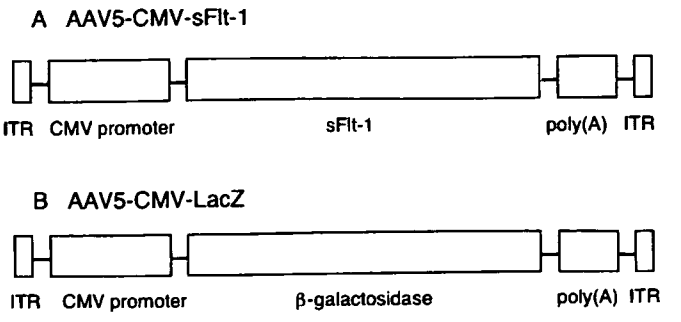

Figure 1. Structure of rAAV vectors. (A) rAAV5-CMV-*sflt-1* vector. (B) rAAV-CMV-*lacZ* vector. ITR, inverted terminal repeat of AAV serotype 5; CMV, human cytomegalovirus promoter; GH, human growth hormone first intron enhancer; Poly (A), SV40 early poly A.

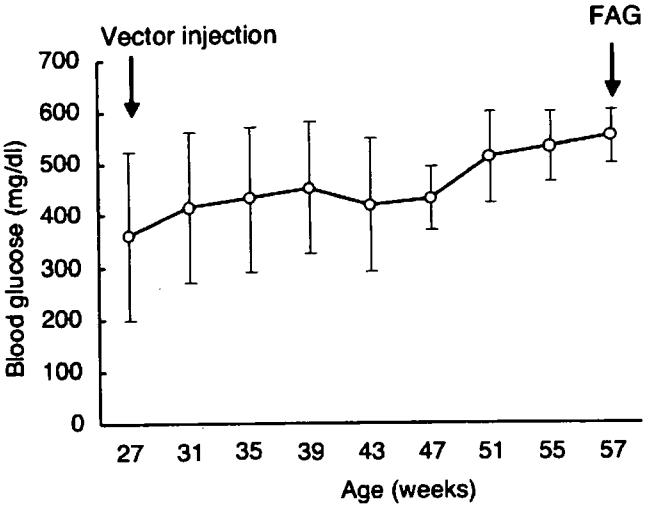

Figure 2. Blood glucose levels of animals during the study. All rats developed diabetes mellitus by the time of subretinal vector administration, and high glucose levels continued throughout the study. Data were shown as mean ± SD, (n=8). FAG, fluorescein angiography.

(Bausch & Lomb, Rochester, NY), mounted on a 10-μl Hamilton syringe, was inserted into the subretinal space through the sclera and ~10 μl of viral suspension was injected. Treated eyes received rAAV5-CMV-*sflt-1* ($4x10^{10}$ vector genome/eye) plus rAAV5 expressing ß-galactosidase (rAAV5-CMV-*lacZ*, $1x10^{10}$ vector genome/eye). Control eyes received only rAAV-CMV-*lacZ* ($1x10^{10}$ viral genome/eye).

Fluorescein-dextran microscopy and quantification of DR. Thirty weeks after the vector administration, the progression of DR was evaluated using fluorescein angiography (FAG). Cardiac perfusion was performed with 1 ml of PBS containing 50 mg of fluorescein-labeled dextran (fluorescein isothiocyanate-dextran; MW, $2x10^6$ daltons; Sigma, St Louis, MO), after administration of a lethal dose of pentobarbital sodium. The eyes were enucleated, the cornea and lens were removed, and the retina dissected from the eyecup. The retina was cut radially and flat-mounted on a glass slide without fixation. A drop of aqueous mounting medium (Crystal/mount, Biomeda

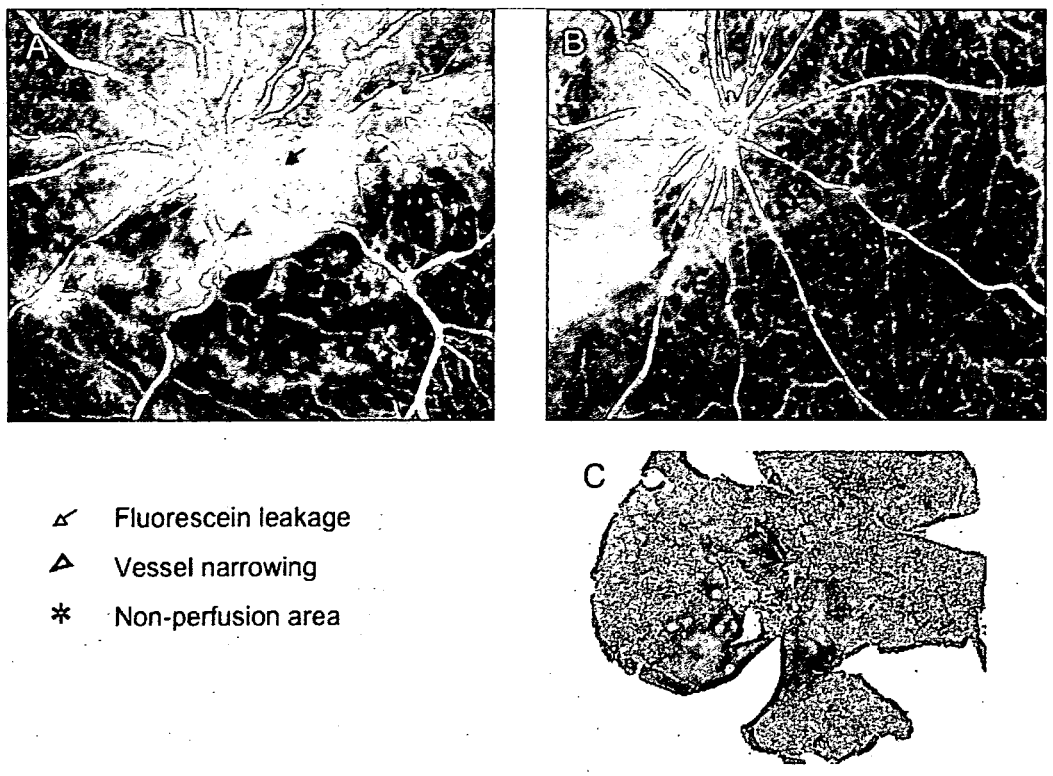

Figure 3. Fluorescein microangiography (FAG) of the rats 30 weeks after vector administration. (A) FAG from AAV5-CMV-*lacZ* injected rats. (B) FAG from rAAV5-CMV-*sflt-1* plus rAAV5-CMV-*lacZ* injected rats. The leakage from the fluorescein spot and avascular area are less extensive in B than in A, thus indicating that the progression of diabetic retinopathy is less marked in the rats treated with rAAV5-CMV-*sflt-1*. (C) A typical X-gal staining of the rat retina showing the distribution of the transduced tissue after subretinal injection of the rAAV-CMV-*lacZ* vector.

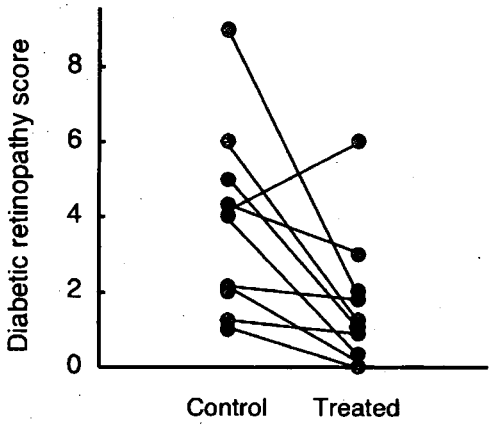

Figure 4. Diabetic retinopathy score of the rats evaluated at the end of the study. The Wilcoxon signed-ranks test demonstrates that the scores for the treated eyes are significantly less than those for the control eyes (n=8, $p<0.05$).

Corp, Foster City, CA) was applied over the retina and allowed to dry. Then the whole flat-mounted retina was examined by fluorescent microscopy (Nikon Labphoto, Nikon, Tokyo, Japan). Without providing information on the vectors injected, the status of DR was determined using the following three parameters: the presence of an avascular area, extensive hyperfluorescence, and arterial narrowing. Each parameter was scored from 0 (none) to 3 (severe) based upon the findings of FAG. The score for each eye was compared and analyzed using the Wilcoxon signed-ranks test. Rats that did not show DR in either of the eyes were excluded from the study. To confirm the subretinal injection of vector solution, X-gal staining of the eye was performed after FAG.

Results

Effect of sFlt-1 in vitro. To prove the biological activity of the protein produced from the *sflt-1* cDNA *in vitro*, we incubated HUVEC with different dilutions of media from 293 cells transfected with the plasmid. The conditioned media from transfected cells inhibited proliferation of HUVEC in a dose-dependent manner, whereas media from untransfected cells had no effect on HUVEC proliferation (data not shown).

Development of DM in SDT rats. All rats developed DM by 35 weeks of age and high blood glucose levels continued throughout the study (Fig. 2). At the end of the study, HbA1c levels in all rats were high (9.4±0.95%; means ± SD), and plasma sFlt-1 was not detected. No adverse effects of sFlt-1 gene therapy were observed throughout the study.

Evaluation of efficacy of gene transfer into the retina. Thirty weeks after the vector administration, FAG was performed to determine the progression of DR. DR was diagnosed using three parameters, arterial narrowing, pooling of fluorescein and a non-perfusion area, and the severity of these parameters were evaluated. The scores of DR in the treated eyes were significantly less than those in the control eyes (Fig. 3A and B; Fig. 4). X-gal staining demonstrated that the LacZ protein was produced in the retinal tissue after transduction with the

rAAV5 vector, and the transgene expression persisted for over 30 weeks after vector administration (Fig. 3C).

Discussion

VEGF is supposedly one of the most essential factors in retinal neovascularization during DR progression. The inhibition of retinal neovascularization by *sflt-1* gene transfer in animal models has been demonstrated in earlier reports (30,31). However, since the mechanisms underlying neovascularization in these models were not related to hyperglycemia, the effectiveness of sFlt-1 in inhibiting DR had not been estimated. To shed light on this issue, a more clinically relevant model has long been awaited. The recently developed SDT rat model is a candidate for this purpose. This model is unique because its diabetic status mimics human NIDDM rather than IDDM. The animals can live for 1 year after the onset of DM without insulin, with a gradual maturation of DR. Therefore, this model is a valuable tool because it can reflect mid- to late-stage human DR associated with NIDDM (32,33). However, this model has certain drawbacks as well. First, the disease progression is much slower than that in other 'conventional' animal models, and DR can be observed mainly after 55 weeks of age. Therefore, one series of experiments requires a long time period. Second, these animals are prone to death, probably due to the complications of DM. Unfortunately, the number of animals decreases before they develop sufficient disease severity. Therefore, to ensure valid results, the sample size of each group needs to be sufficiently large.

This study aimed to demonstrate the efficacy of gene therapy in preventing DR disease progression. For this purpose, we injected the vector soon after the onset of DM, and the efficacy was evaluated at the age of full-blown DR. Considering that a preventive action was significant in this study, a more precise examination of whether short-term *sflt-1* expression is sufficient to prevent DR progression or has an effect in reversing the DR status should be considered for future study. A more difficult task includes determining effective methods to develop this strategy into a clinically realized therapy. Generally, if the therapeutic efficacy is proven in small animals, additional experiments need to be performed on larger animals prior to conducting clinical trials. Regarding DR, no appropriate model has been found in species of large animals. Proliferative DR-like changes were observed in a galactose-fed dog model (44). Nevertheless, this model requires up to 7 years to establish mature DR, which is impractical in a preclinical study. Development of novel large animal models for this purpose is ideal but not practical due to the uncertainty of success in establishing such models during a defined time range. Resolving this problem may not be easy; however, we believe that before clinical trials are considered, further studies using large animals are essential.

In this study, the area of transgene expression was sufficiently wide to protect vision, and the expression continued for over 30 weeks after the injection, indicating that rAAV-mediated ocular gene transfer via a single injection of vector solution could lead to a long-term therapeutic effect. The area of *sflt-1* expression should be comparable to that of X-gal staining, although human sFlt-1 in the retina was undetectable by immunohistochemistry. This may have occurred probably due to technical difficulties in localizing the soluble antigen (31). Therefore, ocular gene transfer under the present experimental conditions is a practical approach. Nonetheless, DR progression was suppressed partially and not completely. At present, it is unclear whether the incomplete suppression was due to the residual actions of VEGF or the uninhibited activity of an alternative angiogenic factor (45). Regarding the latter, a combination of transgenes that act on different aspects of angiogenesis may increase the efficacy of gene therapy for DR prevention.

In patients with DM, VEGF is closely involved in the degree of complication. Elevated VEGF levels in the retina may worsen the DR status and cause visual loss (11,12), while high systemic VEGF levels induce neovascularization, improving ischemic conditions. If circulating sFlt-1 levels affect systemic VEGF levels, DM patients may develop ischemic heart disease, diabetic neuropathy, and diabetic gangrene. To avoid these adverse effects, local sFlt-1 delivery and VEGF inhibition is necessary. In this study, plasma sFlt-1 levels were not elevated after subretinal vector administration, and no adverse effects of *sflt-1* gene transfer were observed. Therefore, the subretinal administration of a vector solution and neutralization of the VEGF activity *in situ* appear to be appropriate measures that should be adopted to achieve our goal.

In conclusion, we demonstrated the successful prevention of DR in SDT rats by using an rAAV vector-encoding *sflt-1* gene. These findings strongly suggest the efficacy of sFlt-1 for DR and the usefulness of rAAV5 for ocular gene transfer. Further studies are necessary to develop and optimize ocular gene therapy for human DR.

Acknowledgements

We thank the Association for the Spontaneously Diabetic Torii Rat for providing the SDT rats. We also thank J.A. Chiorini for providing the systems to produce the AAV5-based vectors. This study was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan; the 'High-Technology Research Center' Project for Private Universities: a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, 2003-2007; and the 21st Century Centers of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology.

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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

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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.
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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 = $(\text{short diameter})^2 \times (\text{long diameter}) \times 0.5$.[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).

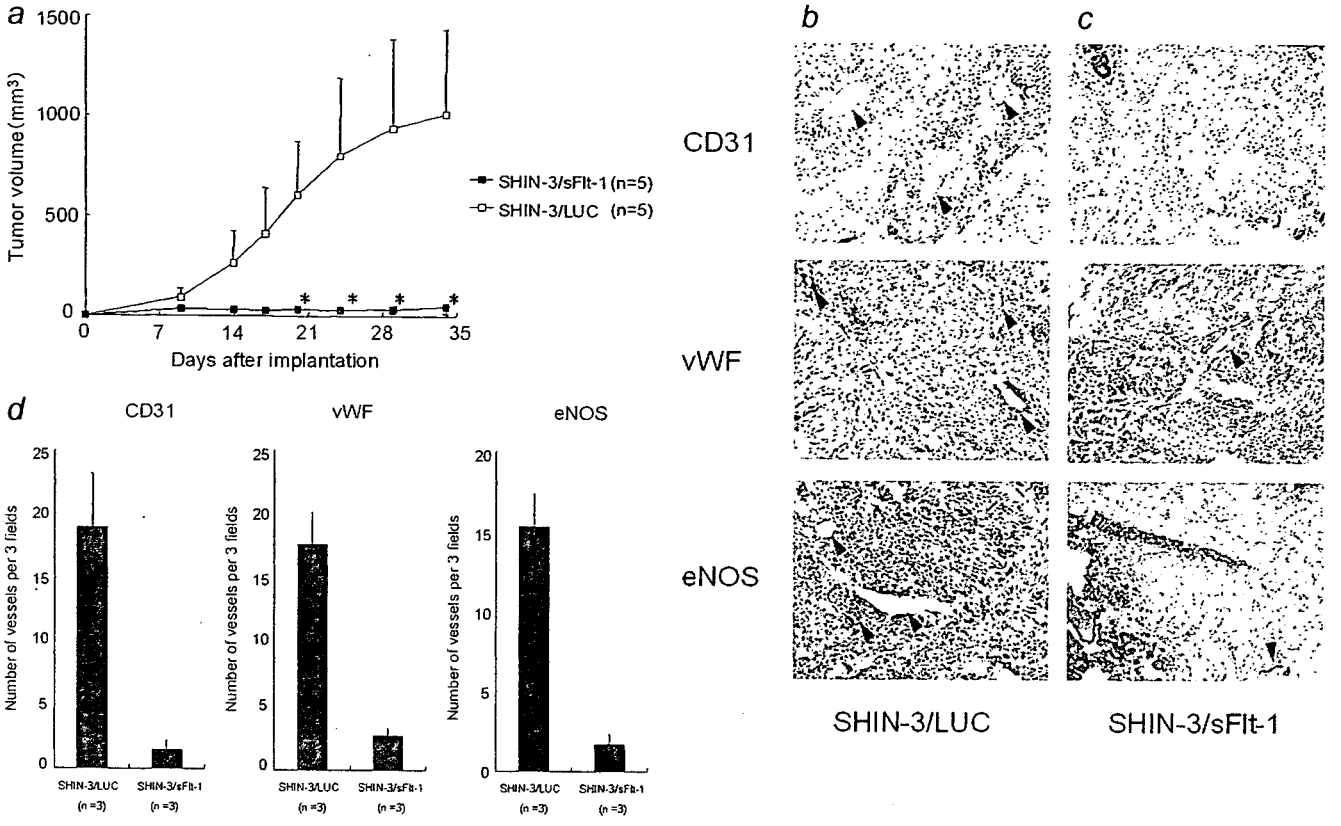

FIGURE 1 – (*a*) *In vivo* tumor growth of sFlt-1-expressing SHIN-3 cells. Tumor cells were subcutaneously injected into the back of mice, and the tumor size was measured every 3 days. The tumor size of SHIN-3/sFlt-1 (■) was significantly smaller than that of SHIN-3/LUC (□, $p < 0.01$ (*)). The tumor 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

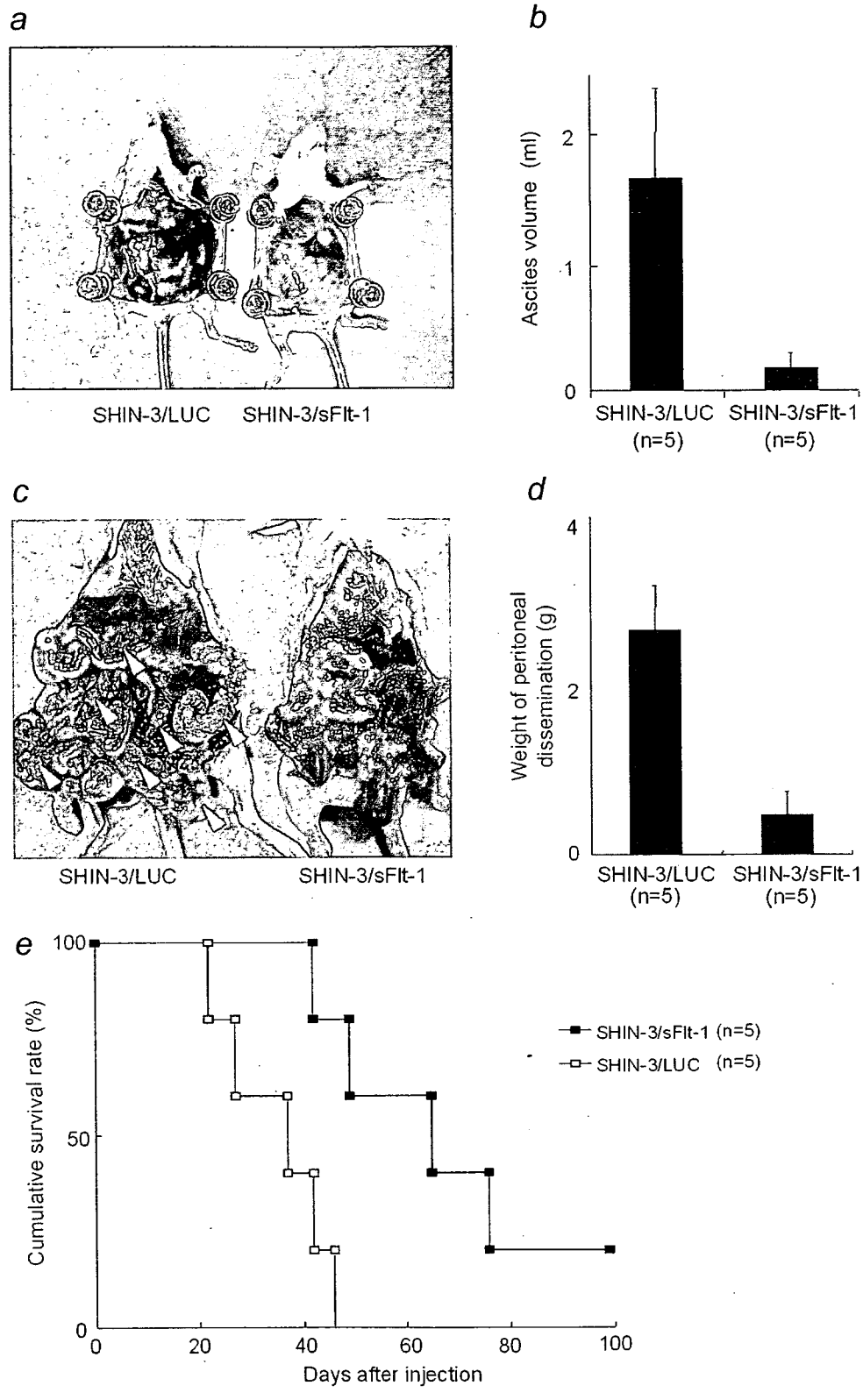

FIGURE 2 – (*a* and *b*) Ascites fluid accumulation on the 23rd day after the intraperitoneal inoculation of cancer cells. Large amounts of bloody ascitic fluid were observed in the SHIN-3/LUC group, whereas in the SHIN-3/sFlt-1 group, the accumulation of ascitic fluid was significantly suppressed (1.67 ± 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.

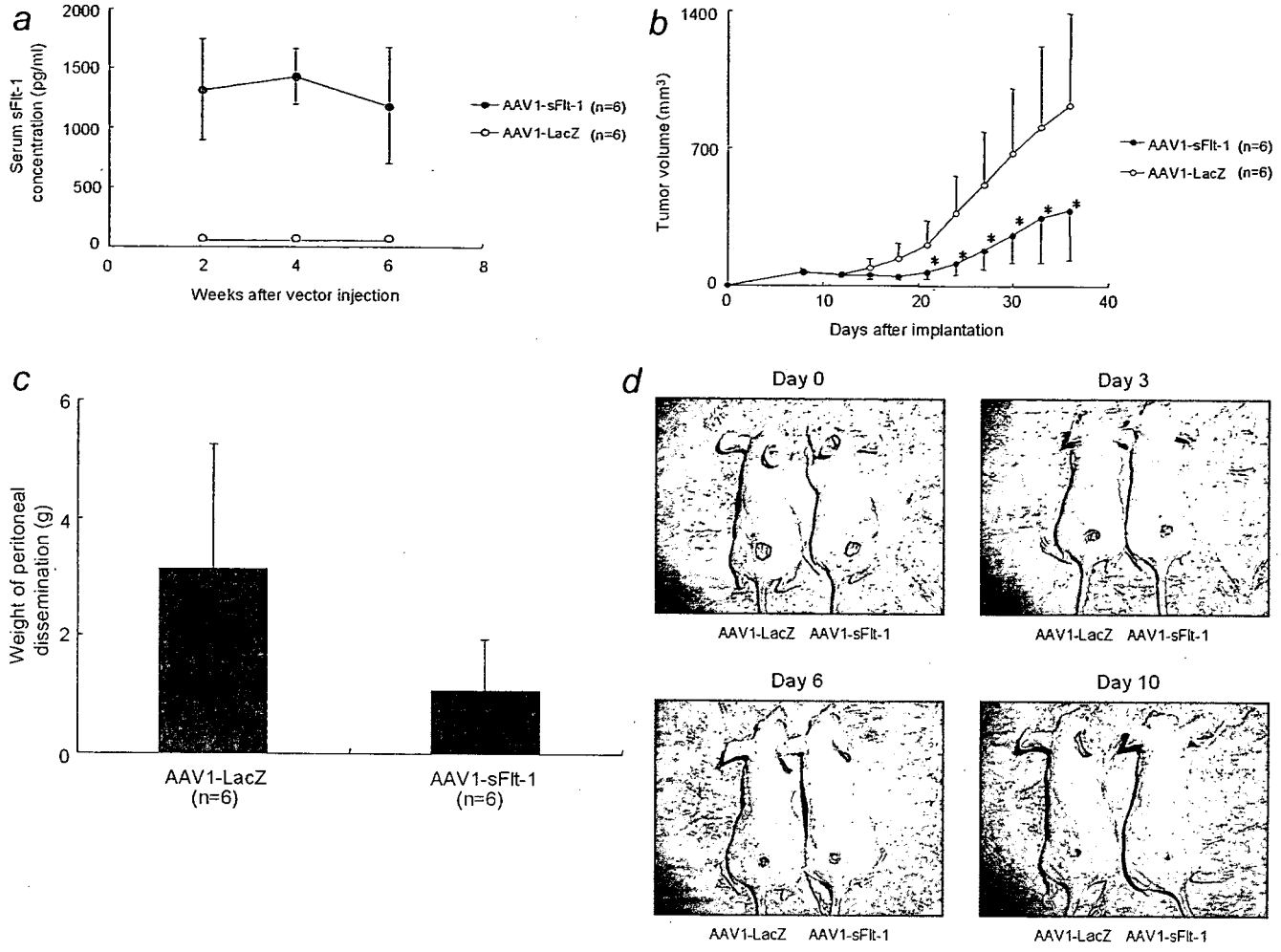

FIGURE 3 – (*a*) Serum sFlt-1 concentrations. In the mice that were intramuscularly injected with AAV1-sFlt-1, the serum sFlt-1 concentration was higher than 1,000 pg/ml. In contrast, in the mice that were intramuscularly injected with AAV1-LacZ, the serum sFlt-1 concentration was below the detection 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/μl)	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/μl)	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.

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Tendon Healing In Vitro: Adeno-Associated Virus-2 Effectively Transduces Intrasynovial Tenocytes with Persistent Expression of the Transgene, but Other Serotypes Do Not

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Background: Transfer of exogenous growth factor genes to injured tendons offers a promising method for strengthening tendon repairs. Adeno-associated virus vectors have advantages of being both nonpathogenic and nontoxic. The authors explored the efficiency of transduction of intrasynovial tenocytes with different serotypes of adeno-associated virus (AAV) and the persistency of its expression of a growth factor transgene.

Methods: Tenocytes were obtained from cultures of rat intrasynovial tendons and distributed to 82 wells in eight culture plates and to 30 culture dishes. The tenocytes in the wells were treated with AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, and AAV8 vectors containing the *lacZ* gene, and plasmid vectors (pCMVβ-lacZ). The tenocytes were stained with in situ β-galactosidase 5 days later. The basic fibroblast growth factor (*bFGF*) gene was cloned to the AAV2 vector to construct the AAV2-bFGF vector, which transduced tenocytes in culture dishes. Expression of the transgene was measured over 3 weeks and analyzed statistically.

Results: AAV2 effectively delivered exogenous genes to proliferating intrasynovial tenocytes. In contrast, other tested adeno-associated viruses transduced tenocytes minimally or not at all. The efficiency of gene transfer by AAV2, indicated by the percentage of cells with positive β-galactosidase staining, was significantly greater than that by a plasmid vector ($p = 0.001$). Expression of the *bFGF* gene in tenocytes transduced with the AAV2-bFGF was significantly higher than that in the control over the 3-week period ($p < 0.01$).

Conclusions: Gene transfer to tenocytes by AAV2 is more efficient than that by a plasmid vector. However, other adeno-associated virus serotypes cannot effectively transduce tenocytes. The *bFGF* gene can be delivered to intrasynovial tenocytes by the AAV2 vector effectively, and the gene transfer significantly increases expression of bFGF gene over 3 weeks. (*Plast. Reconstr. Surg.* 119: 227, 2007.)

Increasing the strength of tendon healing to reduce repair rupture and to facilitate a more aggressive motion regimen has been a central goal of hand surgeons over recent decades.[1–5] Delivery of exogenous genes to lacerated intrasynovial flexor tendons offers a new approach for enhancing tendon healing, but methods of gene delivery have rarely been explored.[6–9] Exogenous genes have been transferred to the tenocytes through adenoviral or plasmid vectors in previous investigations.[6–9] Lou et al. reported transfer of the *Escherichia coli lacZ* gene, a focal adhesion kinase gene, and bone morphological protein-12 gene to chicken tendons using adenoviral vectors.[6–8] Wang et al. reported transfer of platelet-derived growth factor-B (*PDGF-B*) gene to tenocytes using plasmid vectors.[9] Transfer of the *PDGF-B* gene has effectively promoted the expression of type I collagen gene and increased the overall expression of the target gene in the tenocytes.[9] There is evidence that gene therapy could improve healing of lacerated intrasynovial tendons. However, adenoviral vectors are immunogenic and produce strong adverse tissue reactions,[10,11] and the liposome that aids in gene

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Received for publication March 11, 2005; accepted June 1, 2005.

DOI: 10.1097/01.prs.0000244861.57040.3f

transfers to target cells may cause some adverse tissue reactions or damage.[12,13] Over the past several years, adeno-associated virus vectors have been used increasingly as vehicles that deliver genes to cells or tissues.[14,15] They are nonpathogenic and nontoxic, unlike the two methods mentioned above.[10,11] However, transfer of a growth factor gene through adeno-associated virus vectors to intrasynovial tenocytes has not yet been attempted. Furthermore, because serotypes of adeno-associated viruses vary in their preferences for target cells, their effectiveness as carriers of exogenous genes to tenocytes remains unknown. As an essential step in exploring this promising vector system to deliver genes of interest to healing tendons, we investigated the efficiency of gene transfer to tenocytes with different serotypes of adeno-associated viruses and persistency of expression of a growth factor [basic fibroblast growth factor (*bFGF*)] gene over a critical period of healing in an in vitro model of proliferating tenocytes. We also compared the efficiency of gene transfer by adeno-associated virus vectors to that by plasmid vectors, a previously documented method of gene delivery to tenocytes.[9]

MATERIALS AND METHODS

Flexor digitorum profundus tendons from six adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were used for this study. Animal experimentation complied with the guidelines of the institutional animal care and use committee. The rats, weighing 250 to 300 g each, were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg). Both front feet were sterilized, and the flexor digitorum profundus tendon equivalents located deeper to the flexor digitorum superficialis tendon equivalents were identified in the middle digits. Under an operating microscope, a tendon segment 1 cm long was harvested with a surgical blade at the level of the proximal phalanx, which corresponds to the tendon in zone II of human hands.

Culture of Tendon Explants and Tenocytes

The tendon segments were cut into pieces 5 mm long and placed in six 60-mm culture dishes for explant culture in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO) and penicillin (100 U/ml) /streptomycin (100 μg/ml). The culture dishes were placed in a humidified, 37°C, 5% carbon dioxide incubator. Culture for approximately 10 to 14 days allowed the migration of sufficient tenocytes out of the explants and proliferation to approximate monolayer confluence. The explants were removed and the tenocytes on the dishes were passaged to eight 100-mm culture dishes and grown to confluence. The tenocytes were then seeded onto thirty 100-mm culture dishes at a seeding density of 1×10^6 and 82 wells in eight 24-well plates at a density of 5×10^4.

Construction of the Recombinant AAV2-bFGF Gene Transfer Unit

The AAV2-bFGF gene transfer unit was constructed with a series of genetic cloning followed by production of infectious AAV2-bFGF viral particles. Adeno-associated viruses are single-stranded DNA viruses, and their viral genome consists of *rep* genes (encoding regulatory protein) and *cap* genes (encoding the capsid proteins). The genome is flanked by inverted terminal repeats that function as viral origins of replication. AAV2-bFGF and AAV2-lacZ were constructed for this study. The AAV2-bFGF contained the rat *bFGF* gene (GenBank accession no. X07285) and harbors a nuclear localization signal under the regulation of the cytomegalovirus (CMV) immediate early promoter.[16] The rat *bFGF* gene was preserved in nonexpressing status in a plasmid vector. A segment containing the *bFGF* gene was cut through EcoR1 (sequences, GAATTC) and Xho1 (sequences, CTCGAG) restriction sites and was cloned to the commercially available pBluescript II KS vector (Stratagene, La Jolla, Calif.) at EcoR1 and Xho1 restriction sites to lengthen the segment because the ideal length of the inserted expression cassette in the AAV2 vector is between 1 and 3 kb. Subsequently, a 2.7-kb fragment containing the *bFGF* gene sequences was cut from the pBluescript II KS vector and ligated to the multiple cloning site (MCS) of a pAAV2-MCS vector (Stratagene) using the LigaFast Rapid DNA Ligation System (M8221; Promega Corp., Madison, Wis.) (Fig. 1).

Plasmid AAV2-bFGF was obtained from the above preparation and then co-transfected into a cell line (293 cells, human embryonic kidney cells) with a helper plasmid (pHelper vector) and a pAAV-RC vector. The latter carries the subtype 2 adeno-associated virus *rep* and *cap* genes but lacking the adeno-associated virus inverted terminal repeats (Fig. 1). After 72 hours, the cells were harvested and lysed in Tris HCl buffer through cycles of freezing and thawing, sucrose precipitation, and cesium chloride density-gradient ultra-

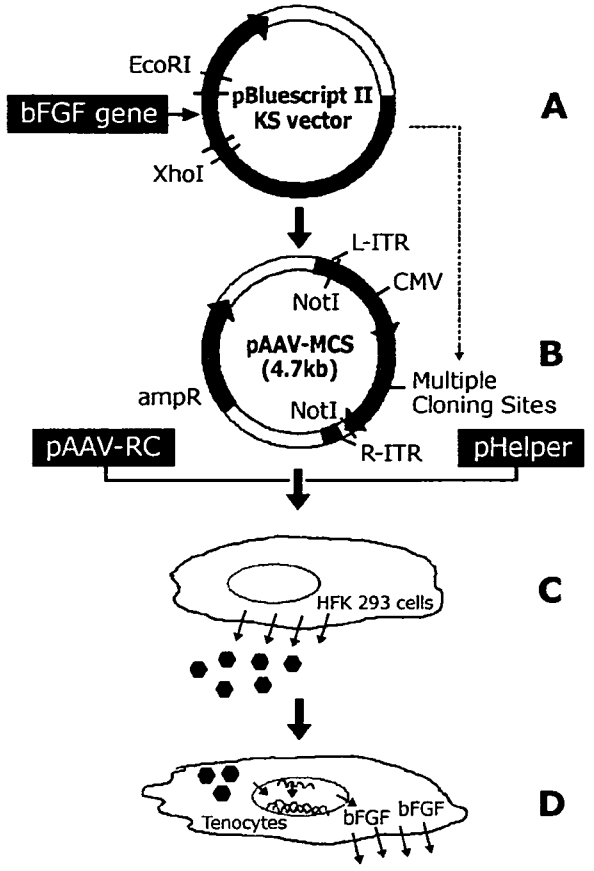

Fig. 1. Method of preparation of AAV2-bFGF vectors for in vitro transduction of tenocytes. *ampR*, ampicillin resistance gene. The CMV promoter gene locates upstream of the inserted *bFGF* transgene and promotes expression of the *bFGF* gene.

centrifugation to isolate AAV2-bFGF. The particle titer of the AAV2-bFGF was determined by real-time polymerase chain reaction (PRISM 7000 Sequence Detection System; Applied Biosystems, Foster City, Calif.).

Delivery of Exogenous Genes to Tenocytes through Different Adeno-Associated Virus Vectors

Gene transfer through AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, and AAV8

We used proliferating tenocytes in 82 culture wells for this study section. Tenocytes in 70 wells were co-cultured with adeno-associated viruses of different serotypes harboring lacZ genes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, and AAV8. On the day after cell plating, the cells reach approximately 50 to 70 percent confluence. The viruses were added to the culture in 70 wells at multiplicities of infection of 1, 10, 20, 50, and 100 in 200 μl of serum-free Dulbecco's modified Eagle's medium.[14-16] Each concentration of viral particles for respective adeno-associated virus vectors was added to two wells. Three hours later, the infection was terminated and 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was added. The plates were placed back into incubator and the cells were cultured for 5 days.

The culture was then terminated and the cells were stained with the reagents in an in situ β-galactosidase (X-gal) staining kit (Stratagene). After removal of culture medium, 0.5 ml of 1× fixing solution was added to each well and the plates were incubated at room temperature for 10 minutes. The fixing solution was then removed and the cells were gently washed twice with 0.5 ml of 1× phosphate-buffered saline. Finally, 0.25 ml of freshly prepared 1× staining solution was added to each well. The culture plates were placed in a humidified incubator at 37°C overnight, and the stained cells in four randomly selected fields in each well were counted under an inverted microscope.

Gene Transfer through a Plasmid Vector

The plasmid vector pCMVβ-lacZ was used to transfect the cells in an additional six wells. The cells in the remaining six wells served as the non-treatment control. We added 0.4 μg of pCMVβ-lacZ, 1.6 μg of lipofectin, and 300 μl of serum-free Dulbecco's modified Eagle's medium to each well. Treatment of tenocytes with this concentration of plasmid vectors transfected the tenocytes and increase the expression of transgene in our previous study.[9] The tenocytes were cultured for 14 hours in a humidified, 37°C, 5% carbon dioxide incubator. The medium was removed and 10% Dulbecco's modified Eagle's medium was added to wells. The culture was terminated at 5 days and the cells were stained with an in situ β-galactosidase staining kit (Stratagene) with the methods identical to those for the cells infected with AAV-lacZ.

Delivery of the *bFGF* Gene to Proliferating Tenocytes through AAV2 Vectors

Gene Transfer through AAV2 Vectors

We used tenocytes cultured in 30 dishes in this part of the study. We delivered the *bFGF* genes to tenocytes in 15 dishes when cultured tenocytes reached 50 to 70 percent confluence. The AAV2-bFGF was added at multiplicities of infection of 100 for 3 hours. Serum-free Dulbecco's modified Eagle's medium was added to the wells after termination of gene transfer. The tenocytes in the

other 15 dishes served as controls. The medium was changed every 2 days. Five experimental dishes and five control dishes were designated to observation periods of 1, 2, and 3 weeks, respectively. The cells used for a 2-week period were passaged once and those for a 3-week period were passaged twice to maintain proliferating status of cultured tenocytes.

Assessment of Increases in Expression of the *bFGF* Gene and Duration

The cells were homogenized on ice and total RNA was isolated by chloroform extraction and isopropanol precipitation, washed in ethanol, and resuspended in diethyl pyrocarbonate (DEPC) water. Reverse transcription reaction to transcript mRNA into cDNA was performed with reagents of the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, Calif.). For each sample, 1 μl of Oligo dT, 1 μl of dNTP mix, and 3 μg of total RNA were added and diluted with DEPC water to a total of 10 μl per tube. The tube was heated at 65°C for 5 minutes and iced. A reaction mixture containing 2 μl of 10× reverse-transcriptase buffer, 4 μl of 25 mM magnesium chloride, 2 μl of 0.1 M DTT, and 1 μl of RNase OUT were added to each tube. After adding 1 μl of Superscript II RT and reaction at 42°C for 50 minutes, the transcription reaction was inactivated by heating at 70°C for 15 minutes. The *E. coli* RNase was added before termination.

The oligonucleotide primers were designed to detect the expression of the *bFGF* gene in the transduced tenocytes and the nontreatment control by amplifying a segment in the *bFGF* gene measuring 207 base pairs long. The primers are 5′-TTGTGTCCATCAAGGGAGTG-3′ (forward) and 5′-CCGTTTTGGATCCGAGTTTA-3′ (reverse) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Because the transferred *bFGF* gene was from rats, the detected *bFGF* gene expression is that of both the transgene and the *bFGF* gene from the host genome. The polymerase chain reaction was performed with a GeneAmp Gold PCR Reagent Kit (Applied Biosystems) at 94°C for 5 minutes, 35 cycles of 94°C for 15 seconds (template denaturation), 56°C for 30 seconds (annealing), 72°C for 30 seconds (primer extension), followed by 72°C for 7 minutes. Multiplex polymerase chain reaction was performed using the glyceraldehyde-3′-phosphate dehydrogenase (*GAPDH*) gene as an internal control to standardize comparison.[9,17] Primers for amplifying a *GAPDH* gene segment of 346 base pairs were as follows: 5′-CGTGGAGTCTACTGGCGTCT-3′ (forward) and 5′-GGATGCAGGGATGATGTTCT-3′ (reverse). The primers for the *GAPDH* gene were added to the reaction at the eleventh cycle. Products of polymerase chain reaction were on 1% agarose gel electrophoresis, recorded on an ultraviolet illuminator, and sequenced.

Statistical Analysis

Expression of the *bFGF* gene over time was expressed as the densitometric volume of the band in contrast to that of the *GAPDH* gene. The cells positively stained with X-gal were counted in each observation field under the microscope. Cells in four random fields in each well were counted. The percentage of positively stained cells in these fields was calculated and expressed as mean ± SD. The differences in transduction rate were statistically analyzed by one-way analysis of variance. When the analysis of variance indicated significance, the Newman-Keuls test was used as the post hoc test. The unpaired t test was used to detect the difference in the data of gene expression at each time period. A value of $p < 0.05$ was considered significant.

RESULTS

Efficiency of Gene Transduction of Proliferating Tenocytes by AAV2 Vector

X-gal staining of lacZ-transduced cells showed blue nuclei. The transduction rate is the percentage of X-gal positively stained cells in the entire cell population. The lacZ-positive cells were 8.1 ± 2.6 percent in the cultured tenocytes transduced by AAV2-lacZ, 4.3 ± 1.9 percent in the tenocytes transfected by pCMVβ-lacZ, and none in the control tenocytes (Fig. 2). The efficiency of gene transfer was 2-fold higher with the AAV2 vector than with the plasmid vector. The increase in the *LacZ*-positive cells with the AAV2 vector was statistically significant compared with that with the plasmid vector ($p < 0.001$).

Transduction Rates of other Serotypes of Adeno-Associated Viruses in Tenocytes

We further tested the transduction rate of AAV2 and other serotypes (AAV1, AAV3, AAV4, AAV5, AAV7, and AAV8) of adeno-associated virus vectors and at different concentrations of viral particles (multiplicities of infection, 1, 10, 20, 50, and 100). There were almost no tenocytes positively stained with X-gal when the adeno-associated viruses were at concentrations ranging from

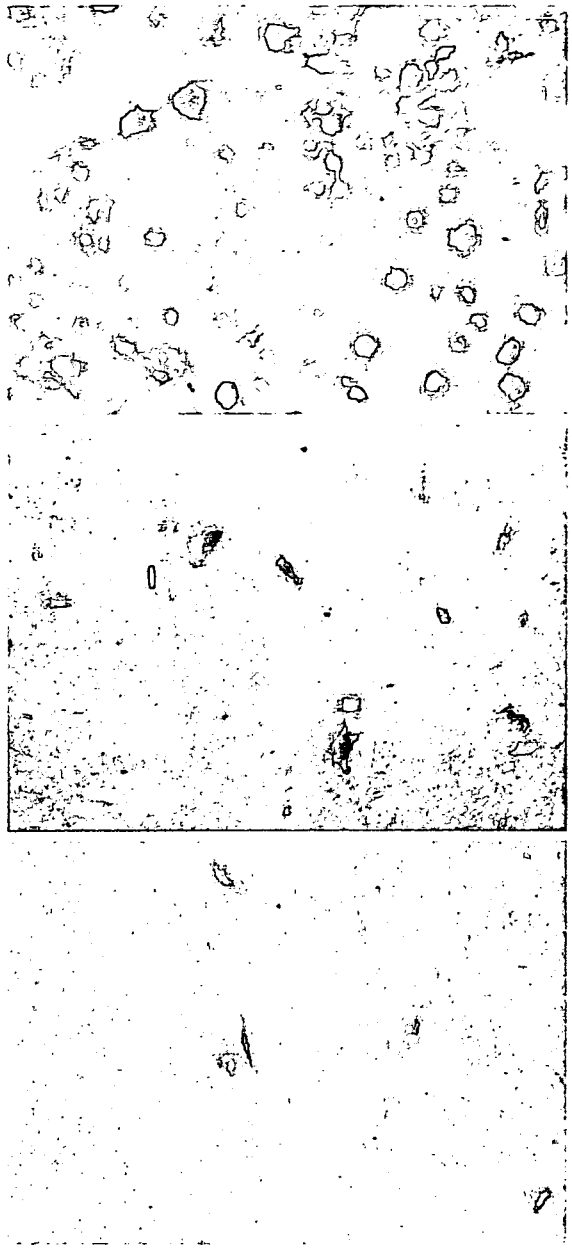

Fig. 2. X-gal staining of tenocytes transduced with AAV2-lacZ (*center*) and transfected with pCMVβ-lacZ (*below*). (*Above*) The staining of cells (Cos 7) known to highly permissive to AAV2 was used as a positive control. AAV2 could transduce tenocytes more effectively than the plasmid vector, but the transduction rate of tenocytes by AAV2 was not as high as that of highly permissive cells (original magnification, ×400). (*Above*) Highly permissive cells (positive control). (*Center*) Tenocytes transduced by AAV2. (*Below*) Tenocytes transfected by plasmids.

1 to 20. When concentrations were 50, positively stained cells could be observed only in the tenocytes transduced with AAV2. At multiplicities of infection of 100, the AAV2 transduced the tenocytes at the highest transduction rates among the tested virus serotypes (Fig. 3).

Expression of the *bFGF* Gene in AAV2-bFGF–Transduced Tenocytes

The expression of the *bFGF* gene demonstrated in reverse-transcriptase polymerase chain reaction was elevated significantly at each time point of the observation period compared with the gene expression in the control ($p < 0.01$, at all observation periods). The percentage increase of the levels of expression of the *bFGF* gene was 138, 153, and 146 percent at 1, 2, and 3 weeks, respectively, after gene therapy (Figs. 4 and 5).

DISCUSSION

Characterizing the effects of direct application of growth factors to tenocytes has been the focus of investigations for years.[18-21] Transfer of exogenous growth factor genes to intrasynovial tenocytes has been attempted only recently.[9] However, the most appropriate method of gene delivery to tenocytes has been unclear. Among viral vectors, adeno-associated virus vectors appear to be good candidates for gene therapy strategies aimed at intrasynovial tendon healing. They are free of viral coding sequences; thus, transduced cells will not synthesize any viral proteins.[11,22], and their vectors cause minimal tissue reactions (or none at all),[11,14,22] in contrast to adenoviral vectors, which cause dramatic adverse tissue reactions.[10,11] In this study, we used adeno-associated virus vectors for gene delivery to intrasynovial tendon cells. We found a significant increase in expression of the *bFGF* gene of the cultured tenocytes treated with AAV2-bFGF compared with the tenocytes without AAV2-bFGF treatment. The efficiency of gene transfer by AAV2 was twice that of a plasmid vector. The level of expression of the *bFGF* gene was elevated through the 3-week observation period after transfer of the *bFGF* gene.

Previous investigators showed that supplementation of bFGF to cultured tenocytes increased the proliferation rate and promoted expression of both the type I collagen gene and the nuclear transcription factor-kappa B gene in signaling pathways.[18,20] Basic fibroblast growth factor is a potent stimulator of proliferation and matrix synthesis of the tendons.[18,20] Our current study shows that AAV2 is efficient for delivery of growth factor genes to tenocytes. Coupled with its known characteristics of being nonpathogenic and eliciting a minimal or no immune response,[22] AAV2 appears

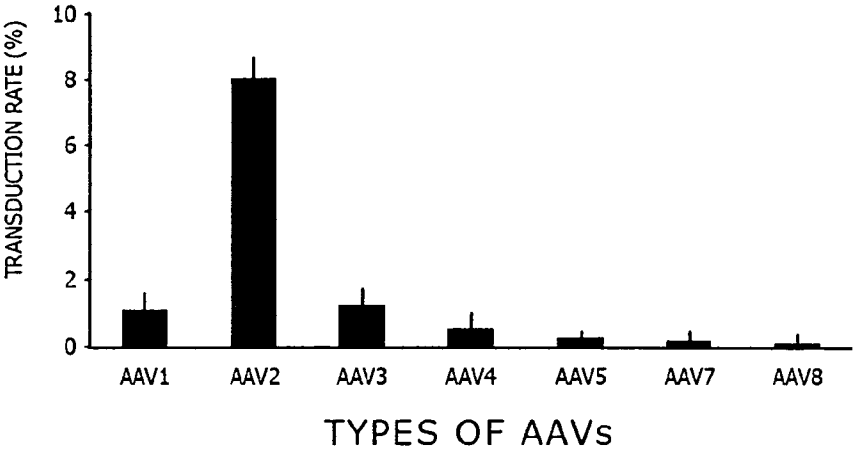

Fig. 3. Transduction rates of different serotypes of adeno-associated viruses at the highest concentration (multiplicities of infection, 100) at 5 days after transduction. For each viral serotype, four randomly selected fields from each of the two wells were observed. Each observation field under the 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