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

JUNICHI IDENO[1,2], HIROAKI MIZUKAMI[1], AKIHIRO KAKEHASHI[3], YUKA SAITO[3],
TAKASHI OKADA[1], MASASHI URABE[1], AKIHIRO KUME[1], MASATOSHI KUROKI[4],
MASANOBU KAWAKAMI[4], SHUN ISHIBASHI[2] and KEIYA OZAWA[1]

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, [2]Department of Medicine, Division of Endocrinology
and Metabolism, Jichi Medical University, Tochigi, Japan; [3]Department of Ophthalmology, [4]Department of
Comprehensive Medicine I, Omiya Medical Center, Jichi Medical University, Saitama, Japan

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.

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). Glyco-sylated 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
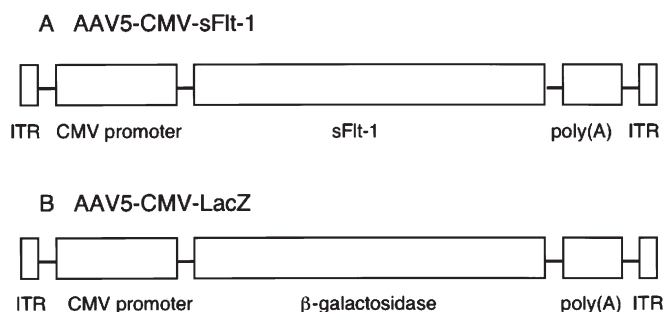

Figure 1. Structure of rAAV vectors. (A) rAAV5-CMV-*sflt-1* vector. (B) rAAV-CMV-*lacZ* vector. ITR, inverted terminal repeat of AAV serotype 5; CMV, human cytomegalovirus promoter; GH, human growth hormone first intron enhancer; Poly (A), SV40 early poly A.
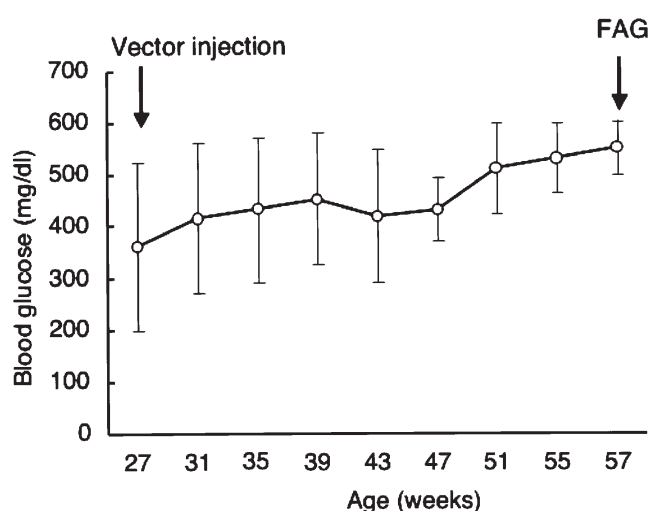

Figure 2. Blood glucose levels of animals during the study. All rats developed diabetes mellitus by the time of subretinal vector administration, and high glucose levels continued throughout the study. Data were shown as mean ± SD, (n=8). FAG, fluorescein angiography.

(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* (4×10^{10} vector genome/eye) plus rAAV5 expressing ß-galactosidase (rAAV5-CMV-*lacZ*, 1×10^{10} vector genome/eye). Control eyes received only rAAV-CMV-*lacZ* (1×10^{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 isothio-cyanate-dextran; MW, 2×10^6 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
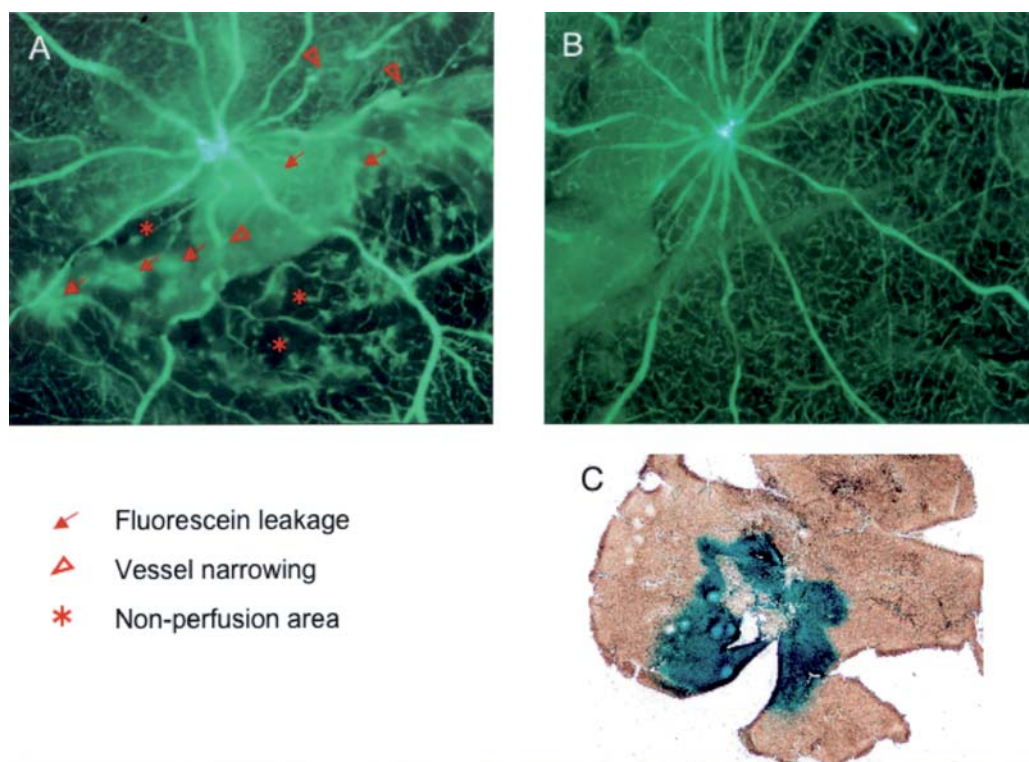
Figure 3. Fluorescein microangiography (FAG) of the rats 30 weeks after vector administration. (A) FAG from AAV5-CMV-*lacZ* injected rats. (B) FAG from rAAV5-CMV-*sflt-1* plus rAAV5-CMV-*lacZ* injected rats. The leakage from the fluorescein spot and avascular area are less extensive in B than in A, thus 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.
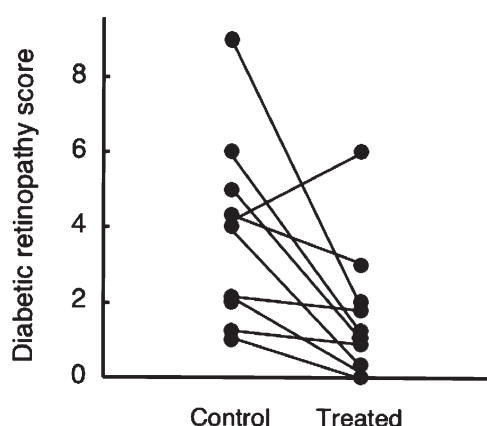

Figure 4. Diabetic retinopathy score of the rats evaluated at the end of the study. The Wilcoxon signed-ranks test demonstrates that the scores for the treated eyes are significantly less than those for the control eyes (n=8, p<0.05).

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.

References

1. Hyman L: Epidemiology of eye disease in the elderly. Eye 1: 330-341, 1987.
2. Klein BE and Klein R: Ocular problems in older Americans with diabetes. Clin Geriatr Med 6: 827-837, 1990.
3. King H and Rewers M: Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO *Ad Hoc* Diabetes Reporting Group. Diabetes Care 16: 157-177, 1993.
4. Hotta N, Nakamura J, Sakakibara F, *et al*: Electroretinogram in sucrose-fed diabetic rats treated with an aldose reductase inhibitor or an anticoagulant. Am J Physiol 273: E965-E971, 1997.
5. Robison WG Jr, Nagata M, Tillis TN, Laver N and Kinoshita JH: Aldose reductase and pericyte-endothelial cell contacts in retina and optic nerve. Invest Ophthalmol Vis Sci 30: 2293-2299, 1989.

6. Aiello LP, Bursell SE, Clermont A, *et al*: Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C *in vivo* and suppressed by an orally effective beta-isoform-selective inhibitor. Diabetes 46: 1473-1480, 1997.

7. Lee TS, MacGregor LC, Fluharty SJ and King GL: Differential regulation of protein kinase C and (Na,K)-adenosine triphosphatase activities by elevated glucose levels in retinal capillary endothelial cells. J Clin Invest 83: 90-94, 1989.

8. Nakamura J, Kato K, Hamada Y, *et al*: A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes 48: 2090-2095, 1999.

9. Hammes HP, Wellensiek B, Kloting I, Sickel E, Bretzel RG and Brownlee M: The relationship of glycaemic level to advanced glycation end-product (AGE) accumulation and retinal pathology in the spontaneous diabetic hamster. Diabetologia 41: 165-170, 1998.

10. Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K and Horiuchi S: The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. Diabetologia 40: 764-769, 1997.

11. Aiello LP: Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. Ophthalmic Res 29: 354-362, 1997.

12. Aiello LP, Avery RL, Arrigg PG, *et al*: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331: 1480-1487, 1994.

13. Ozaki H, Seo MS, Ozaki K, *et al*: Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. Am J Pathol 156: 697-707, 2000.

14. Ferrara N, Houck K, Jakeman L and Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 13: 18-32, 1992.

15. Jakeman LB, Winer J, Bennett GL, Altar CA and Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89: 244-253, 1992.

16. Senger DR, Perruzzi CA, Feder J and Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 46: 5629-5632, 1986.

17. Thieme H, Aiello LP, Takagi H, Ferrara N and King GL: Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. Diabetes 44: 98-103, 1995.

18. Adamis AP, Miller JW, Bernal MT, *et al*: Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118: 445-450, 1994.

19. Boulton M, Gregor Z, McLeod D, *et al*: Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management. Br J Ophthalmol 81: 228-233, 1997.

20. Okamoto N, Tobe T, Hackett SF, *et al*: Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neo-vascularization. Am J Pathol 151: 281-291, 1997.

21. Adamis AP, Shima DT, Tolentino MJ, *et al*: Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 114: 66-71, 1996.

22. Robinson GS, Pierce EA, Rook SL, Foley E, Webb R and Smith LE: Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. Proc Natl Acad Sci USA 93: 4851-4856, 1996.

23. Aiello LP, Pierce EA, Foley ED, *et al*: Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92: 10457-10461, 1995.

24. Goldman CK, Kendall RL, Cabrera G, *et al*: Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc Natl Acad Sci USA 95: 8795-8800, 1998.

25. Hasumi Y, Mizukami H, Urabe M, *et al*: Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. Cancer Res 62: 2019-2023, 2002.

26. Kendall RL and Thomas KA: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90: 10705-10709, 1993.

27. Kong HL, Hecht D, Song W, *et al*: Regional suppression of tumor growth by *in vivo* transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. Hum Gene Ther 9: 823-833, 1998.

28. Shiose S, Sakamoto T, Yoshikawa H, *et al*: Gene transfer of a soluble receptor of VEGF inhibits the growth of experimental eyelid malignant melanoma. Invest Ophthalmol Vis Sci 41: 2395-2403, 2000.

29. Kendall RL, Wang G and Thomas KA: Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 226: 324-328, 1996.

30. Bainbridge JW, Mistry A, De Alwis M, *et al*: Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. Gene Ther 9: 320-326, 2002.

31. Honda M, Sakamoto T, Ishibashi T, Inomata H and Ueno H: Experimental subretinal neovascularization is inhibited by adenovirus-mediated soluble VEGF/flt-1 receptor gene transfection: a role of VEGF and possible treatment for SRN in age-related macular degeneration. Gene Ther 7: 978-985, 2000.

32. Shinohara M, Masuyama T, Shoda T, *et al*: A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. Int J Exp Diabetes Res 1: 89-100, 2000.

33. Kakehashi A, Saito Y, Mori K, *et al*: Characteristics of diabetic retinopathy in SDT rats. Diabetes Metab Res Rev (Epub ahead of print March 30, 2006).

34. Dudus L, Anand V, Acland GM, *et al*: Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. Vision Res 39: 2545-2553, 1999.

35. Martin KR, Klein RL and Quigley HA: Gene delivery to the eye using adeno-associated viral vectors. Methods 28: 267-275, 2002.

36. Auricchio A, Behling KC, Maguire AM, *et al*: Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. Mol Ther 6: 490-494, 2002.

37. Lotery AJ, Yang GS, Mullins RF, *et al*: Adeno-associated virus type 5: transduction efficiency and cell-type specificity in the primate retina. Hum Gene Ther 14: 1663-1671, 2003.

38. Rabinowitz JE, Rolling F, Li C, *et al*: Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 76: 791-801, 2002.

39. Yang GS, Schmidt M, Yan Z, *et al*: Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. J Virol 76: 7651-7660, 2002.

40. Yoshimura I, Mizuguchi Y, Miyajima A, Asano T, Tadakuma T and Hayakawa M: Suppression of lung metastasis of renal cell carcinoma by the intramuscular gene transfer of a soluble form of vascular endothelial growth factor receptor I. J Urol 171: 2467-2470, 2004.

41. Zhang M, Volpert O, Shi YH and Bouck N: Maspin is an angiogenesis inhibitor. Nat Med 6: 196-199, 2000.

42. Hermens WT, ter Brake O, Dijkhuizen PA, *et al*: Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther 10: 1885-1891, 1999.

43. Zolotukhin S, Byrne BJ, Mason E, *et al*: Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6: 973-985, 1999.

44. Kadof PF, Takahashi Y, Wyman M, Ferris F III: Diabetes-like proliferative retinal changes in galactose-fed dogs. Arch Ophthalmol 113: 352-354, 1995.

45. Casey R and Li WW: Factors controlling ocular angiogenesis. Am J Ophthalmol 124: 521-529, 1997.