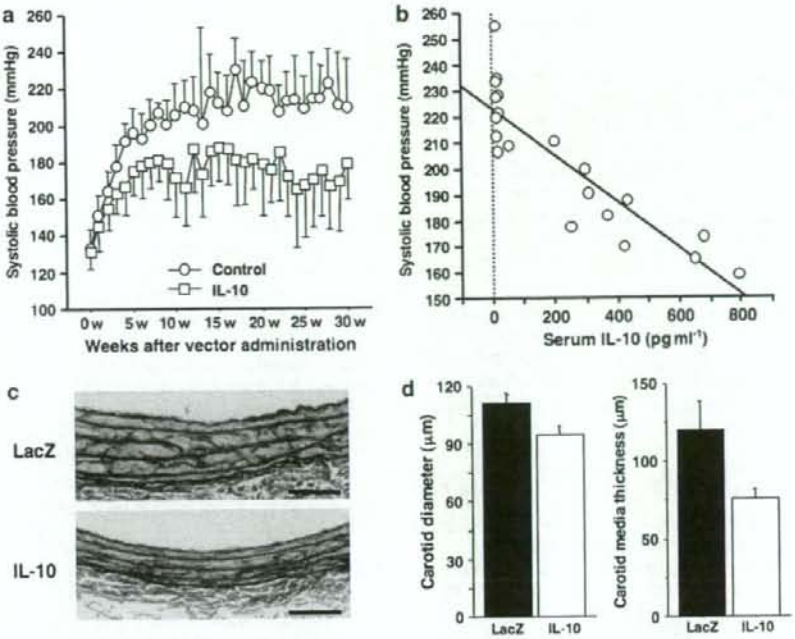

Figure 6 Decrement of blood pressure and morphological changes of carotid artery in the IL-10-transduced SHR-SP. (**a**) Systolic blood pressure of SHR-SP. The transduction protocol is identical to that used in Figure 2. Systolic blood pressure was evaluated by the tail-cuff method. Data are shown as mean ± s.d. (**b**) Correlation between serum IL-10 concentration and blood pressure at 9 weeks after transduction ($n = 20$; $r = 0.882$; $P < 0.0001$). (**c**) Morphological change of the carotid artery after vector administration. Histological changes in the carotid artery of LacZ-transduced group and IL-10-transduced group were evaluated by elastica van Gieson staining at 6 months after gene delivery. Scale bars: 100 μm. (**d**) Quantitative analysis of carotid diameter and media thickness at 6 months after gene delivery. Both the carotid diameter and media thickness were significantly decreased in the IL-10-transduced group than in the LacZ-transduced group ($n = 5$ for each group, $^*P < 0.01$). IL, interleukin; SHR-SP, stroke-prone spontaneously hypertensive rat.

We further investigated the role of TGF-β in renal arteriolosclerosis and nephrosclerosis. TGF-β, a multifunctional growth factor, plays an important role in tissue repair and fibrosis by regulating cell proliferation and differentiation. Recent evidence supports the notion that overproduction of TGF-β may cause the vascular remodeling and other long-term sequelae of hypertension, including nephrosclerosis.[34,35] TGF-β may regulate blood pressure levels by stimulating endothelin-1 mRNA expression and releasing renin from the juxtaglomerular cells of the kidney.[34,36] It can also increase vascular compliance by promoting deposition of extracellular matrix components in the vessel walls.[37] Moreover, treatment of the Dahl salt-sensitive rat strain with an anti-TGF-β antibody significantly reduces blood pressure, proteinuria and albuminuria.[38] In the SHR-SP, glomerular and tubulointerstitial TGF-β expression, as well as cellular phenotypic modulation, accelerates the progression of renal fibrosis and nephrosclerosis.[39] Enhanced TGF-β expression also promotes the hypoxia-induced tubulointerstitial transdifferentiation of proximal tubular cells.[40] We found evidence for renal arteriolosclerosis and nephrosclerosis, along with enhanced TGF-β expression, in the renal epithelial cells and sera of the SHR-SP. In contrast, the IL-10-transduced rats had preserved renal structures and decreased TGF-β expression compared with the controls. These results indicate that IL-10-mediated TGF-β regulation may be involved in the physiological protective mechanism against renal arteriolosclerosis and the emergence of hypertension.

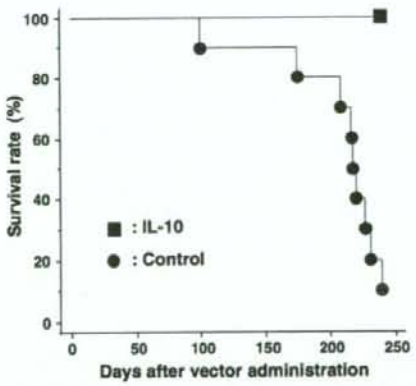

Figure 7 Improved survival of the IL-10-transduced SHR-SP. Survival after transduction of the SHR-SP was estimated by Kaplan–Meier analysis. The experimental protocol is identical to that used in Figure 2. The SHR-SP transduced with AAVRIL10 showed significantly prolonged survival ($P < 0.001$). IL, interleukin; SHR-SP, stroke-prone spontaneously hypertensive rat.

The cholesterol-lowering effect of sustained, systemic IL-10 expression we observed is consistent with our earlier study.[7] The decreased levels of glucose and triglyceride that appeared in the control group may be associated with malnutrition after stroke episodes. Decreased serum albumin levels in this group might result from proteinuria caused by hypertensive renal dysfunction. Importantly, serological studies showed no apparent adverse effects in the IL-10-transduced group.

As a first step toward the future therapeutic investigation, we tried rAAV-mediated IL-10 transduction to prevent vascular remodeling and inflammatory lesions in this study. Here, we have shown protective function of IL-10 against malignant hypertension, although it would be more important to investigate the therapeutic effect on developed hypertension. This protective approach also provides significant insights into the prevention strategy of disease onset in patients with genetic predisposition or intractable polygenic disorders.

In conclusion, we have provided the first evidence that AAV vector-mediated stable IL-10 expression prevents arteriolosclerosis and end-organ damage in the SHR-SP, leading to decreased stroke episodes and prolonged survival. Although the mechanisms underlying the antihypertensive effect of IL-10 require further clarification, its vasculoprotective effect might involve an anti-inflammatory process. Our results suggest that IL-10-mediated vascular protection would be an alternative effective therapeutic strategy to prevent the progression of refractory hypertensive disorders.

Materials and methods

Cloning of rat IL-10 and plasmid construction

Rat IL-10 was cloned from cDNA of rat splenocytes by PCR using the following primers: 5′-GCACGAGAGC CACAACGCA and 5′-GATTTGAGTACGATCCATT TATTCAAAACGAGGAT. The 1.3-kb PCR product was cloned into pCR2.1 by using a TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). The cloned PCR-amplified fragment was verified by sequencing. The resultant plasmid, pCR2.1RatIL-10, was digested with *Eco*RI, and then the rat IL-10 gene fragment was inserted into the *Eco*RI site of p3.3CAG-WPRE, which contains a CAG promoter and woodchuck post-transcriptional regulatory element (WPRE). Finally, the entire expression cassette was inserted between the AAV2-derived inverted terminal repeats (ITRs) in a pUC-based proviral plasmid, pAAVLac Z, to form pWCAGRIL10W.

Recombinant adeno-associated virus production

Recombinant AAV was propagated according to a three-plasmid transfection adenovirus-free protocol.[41] Briefly, 60% confluent HEK293 cells were co-transfected with the proviral plasmid (pWCAGRIL10W or pAAVLac Z), AAV-1, chimeric helper plasmid, p1RepCap, and adenoviral helper plasmid, pAdeno, to produce rAAV expressing either rat IL-10 (AAV1RIL10) or *Escherichia coli* β-galactosidase gene (AAV1LacZ). Resultant crude virus lysates were purified through two rounds of CsCl two-tier centrifugation.[16] The physical titer of the viral stock was determined by dot blot hybridization with plasmid standards.

Western blot analysis and functional analysis of rat IL-10 in vitro

HEK293 cells were infected with AAV1RIL10 or AAV1-LacZ at 1×10^4 g.c. per cell. The supernatant and cell lysate were harvested 72 h after infection. Cells were lysed in a lysis buffer (10 mM Tris-HCl, 150 mM NaCl and 1% NP-40 (pH 7.6)) with Complete Mini (Roche Diagnostics, Mannheim, Germany). The supernatant was concentrated 10-fold using centricon YM-10 (Millipore, Bedford, MA, USA). Ten micrograms of cell lysate or 10 μl of concentrated conditioned medium was subjected to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with a 1:1000 dilution of mouse anti-rat IL-10 polyclonal antibody (Genzyme Techne, Minneapolis, MN, USA). The membrane was then rinsed and incubated with a 1:1000 dilution of peroxidase-linked anti-mouse IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoreactive bands were visualized using the ECL Western blotting kit (Amersham). The biological activity of rat IL-10 was determined as follows: HEK293 cells were transduced with AAV1RIL10 at 1×10^4 g.c. per cell. Seventy-two hours after infection, this supernatant was recovered and the concentration of IL-10 in the supernatant was determined using the Rat Biotrak ELISA System (Amersham). Rat primary splenocytes were incubated with the IL-10-containing supernatant. Thirty minutes after incubation, lipopolysaccharides were added at a concentration of 10 ng ml^{-1}. Twenty-four hours later, the concentration of IFN-γ in the supernatant was determined using the Rat Biotrak ELISA System (Amersham).

Intramuscular injection of rAAV and physiological analysis

Male SHR-SP at 6 weeks of age were purchased from Japan SLC (Shizuoka, Japan) and used in the transduction study. The rats were housed under controlled conditions of constant temperature and humidity and exposed to 12-h light/dark cycle. The rats had free access to chow and tap water. All animal studies were performed in accordance with the guidelines issued by the committee on animal research of Jichi Medical School and approved by its ethics committee.

Male SHR-SP were injected with AAV1RIL10 (1×10^{11} or 1×10^{12} g.c. per body; $n = 5$ for each group), AAV1-LacZ (1×10^{11} g.c. per body; $n = 5$) or saline ($n = 5$) into the bilateral anterior tibial muscles at 6 weeks of age. Control group comprised AAV1LacZ-injected animals and saline-injected animals. From 8 weeks of age, rats were fed a controlled diet (Funahashi SP diet; Funahashi, Chiba, Japan). The systolic blood pressure of rats was measured weekly using a manometer tachometer (Natsume KN-210; Natsume Seisakusho, Tokyo, Japan) with a tail-cuff method. An average of five readings was recorded for each animal after they had acclimatized to the environment. Urine was collected from rats in metabolic cages for a 24-h period at 8, 12, 16 and 24 weeks after gene delivery. Urinary protein levels were determined by the Lowry method. Rats were monitored on a daily basis for behavioral signs of stroke. Stroke-associated symptoms, such as seizure, hindlimb

paralysis and decreased activity, were also assessed as physiological parameters. When any one of these symptoms occurred in the SHR-SP, animals were regarded as stroke positive. Six months after gene delivery, neurological deficits were evaluated according to the following scoring system:[42] (0) normal; (1) slight decrease in motor activity; (2) marked decrease in motor activity or hyperirritability; (3) no walking (decreased responsiveness); (4) inability to stand without support or paralysis of hindlimbs.

Serum biochemistry

Serum samples were collected from the tail vein and stored at −80 °C. Serum biochemistry values, including the concentration of albumin, AST, ALT, total cholesterol, triglyceride, glucose, blood urea nitrogen and creatinine, were estimated using standard procedures. IFN-γ, IL-4 and IL-10 concentrations in sera were measured by using a BIOTRAK ELISA system (Amersham). The concentration of TGF-β was determined by commercial enzyme-linked immunosorbent assay (BioSource International, Camarillo, CA, USA).

Histological examination

Seven months after gene delivery, anesthetized rats were perfused with 50 ml of saline, followed by 100 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain, kidney, descending aorta and carotid artery were fixed in the same fixative and finally embedded in paraffin. Three-micrometer thick sections were stained with hematoxylin and eosin, periodic acid Schiff, oil red O and elastica van Gieson by standard methods for light microscopy.

Immunohistochemistry

Immunohistochemical staining was performed using a standardized streptavidin-bioin-peroxidase method. A mouse monoclonal antibody against rat ED1 (1:100; Serotec, Oxford, UK), a mouse monoclonal antibody against rat CD11b (1:100; Serotec), a rabbit polyclonal antibody against human collagen type IV (1:100; Progen, Heidelberg, Germany), a mouse monoclonal antibody against TGF-β (1:100; Chemicon, Temecula, CA, USA) and a mouse monoclonal antibody against NF-κB p65 subunit (1:50; Chemicon) were used as primary antibodies. Seven months after gene delivery, anesthetized rats were perfused, as described above. Four hours after fixation, the brain and kidney were transferred to 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for cryoprotection and stored at 4 °C overnight. The tissue was frozen in OCT compound (Tissu-Tek; Sakura Finetek, Torrance, CA, USA) at −20 °C and 10-μm thick sections were sliced with a cryostat. The sections were washed and permeabilized with phosphate-buffered saline (PBS) containing 0.5% Triton-X for 10 min, followed by incubation in PBS containing 50 mM glycine. Slides were then washed three times with PBS and blocked with PBS containing 1% bovine serum albumin for 20 min. Internal peroxidase activity was quenched by incubation in PBS buffer containing 0.3% hydrogen peroxide with 0.1% sodium azide. After washing with PBS for three times, the sections were incubated with primary antibodies overnight at 4 °C followed by incubation with biotinylated anti-rabbit or anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) and horse radish peroxidase-labeled streptavidin (Vector Laboratories). The reaction was visualized by using the Vector SG kit (Vector Laboratories), and nuclear fast red was used for counterstaining.

Acknowledgements

We thank Dr James M Wilson for providing p1RepCap (identical to p5E18RXCI). We thank Avigen Inc. (Alamada, CA, USA) for providing pAAVLacZ and pAdeno. We thank Dr Thomas Hope for providing WPRE DNA. We also thank Ms Miyoko Mitsu and Ms Naomi Inaba for their encouragement and technical support. This work was supported in part by grants from the Ministry of Health, Labour and Welfare of Japan: grants-in-aid for Scientific Research; grant for twenty-first century COE program; and 'High-Tech Research Center' Project for Private Universities, matching fund subsidy, from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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