Potential role of recombinant adeno-associated virus human thioredoxin-PR39 in cell and vascular protection against hypoxia

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Abstract. The aim of the present study was to successfully construct a recombinant adeno-associated virus (rAAV) vector containing the human thioredoxin (hTRX)-PR39 chimeric gene (rAAV/hTRX-PR39), and verify that the vector was able to maintain a sustained, stable and efficient expression to achieve protein production in the cell. In the present study, a chicken embryo model was utilized to analyze the therapeutical effect of rAAV/hTRX-PR39 in cerebral ischemia diseases. ECV304 cells were transfected with rAAV/hTRX-PR39 and incubated under conditions of 20, 5 and 1% O_2. Subsequently, the expression levels of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, fibroblast growth factor receptor (FGFR)-1 and syndecan-4 were detected by reverse transcription-quantitative polymerase chain reaction. Under hypoxic conditions, the mRNA expression levels of VEGF, VEGFR-1, VEGFR-2, FGFR-1 and syndecan-4 were found to increase in the PR39-transfected group when compared with the control group, while no statistically significant difference was observed between the PR39-transfected group and the control group under conditions of 20% O_2. In addition, hTRX-PR39 was shown to increase the density of the vasculature and the survival rate of the chick embryos. Under hypoxic conditions, it was hypothesized that rAAV/hTRX-PR39 was capable of promoting angiogenesis, which may subsequently protect the cells from impairment by hypoxia. In conclusion, rAAV/hTRX-PR39 was demonstrated to promote vascularization and cell survival in hypoxia; thus, rAAV/hTRX-PR39 may have potential for use in therapy targeting cerebral ischemia.

Introduction

Cerebral infarction is a severe disease of the central nervous system that has a high incidence rate. Thrombolytic therapy is considered to be the only safe and effective method to recover the blood supply; however, an effective method of providing neuronal protection against ischemia and ischemia-reperfusion injury has not yet been identified. In addition, a method for inducing angiogenesis, which may aid the establishment of collateral circulation and secondary prevention, has not yet been developed (1).

Gene therapy has been considered to be prospective in the treatment of infarction and neuron protection (2,3). A number of studies assessing monogenic function have been conducted, where the protective roles of neurotrophic factors, antiapoptosis genes and angiogenic growth factors have been investigated (4). However, the mechanisms underlying cerebral ischemic diseases are complicated and involve a variety of courses; thus, it was proposed that regulating two genes that exhibit positive feedback on each other may aid further understanding (5). Human thioredoxin (hTRX) is a type of stress-induced protein that protects neurons against oxidative stress (6,7). hTRX has been demonstrated to possess the ability to scavenge free radicals, and also ease the inflammatory response by regulating nuclear factors or mitogen-activated protein kinases. In addition, since hTRX is a type of natural human protein with low immunogenicity, hTRX can be used as a frame protein to construct a gene fusion system, in which the solubility and activity of the expression product can be increased significantly (8,9).

Antibacterial peptide (PR39), a proline- and arginine-rich peptide consisting of 39 amino acids, is considered to be the switch for angiogenesis (10). PR39 is able to inhibit the degradation of hypoxia inducible factor (HIF)-1α, which subsequently elevates the expression of vascular endothelial growth factor (VEGF), kinase insert domain containing-receptor, fms-like tyrosine kinase and fibroblast growth factor receptor (FGFR)-1 to promote vascularization (11,12). This mechanism

of action is similar to the mechanism of vascularization that is observed under conditions of hypoxia. Sun *et al* indicated that adeno-associated virus (AAV)-PR39 may serve as a novel therapeutic agent for the treatment of myocardial infarction (13). As a short peptide, PR39 is unstable; thus, hTRX may be used to provide a frame structure for PR39. Following the insertion of the hTRX frame structure, the aptamer (PR39) is more stable compared with the free peptide. Furthermore, the cell-penetrating ability of hTRX (14,15) may enable PR39 to pass through the blood-brain barrier, which is conducive to enabling the full function of PR39.

In the present study, it was hypothesized that the recombinant gene, hTRX-PR39, may exhibit multiple functions in the protection of neurons and the vasculature. Thus, the aim of the present study was to investigate the therapeutic roles of hTRX-PR39 in hypoxia.

Materials and methods

Recombinant virus construction. The pGEM-T-hTRX-PR39 cloning vector containing hTRX-PR39 full-length gene sequence was constructed as previously described (16). First, the forward and reverse primers of PR39 were designed and synthesized. Using PCR, the fragment encoding PR39 was produced, including *Eco*R721 and *Bam*HI restriction enzyme sites, and the new hTRX cDNA including *Eco*R721 and *Eco*RI restriction enzyme sites was generated. Next, the synthesized fragments were cloned into a pGEM-T vector. The positive clone was identified using restriction enzymes, and the cloned amplified fragments were sequenced by the dideoxy-mediated chain-termination method. The cloned hTRX and PR39 cDNA were compared with the GenBank sequence (http://www.ncbi.nlm.nih.gov/genbank/) using DNASIS software (MiraiBio Group, San Francisco, CA, USA). Subsequently, pGEM-T-hTRX and pGEM-T-PR39 were digested by *Bam*HI and *Eco*RI, the PR39 *Bam*HI, *Eco*RI was cloned into the recombinant vector pGEM-T-hTRX *Bam*HI, *Eco*RI. Thus, the recombinant vector pGEM-T-hTRX-PR39 was produced. The pSSCMV viral vector, adenovirus plasmid pAAV/Ad, *Escherichia coli* TOP10, ECV304 and HEK293 cell lines were provided by Xi'an Huaguang Biological Engineering Co., Ltd. (Xi'an, China) (17).

Transfection. The recombinant virus was seeded into the culture medium of ECV304 cells (Xi'an Huaguang Biological Engineering Co., Ltd.) and incubated for 24 h. For the control, adenoviruses were seeded into the culture medium of ECV304 cells instead of the recombinant virus. The control and transfection groups were subsequently divided into three subgroups that were separately incubated in a hypoxic (1 and 5% O_2) or normoxic environment (20% O_2) for 72 h.

Reverse transcription-quantitative polymerase chain reaction (PCR). Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed to cDNA using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase PCR kit (Promega Corporation, Madison, WI, USA). Briefly, 3 μl RNA was reverse transcribed to cDNA at 37˚C for 1 h in a 20-μl reaction system that contained 1 μl M-MLV reverse transcriptase,

4 μl 5X M-MLV buffer, 0.5 μl RNase inhibitor, 1 μl oligo-dT and 1 μl dNTP (Promega Corporation, Madison, WI, USA). For quantitative PCR, the PCR amplification mixture (20 μl) consisted of 2 μl cDNA mixture, 10 μl SYBR Green (Takara Biotechnology Co., Ltd., Dalian, China), 2 μl primers and 6 μl deionized water. β-actin was used as a control. The amplification conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of 95˚C for 10 sec, 58˚C for 30 sec and 72˚C for 30 sec. Nested PCR was performed using a 2-μl sample of the PCR product as a template under the aforementioned PCR conditions. Bio-Rad IQ5.0 Optical System software (Bio-Rad Laboratories, Hercules, CA, USA) was used for the detection of the quantitative PCR products that were specific for VEGF, vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, FGFR-1, syndecan-4, PR39 and β-actin. The primer sequences used for PCR are shown in Table I.

Effects of AAV-hTRX-PR39 transfection on the hypoxic chick embryo. Effects of AAV-hTRX-PR39 on the hypoxic chick embryo were analyzed using a chick embryo (Xi'an Huaguang Biological Engineering Co., Ltd.) chorioallantoic membrane (CAM). In total, 120 fertilized chicken eggs (age, seven days; weight, 50-55 g) were incubated under 65-70% relative air humidity at 37˚C. On day four of incubation, 100 μl AAV-hTRX-PR39 or adenovirus (control) was gently pipetted onto the CAM surface using a transfer pipette. The eggs were subsequently placed in an incubator for three days. Next, the CAMs were incubated for 8 h in a hypoxic (1 and 5% O_2) or normoxic (20% O_2) environment, after which they were incubated for 11 days in a normoxic environment. The CAMs were subsequently photographed using an Olympus DP73 digital camera (Olympus Corporation, Tokyo, Japan), as presented in Fig. 1. The vessel density of the CAMs was analyzed using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). For each study group, 10-15 domains were selected for vessel quantification, and the mean values of the vessel density were calculated. All animal studies were approved by the Shandong University Institutional Animal Care and Use Committee.

Statistical analysis. Data are expressed as the mean ± standard deviation, and were analyzed using analysis of variance and the homogeneity of variance test, according to a completely randomized design. Comparisons between groups were performed using the two-sample t-test. SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of VEGF, VEGFR-1, VEGFR-2, FGFR-1, syndecan-4 and PR39. Under normoxic conditions of 20% O_2, the mRNA expression levels of PR39, VEGF, VEGFR-1, VEGFR-2, FGFR-1 and syndecan-4 exhibited no statistically significant difference when comparing the hTRX-PR39-transfected ECV304 cells and the control group ($P>0.05$). However, under hypoxic conditions of 1% O_2, the quantitative PCR results demonstrated increased mRNA

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
VEGF	TCTACCTCCACCATGCCAAGT	GCTGCGCTGATAGACATCCA	104
VEGFR-1	TCCCTTATGATGCCAGCAAGT	CCAAAAGCCCCTCTTCCAA	79
VEGFR-2	CTTCGAAGCATCAGCATAAGAAACT	TGGTCATCAGCCCACTGGAT	156
FGFR-1	ACTCTGTGGTGCCTTCTGAC	CATTTCCTTGTCGGTGGTAT	317
Syndecan-4	CTGCTGCTGTTCTTCGTAGG	CTTTGAGCTGTCTGGCTCTG	153
PR39	CTCTACCGCCTCCTGGAGCT	GGCCCTTCATAATATCCCCCA	117

VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor, FGFR, fibroblast growth factor receptor.

Table II. Expression levels of the angiogenic growth factors in the control and PR39-transfected groups.

	20% O_2		1% O_2	
Gene expression	Control 1 group	PR39	Control 2 group	PR39
VEGF	0.3±0.06	0.33±0.08	0.47±0.22	0.75±0.25[a]
VEGFR-1	0.14±0.07	0.15±0.10	0.31±0.09	0.49±0.21[a]
VEGFR-2	0.16±0.05	0.15±0.11	0.35±0.10	0.54±0.12[a]
FGFR-1	0.23±0.06	0.28±0.16	0.42±0.14	0.67±0.20[a]
Syndecan-4	0.16±0.05	0.15±0.04	0.29±0.06	0.39±0.11[a]
PR39	0.00±0.00	0.10±0.12	0.00±0.00	1.43±0.25[a]

[a]$P<0.05$, vs. control 2 group. VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor.

Table III. Effects of AAV-hTRX-PR39 transfection on the hypoxic chick embryo.

Group	Survival rate (n)	Wet weight (g)	Density of vessels (%)
1% O_2			
Control 1	0/20[c]	8.5±3.56[c]	2.21±0.4[c]
PR39	16/20[a]	32.5±4.5[a]	10.6±0.6[a]
5% O_2			
Control 2	3/20[c]	20.4±8.56[c]	5.65±0.6[c]
PR39	17/20[b]	34.31±6.51[b]	11.9±0.5[b]
20% O_2			
Control 3	20/20	38.8±4.27	12.5±0.5
PR39	20/20	34.2±5.44	10.1±0.5

[a]$P<0.05$, vs. control 1 group; [b]$P<0.05$, vs. control 2 group; and [c]$P<0.05$, vs. control 3 group. AAV, adeno-associated virus; hTRX, human thioredoxin.

expression levels of VEGF, VEGFR-1, VEGFR-2, FGFR-1 and syndecan-4, as well as sharply increased expression levels of PR39, in the hTRX-PR39-transfected group ($P<0.05$), as compared with the control group. The results are shown in Table II. The 5% O_2 condition was not examined as the 20% and 1% O_2 conditions successfully demonstrated that transfection with rAAV/hTRX-PR39 increased the expression levels of

VEGF, VEGFR-1, VEGFR-2, FGFR-1 and syndecan-4 under hypoxic condition (1% O_2).

Effects of AAV-hTRX-PR39 transfection on the hypoxic chick embryo. Under 20% O_2 conditions, the survival rate of the chick embryos was 100% in the transfected and non-transfected groups ($P>0.05$). By contrast, under hypoxic conditions (5 or
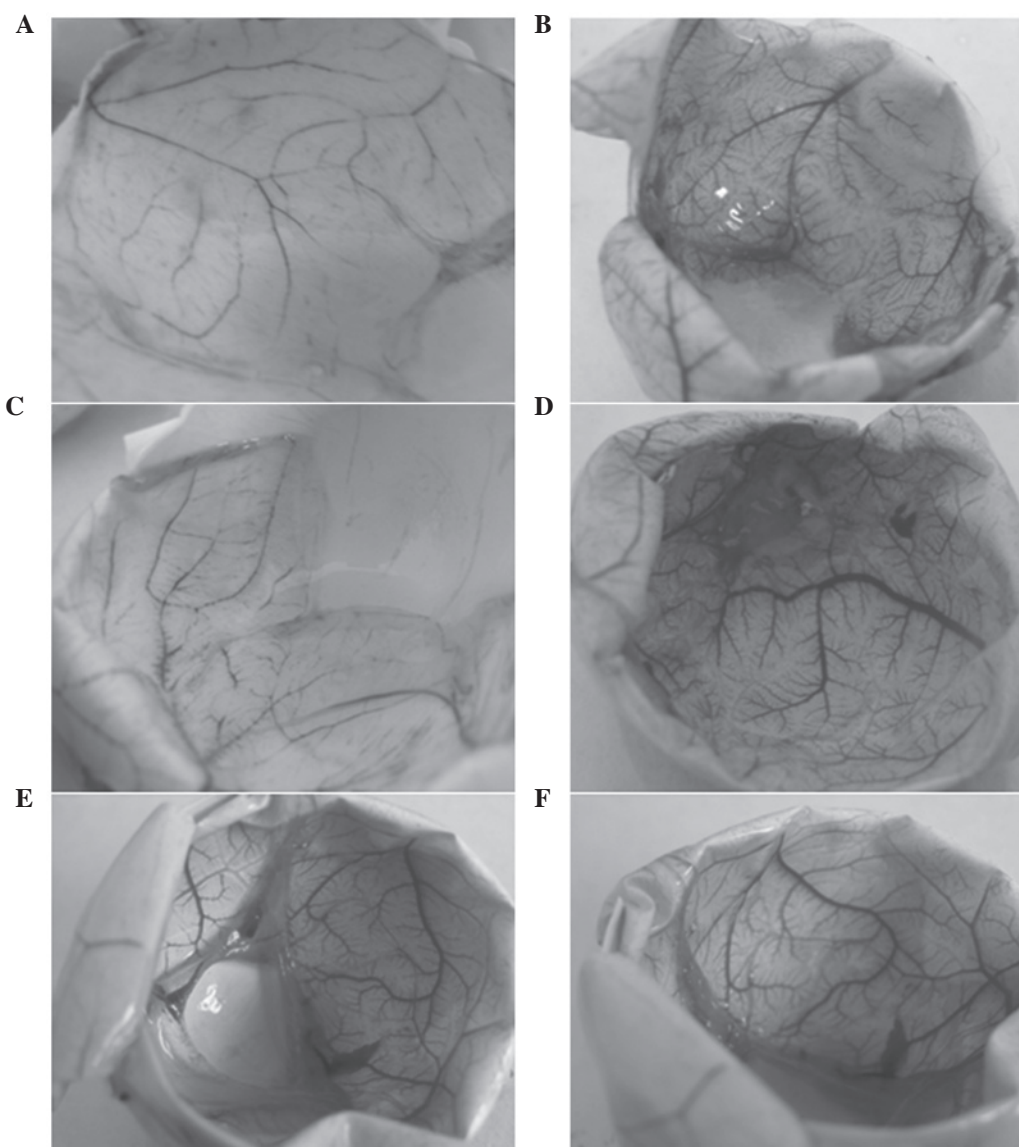
Figure 1. Effect of adeno-associated virus-human thioredoxin-PR39 transfection on the hypoxic chick embryo in various O_2 environments. (A) 1% O_2, (B) 1% O_2PR39, (C) 5% O_2, (D) 5% O_2PR39, (E) 20% O_2 and (F) 20% O_2PR39.

1% O_2), the survival rate of the chick embryos was shown to increase in the transfected group when compared with the non-transfected group (5% O_2, 17/20 vs. 3/20; 1% O_2, 16/20 vs. 0/20; P<0.05).

As shown in Table III, the wet weight of the chick embryos under normoxic conditions did not significantly differ between the transfected or non-transfected groups (P>0.05). However, the wet weight of the chick embryos under hypoxic conditions (5 or 1% O_2) increased significantly in the transfected group when compared with the non-transfected group (P<0.05).

Under a normoxic environment, no statistically significant difference was observed with regard to the density of the vessels between the transfected and non-transfected groups (P>0.05). However, the density of the vessels in the chick embryos subjected to mild hypoxia (5% O_2) and severe hypoxia (1% O_2) decreased significantly in the non-transfected group when compared with the 20% O_2 control group. In addition, the density of the vasculature increased significantly in the transfected groups when compared with the respective non-transfected groups (P<0.05). The results are presented in Table III.

Discussion

hTRX is a micromolecular protein that functions as an oxidant and a reductant. The hTRX gene is 13 kb in length and encodes 104 amino acids (18). hTRX is known to play a number of important roles, including regulating the redox reaction, scavenging free radicals and exerting antiapoptosis effects (19-21). Previously, hTRX protein homology was demonstrated to consist of a protein cross-frame feature that provides available sites for the binding of an active aptamer (9,22). Following the insertion of the aptamer into the cross-frame structure, the aptamer becomes more stable compared with the free peptide and is more prone to transfer into cells. PR39, a short peptide that is extremely unstable, is prone to inactivating conformational changes. Thus, therapeutic use of PR39 requires expression as a recombinant protein. The hTRX protein can provide a frame-work for the expression of PR39 as a therapeutic aptamer. As hTRX is a natural human protein with low immunogenicity, hTRX can serve as a cross-frame protein for the construction of a gene fusion expression system, which may significantly

increase the activity of the expression products and activated soluble proteins. Previous studies have demonstrated that hTRX-PR39 can reduce the number of apoptotic ECV304 cells under hypoxic conditions (23,24). Thus, the hTRX-PR39 chimeric protein provides structural compatibility to ensure the directed bioavailability of PR39 at the target site, in addition to the added stability of the protein. Furthermore, in the present study, the mRNA expression levels of PR39 and the various growth factors were only activated under conditions of hypoxia, but not under conditions of 20% O_2, indicating that the application of hTRX-PR39 is controllable.

In the present study, the mRNA expression levels of VEGF, VEGFR-2, FGFR-1 and syndecan-4 were shown to increase in the PR39-transfected groups when compared with the respective control groups, indicating that PR39 may activate these growth factors and receptors. To assess the effect on vascularization, the density of the allantoic sac vasculature were calculated. The results demonstrated that the density of the vasculature was markedly increased in the transfected groups. Accordingly, the survival rates of the chick embryos were also improved in the transfected groups when compared with the respective control groups. Therefore, the results indicated that hTRX-PR39 was able to induce tolerance to hypoxia.

PR39 has been previously demonstrated to prevent the degradation of HIF-1α, which results in the upregulation of HIF-1α-dependent genes, including VEGF and VEGFR-1 (24,25). In addition, PR39 is known to upregulate the expression of the FGFRs, FGFR-1 and syndecan-4, which subsequently activate FGF signaling (24,26). The improvement in blood supply may further increased the level of PR39 (27,28). In the present study, increases in the expression levels of angiogenic growth factors and vascularization were confirmed in the PR39-transfected groups; thus, it was hypothesized that these factors may be the main mechanisms underlying the protective role of PR39 in hypoxia. In addition, PR39 has been hypothesized to play a role in the protection of IAP-2 (an inhibitor of apoptosis) and the decreased activity of caspase-3, leading to the suppression of apoptosis (29). According to these results, PR39 may stimulate an angiogenic response, which may be used as a therapeutic intervention in ischemic areas. In future studies, hTRX-PR39 should be transfected into ischemic brain tissues to observe the protective role in ischemic stroke.

According to previous studies (30-33) by other groups, and our previous study (34), a recombinant AVV (rAAV) vector, containing the hTRX-PR39 chimeric gene, was constructed to achieve sustained, stable and efficient protein production. Vectors containing hTRX-PR39 were transfected into chick embryonic tissues that had been subjected to hypoxia, and the expression of PR39 was shown to be activated, as well as that of downstream factors. Expression activation was only observed under hypoxic conditions, indicating that the expression is well-controlled. Thus, rAAV/hTRX-PR39 was demonstrated to promote vascularization and cell survival in hypoxia, and may have potential in the therapy of cerebral ischemia.

In conclusion, the hTRX-PR39 chimeric protein provides structural compatibility to ensure the directed bioavailability of PR39 at the target site, in addition to added stability of the protein. Furthermore, transfection with rAAV/hTRX-PR39 was shown to increase the expression levels of VEGF,

VEGFR-1, VEGFR-2, FGFR-1 and syndecan-4, and promote angiogenesis and cell survival under hypoxic conditions. Thus, rAAV/hTRX-PR39 may provide a novel therapeutic method for the treatment of ischemia.

Acknowledgements

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