Recombinant adeno-associated virus delivered human thioredoxin-PR39 prevents hypoxia-induced apoptosis of ECV304 cells

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Abstract

Human thioredoxin and antibacterial peptide, PR39, have been shown to have potent antioxidant effects that may prolong survival of cells during hypoxia. The pSSCMV/human thioredoxin-PR39 vector was successfully constructed in this study and used to infect ECV304 cells. Transfected ECV304 cells were incubated at 1%, 5% hypoxic, and normal oxygen conditions. We found that the number of apoptotic cells after transfection with recombinant adeno-associated virus-human thioredoxin-PR39 was significantly lower than controls, suggesting a protective effect of the recombinant human thioredoxin-PR39 protein on hypoxic cells.

Key Words: human thioredoxin; antimicrobial peptide PR39; fusion gene; recombinant adeno-associated virus; gene therapy; apoptosis; hypoxia

Abbreviation: hTRX, human thioredoxin; PR39, antibacterial peptide 9; rAAV, recombinant adeno-associated virus

INTRODUCTION

Gene therapy for central nervous system diseases often involves functional proteins; however, large molecules cannot cross the blood-brain barrier and thus are ineffective for the treatment of the brain. Therefore, there is an urgent need for improved delivery into the central nervous system. Human thioredoxin (hTRX) is a micromolecular protein that can act as both an oxidant and a reductant. The hTRX gene is 13 kb long and encodes 104 amino acids³-⁵. The hTRX protein homology consists of a protein cross-frame feature that provides available sites for binding active aptamer. The aptamer that has been inserted into the cross-frame becomes more stable than free peptide and is more prone to transfer into cells⁶. As hTRX is a natural human protein with low immunogenicity, it serves as a cross-frame protein to construct a gene fusion expression system and can significantly increase the activity of expression products and activated soluble proteins⁴. Antibacterial peptide 9 (PR39) is a short peptide that is extremely unstable and is prone to inactivating conformational changes. Thus, therapeutic use of PR39 requires it to be expressed as a recombinant protein. Currently, studies of PR39 have mainly focused on two issues. (1) Direct synthesis of PR39 shows that it is significantly effective for a short time, but the half-life is relatively short; PR39 is rapidly degraded in vivo and expressed for only a short time, and since the cost of production is high, has limited clinical application. (2) Adenoviruses can mediate PR39 expression in vascular endothelial cells and promote effects on blood vessels in the ischemic myocardium. Adenoviruses can act as gene expression vectors because of their ability to reach high titers, can infect a variety of cells, including non-dividing cells, and exogenous genes can be instantaneously and highly expressed. However, adenoviruses often induce inflammatory or toxic reactions that result in the recombinant gene not being expressed long term⁶⁷. The hTRX protein can provide a framework for the expression of PR39 as a therapeutic aptamer. It has been previously shown that insertion of PR39 into the hTRX framework results in increased stability of PR39 compared with the free peptide with hTRX providing blood-brain barrier penetration⁶-⁷. Thus, the hTRX-PR39 chimeric protein provides structural compatibility to ensure both the directed bioavailability of PR39 at the target site and added stability of the protein. This study aimed to construct a recombinant adeno-associated virus (rAAV) vector containing a hTRX-PR39 chimeric gene to produce sustained, stable, and efficient
expression of gene products in hypoxic ECV304 cells. These studies provide the basis for the investigation of in vivo expression of the chimeric protein to limit the effects of hypoxia.

**RESULTS**

**Successful construction of recombinant plasmid pSSCMV/hTRX-PR39**

Target gene segments of 5,020 and 438 bp were obtained after EcoRI and BamHI digestion of recombinant plasmids indicating that the hTRX-PR39 fragment had been inserted into the pGEM-T easy vector (Figure 1; supplementary Figures 1, 2 online).

**Titers of hTRX-PR39 recombinant AAV**

Viral titers were determined using dot blot assay (Figure 2). For comparison, the concentration of viral DNA was determined to be 10–100 ng/μL, which correlated to $3.46 \times (10^9 - 10^{10})$ virus particles/μL.

**Recombinant hTRX-PR39 expressed in ECV304 cells**

Microscopy of stained cells revealed that a large number of hTRX brown granules (98.5 ± 1.5%) were visible within the cytoplasm of ECV304 cells in the rAAV-hTRX-PR39 treated group, while no hTRX expression was observed in the control group (1.5 ± 0.6%; $P < 0.01$). This is evidence that recombinant fusion protein, hTRX-PR39, was expressed in ECV304 cells (Figure 3).
DISCUSSION

This study showed that rAAV carrying hTRX-PR39 was successfully constructed and expressed in ECV304 cells. Therefore, the hTRX-PR39 fusion gene can be expressed in cells, and rAAV can deliver the gene into cells to produce the bioactive short-peptide in eukaryotic cells. Under normoxic conditions, ECV304 cell morphology and viability was not significantly different between the control group and the rAAV-hTRX-PR39 treated group, and the number of viable cells was much higher than the control group regardless of hTRX-PR39 transfection.

![Table 1](image)

| Oxygen concentration | Control group (× 10^4) | rAAV-hTRX-PR39 group (× 10^4) |
|----------------------|------------------------|------------------------------|
| 24 hours             |                        |                              |
| Normal oxygen        | 30.0±1.2               | 50.0±1.6^b                   |
| 5% oxygen            | 24.0±1.7^a             | 30.0±2.2^b                   |
| 1% oxygen            | 10.0±2.5^a             | 30.0±2.0^b                   |
| 48 hours             |                        |                              |
| Normal oxygen        | 123.0±1.7              | 224.0±1.2^b                  |
| 5% oxygen            | 30.0±1.3^a             | 122.0±1.1^ab                 |
| 1% oxygen            | 5.0±2.7^a              | 104.0±1.9^ab                 |
| 72 hours             |                        |                              |
| Normal oxygen        | 210.0±1.1              | 350.0±1.7^b                  |
| 5% oxygen            | 28.0±2.0^a             | 180.0±2.3^ab                 |
| 1% oxygen            | 1.0±0.5^a              | 185.0±0.2^ab                 |

Data are expressed as mean ± SD and values between groups were compared using two-sample t tests. ^aP < 0.01, vs. normal oxygen group; ^bP < 0.01, vs. non-transfection group. hTRX: human thioredoxin; rAAV: recombinant adeno-associated virus; PR39: antibacterial peptide 9.

![Figure 4](image)

Figure 4 Changes of ECV304 cell morphology at different oxygen concentrations.

In control group, the number of cells was significantly reduced, cell morphology became sharpened, and several processes formed. Severe hypoxia could lead to great cell damage. The cell morphology was improved after recombinant adeno-associated virus (rAAV)-human thioredoxin (hTRX)-antibacterial peptide 9 (PR39) treatment.

![Figure 5](image)

Figure 5 Cell cycle analysis of ECV304 cells cultured with 1% oxygen determined by flow cytometry. Red dots represent propidium iodide stained cells, green dots represent FITC stained cells, and the green area represents apoptotic cells.

(A) Control group apoptosis rate was 32.58 ± 0.39%.
(B) rAAV-hTRX-PR39 group apoptosis rate was 7.35 ± 0.43%.

rAAV: Recombinant adeno-associated virus; hTRX: human thioredoxin; PR39: antibacterial peptide 9; FITC: fluoresceine isothiocyanate.
Under hypoxic (1% and 5% oxygen) conditions, the number of cells was significantly reduced in the control group, with cell morphology changing to thin with several processes. However, for the rAAV-hTRX-PR39 treated group, the number of cells only decreased slightly and maintained a typical round shape. After hTRX-PR39 transfection, the number of surviving cells was significantly higher than that of non-transfected cells, and the cell survival rate decreased with increasing oxygen concentration. This is evidence that hTRX-PR39 can inhibit cellular apoptosis and protect cells against hypoxia. Flow cytometric analysis of apoptosis also agreed with these results.

The mechanism of protection that PR39 affords may be protection of IAP-2 (an inhibitor of apoptosis) and decreased activity of caspase-3, leading to suppression of apoptosis. Increasing the concentration of PR39 in hypoxic tissue has been shown to prevent apoptosis and promote vascular growth. Growing evidence indicates that PR39 can reduce infarction size during the early ischemic stage, promote angiogenesis in ischemic regions, establish effective collateral circulation, and prevent microcirculation dysfunction. Likewise, the protective effects of hTRX on hypoxic cells may be related to antioxidant properties, anti-apoptosis, scavenging free-radicals, growth factors, cytokine-like effects, and a synergistic effect with PR39, as observed in the current study. Therefore, rAAV-hTRX-PR39 conferred a protective effect on hypoxic cells at the cellular level.

### MATERIALS AND METHODS

#### Design
A cell biological experiment of vector construction and transfection.

#### Time and setting
Experiments were performed from April 2008 to December 2009 in the Laboratory of Xi’an Huaguang Biological Engineering, China.

#### Materials
The pGEM-T-hTRX-PR39 cloning vector containing hTRX-PR39 full-length gene sequence was constructed as previously described. The pSSCMV viral vector, adenovirus plasmid PFG140, assistant plasmid pAAV/Ad, E. coli TOP10, ECV304 cell lines, and 293 cell lines were provided by Xi’an Huaguang Biological Engineering Co., Ltd., China.

#### Methods

**Construction and identification of pSSCMV/hTRX-PR39 plasmid vector**
The pGEM-T-hTRX-PR39 plasmid was isolated from transformed E. coli using the standard alkaline lysis method. The hTRX-PR39 cDNA fragment was obtained from this plasmid after restriction enzyme digestion with EcoRI and BamHI. The resultant digested fragment was ligated to pSSCMV viral vectors using T4 DNA ligase. The plasmid was used to transform competent E. coli TOP10 using the CaCl2 transformation method.

Transformed bacteria were selected on LB agar plates containing ampicillin, cultured at 37°C for 16 hours. White colonies were used to inoculate liquid LB medium and incubated overnight, and then stored at 4°C. A small amount of plasmid DNA was prepared using the alkaline lysis method and electrophoresed through 0.1% agarose gel after EcoRI digestion. DNA bands were observed using a short-wave ultraviolet analyzer (Shanghai Huayan Instrument Factory, China). The pSSCMV/hTRX-PR39 plasmid was isolated using the alkali rupture method.

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The formation of fine precipitates was visible on the cell surface by optical microscopy (Olympus, Tokyo, Japan). Cells were cultured in Dulbecco’s modified Eagles medium containing 10% fetal bovine serum at 37°C in a CO2 incubator for 72 hours (Figure 6).

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**Recovery of recombinant viral particles and determination of viral titer**

Cells were collected 72 hours after transfection and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid medium, followed by three repeated freezing/thawing cycles of −20 °C freeze and 37 °C water bath. Cells were lysed using an ultrasonic cell crusher (Ningbo Xinyi Ultrasound Equipment, China) and adenovirus was inactivated at 56 °C. Viral titers were determined by dot blot analysis of the DNA content. Transfected ECV304 cells with pSSCMV/hTRX-PR39 recombinant adenovirus vector

Transfected ECV304 cells with pSSCMV/hTRX-PR39 recombinant adenovirus vector

ECV304 cell lines were transferred to coverslips placed in culture flasks along with Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and cultured at 37 °C in a 5% CO₂ incubator (Thermo Electron Corporation, USA). Recombinant virus was seeded into the culture medium and incubated at 37 °C in a 5% CO₂ incubator for 12 hours. Cells were divided into a control (untreated) group and a rAAV-hTRX-PR39 treated group for determination of viral titer.

**Expression of hTRX-PR39 in ECV304**

Cells grown and infected on coverslips were fixed in acetone at room temperature for 15 minutes and rinsed three times with 0.1 M phosphate-buffered saline (pH 7.4) for 5 minutes. Cells were then treated with 50 μL 0.75% H₂O₂ at 37 °C for 30 minutes to block endogenous peroxidase activity. Cells were rinsed again three times with phosphate buffered saline for 5 minutes, and incubated with 50 μL 0.5% Triton X-100 for 30 minutes. Coverslips were then washed three times of phosphate buffered saline for 30 minutes each. Cells were blocked with 50 μL 20% bovine serum albumin at 37 °C in a humidified chamber for 30 minutes to eliminate non-specific staining. Cells were then incubated with mouse anti-hTRX monoclonal antibody (1:500; Xi’an Huaguang Biological Engineering, China) for 4 hours in a humidified chamber, followed by five washes with phosphate buffered saline for 5 minutes. Cells were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:500; Beijing Zhongshan Company, China) at 37 °C for 2 hours in a humidified chamber, followed by three washes with phosphate buffered saline for 5 minutes. Cells were finally stained using 0.5 mg/mL diaminobenzidine and 0.03% H₂O₂ for 10–20 minutes. Gene expression was detected using an inverted microscope (model 1X71; Olympus, Japan) where the number of positive cells among 100 randomly selected cells from 10 fields-of-view at 200 × magnification were counted. Thus, the percentage of positive cells represented the level of protein expression.

**Hypoxic intervention**

Both control group and rAAV-hTRX-PR39 group cells were divided into three sub-groups and incubated at 1% oxygen, 5% oxygen, and normal concentration of oxygen. All cells were grown at 37 °C in 5% CO₂ incubator for 24, 48, 72 hours. Cellular growth was observed using an inverted fluorescence microscope.

**Cell viability**

At 24, 48, 72 hours culture, cells were resuspended and mixed with 60% trypan blue saline solution for 1 minute. Viable cells were quantified by microscopy (model 1X71; Olympus, Japan).

**Cell apoptosis by flow cytometry**

At 24, 48, 72 hours of culture, the ECV304 cells in the control group (1% oxygen condition) and rAAV-PR39 group were washed twice with cold phosphate buffered saline and resuspended to 1 × 10⁶/mL in binding buffer. Three 100 μL aliquots of cells were incubated with 5 μL Annexin V-fluoescence isothiocyanate and 5 μL propidium iodide using gentle oscillation at room temperature for 15 minutes while being protected from light. Cell apoptosis was detected by using 400 μL staining buffer and flow cytometer (Shanghai Jieweifu Industrial Co., Ltd., China).

**Statistical analysis**

Data were expressed as mean ± SD using SPSS analysis of variance and homogeneity of variance test according to completely randomized design. Comparisons between groups were performed using the two-sample t-test. A P-value of < 0.05 was considered statistically significant.

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**Author contributions:** Guangxiao Yang, Quanying Wang and Yifeng Du had full access to the study concept and design, and revised the manuscript. Xiyun Ruan wrote the manuscript and was responsible for funding. Zhenguo Yuan analyzed data and participated in statistical analyses.

**Conflicts of interest:** None declared.

**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 9, 2012 item after selecting the “NRR Current Issue” button on the page.

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