#957

UNIVERSITY OF ILLINOIS
COLLEGE OF MEDICINE AT PEORIA

Treatment with a recombinant adeno-associated virus expressing the TFPI-2 gene simultaneously downregulates MMP-9 and VEGF in a human glioblastoma cell line

Joseph George, Christopher S. Gondi, Meena Gujrati, Dzung H. Dinh, William C. Olivero, and Jasti S. Rao

Departments of Cancer Biology and Pharmacology, Neuropathology and Neurosurgery, University of Illinois College of Medicine, Peoria, IL 61656

Abstract

Human tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type proteinase inhibitor that regulates a variety of serine proteinases. TFPI-2 is abundant in the extracellular matrix and highly expressed in non-invasive cells, but absent or undetectable in highly invasive human glioblastoma cells. Matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor (VEGF) play major roles in tumor cell invasion and angiogenesis. Using a recombinant adeno-associated viral (rAAV) vector carrying the human TFPI-2 gene (rAAV-TFPI-2), we expressed TFPI-2 in the U251 cell line, a highly invasive human glioblastoma cell line. Results from the TUNEL assay, nuclear chromatin staining and FACS analysis revealed increased apoptosis in U251 cells after transfection with the construct. Results from an *in vitro* angiogenesis assay (co-culture of rAAV-TFPI-2-transfected U251 cells with human microvascular endothelial cells) demonstrated the inhibition of capillary-like structure formation in rAAV-TFPI-2-treated cells as compared with control and mock-transfected cells. *In vivo* angiogenesis studies in nude mice depicted decreased microcapillary formation in U251 cells infected with rAAV-TFPI-2. Zymography, western blot analysis and real time RT-PCR for MMP-9 demonstrated downregulation of MMP-9 expression in U251 cells after treatment with rAAV-TFPI-2. In addition, VEGF expression significantly decreased in rAAV-TFPI-2-transfected U251 cells as demonstrated by western blot and real time RT-PCR. The results of the present study indicate that TFPI-2 expression in highly invasive glioblastoma cells effectively prevents tumor invasion and angiogenesis through downregulation of MMP-9 and VEGF and that rAAV-TFPI-2-mediated gene expression offers a potential therapeutic application for cancer gene therapy.

Introduction

Glioblastomas are highly invasive and aggressive primary brain tumors associated with a dismal prognosis. Glioblastomas comprise 23% of primary brain tumors in the United States and are the most commonly diagnosed brain tumor in adults. The median survival of patients with glioblastoma treated by surgery, radiotherapy and chemotherapy is in the range of 12 months. Although the understanding of the pathophysiology of gliomas has increased significantly over the past few years, an effective treatment has not been developed for this type of cancer. Limits to the efficacy of current treatment modalities call for the development of novel therapeutic approaches targeting the specific biological features of glioblastomas.

Tumor invasion, angiogenesis and metastasis are complex mechanism that includes a variety of cellular processes, among which the proteolytic degradation of extracellular matrix has been considered as one of the important events. The degradation of extracellular matrix can be promoted by the imbalance between proteolytic enzymes and their inhibitors. Studies focusing on matrix metalloproteinases (MMPs), especially MMP-9, have demonstrated that the over expression of this proteolytic enzyme actively involves the degradation of extracellular matrix proteins, thereby promoting tumor invasion, angiogenesis and metastasis of most solid tumors including brain tumors. The degradation of extracellular matrix simultaneously stimulates vascular endothelial growth factor (VEGF) and angiogenesis. Furthermore, the rate of tumor invasion and angiogenesis is directly correlated with the activity of MMP-9 in glioblastomas.

Human tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type proteinase inhibitor that acts against a wide range of serine proteases through their non-productive interaction with a P1 residue in its first Kunitz-type domain. A wide variety of cells, including keratinocytes, dermal fibroblasts, smooth muscle cells, synoviocytes and endothelial cells, synthesize and secrete TFPI-2 primarily into their extracellular matrix. TFPI-2 exhibits inhibitory activity toward a broad spectrum of proteinases, including trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein and the factor VIIa-tissue factor complex. However, TFPI-2 exhibits little or no inhibitory activity toward urokinase-type plasminogen activator, tissue type plasminogen activator and -thrombin. Recently, TFPI-2 expression on certain tumors has been shown to play a significant role in inhibiting tumor invasion and metastasis by a mechanism that involves its inhibitory activity. However, little is known about the role of TFPI-2 expression on matrix metalloproteases, in particular MMP-9.

The expression of TFPI-2 gene is absent in highly invasive glioblastomas, including U-251 cell line, due to the aberrant hypermethylation of TFPI-2 promoter CpG islands. Analysis of TFPI-2 protein in human normal brain and in gliomas revealed that the highest levels of TFPI-2 were present in normal brain, lesser amounts in low-grade gliomas and anaplastic astrocytomas, and undetectable amounts in glioblastomas. Previous studies have demonstrated that the expression of TFPI-2 gene in a highly invasive glioblastoma cell line reduces angiogenesis and tumor invasion in experimental animals as well as in cell culture system. The aim of the present investigation was to evaluate the efficacy of restored TFPI-2 gene in the highly invasive U-251 cell line to decrease the expression and activity of MMP-9 and VGEF, and thus to inhibit tumor invasion, angiogenesis both *in vitro* and *in vivo*.

Figure 1

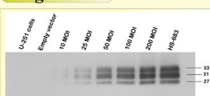

Figure 1. Western blot analysis for the expression of Tissue Factor Pathway Inhibitor-2 (TFPI-2) protein in the extracellular matrix (ECM) of U-251 glioblastoma cells. U-251 cells (procured from National Cancer Institute, Maryland) were transfected with a recombinant adeno-associated virus carrying human TFPI-2 cDNA, at concentrations of 10, 25, 50, 100 and 200 MOI. A 0.8-kb fragment of human TFPI-2 was cloned into a human adeno-associated viral vector (pCMV-MCS) at the BamH1 site. TFPI-2 protein from the ECM extracts of HS-683 cells was used as a positive control. The transfection demonstrated successful restoration of TFPI-2 gene and protein levels in U-251 glioblastoma cells in a dose dependant manner.

Figure 2

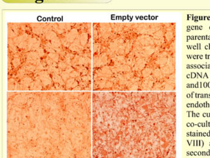

Figure 2. Effect of the restoration of TFPI-2 gene on *in vitro* angiogenesis. U-251 parental cells (5X10^3) were seeded into 8-well chamber slides. After 12 h, the cells were transfected with a recombinant adeno-associated virus carrying human TFPI-2 cDNA (rAAV), at concentrations of 50 and 100 MOI in serum free media. After 24 h of transfection, 2X10^4 human microvascular endothelial cells (HMEC) were co-cultured. The cultures were terminated after 72 h of co-culture, and the cells were fixed and stained with Von Willebrand Factor (Factor VIII) antibody followed by biotinylated secondary antibody. The slides were further treated with horseradish peroxidase labeled streptavidin. The final color was developed using 3% 3-amino-9-ethylcarbzole in *N,N*-dimethylformamide. The cells were viewed under a microscope for the inhibition of capillary-like structure formation. In vitro angiogenesis study demonstrated that the capillary-like network formation by the HMEC cells was completely prevented by rAAV at a concentration of 100 MOI.

Figure 3

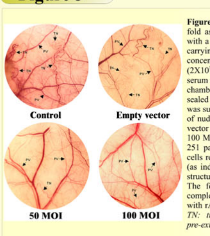

Figure 3. *In vivo* angiogenesis (dorsal skin fold assay). U-251 cells were transfected with a recombinant adeno-associated virus carrying human TFPI-2 cDNA (rAAV) at concentrations of 50 and 100 MOI. The cells (2X10^5) were then suspended in 200 µl of serum free media, injected into the diffusion chamber, and the opening subsequently sealed with sterile bone wax. The chamber was surgically placed under the dorsal skin of nude mice and left for 10 days. Empty vector (AAV-CMV) at a concentration of 100 MOI was used as a vector control. U-251 parental cells and AAV-CMV treated cells resulted in micro-vessel development (as indicated by arrows) with curved thin structures arising from pre-existing vessels. The formation of microvasculature was completely prevented in the cells treated with rAAV at a concentration of 100 MOI. *TN: tumor-induced neovasculature, PV: pre-existing vasculature.*

Figure 4

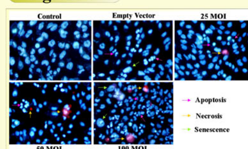

Figure 4. Double nuclear staining of U-251 cells with Hoechst 33342 and propidium iodide. U-251 cells were transfected with a recombinant adeno-associated virus carrying human TFPI-2 cDNA at concentrations of 25, 50 and 100 MOI. Empty vector at a concentration of 100 MOI was used as a vector control. The staining demonstrated sequential increase of apoptosis and necrosis, as well as senescent cells at 48 h after transfection.

Figure 5

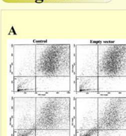

Figure 5A. Flow cytometry dot plot of U-251 cells after transfection with a recombinant adeno-associated virus carrying human TFPI-2 cDNA, at concentrations of 50 and 100 MOI. The cells were treated with 50 µg/ml propidium iodide for 30 min at 4C in the dark. Empty vector at a concentration of 100 MOI was used as a vector control. Column R1 represents the apoptotic cell population. A marked increase of cell population in the R1 area indicates increased apoptosis of U-251 cells after treatment with the virus carrying TFPI-2 gene.

Figure 5B. FACS histogram of U-251 glioblastoma cells after transfection with a recombinant adeno-associated virus carrying human TFPI-2 cDNA, at concentrations of 50 and 100 MOI. An empty vector at a concentration of 100 MOI was used as a vector control. The prominent increase of sub-G1 phase population of the cells indicates increased apoptosis after treatment with the virus carrying TFPI-2 gene.

Figure 5C. Quantitative representation of FACS data for live and apoptotic cells. Data are representative of 4 independent experiments (*$p < 0.001$).

Figure 6

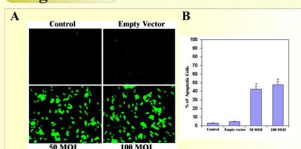

Figure 6A. Fluorescent TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay for apoptotic cells after transfecting U-251 cells with a recombinant adeno-associated virus carrying human TFPI-2 cDNA at concentrations of 50 and 100 MOI. An empty vector (EV) was used as a vector control at a concentration of 100 MOI (x 400). The results of the TUNEL assay demonstrate a dose dependant increase of apoptosis in U-251 cells after transfection with human TFPI-2 cDNA. Furthermore, the results showed a strong positive correlation with FACS analysis data for apoptotic cells.

Figure 6B. Quantitative evaluation of TUNEL assay using ImagePro Discovery software. Data are representative of 3 independent experiments in duplicate (*$p < 0.001$).

Figure 7

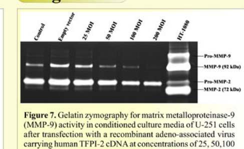

Figure 7. Gelatin zymography for matrix metalloproteinase-9 (MMP-9) activity in conditioned culture media of U-251 cells after transfection with a recombinant adeno-associated virus carrying human TFPI-2 cDNA at concentrations of 25, 50, 100 and 200 MOI. An empty vector was used as a vector control at a concentration of 100 MOI. All samples were treated with 200 nanomoles of Phorbol Myristate Acetate (PMA) per ml of serum free media for 12 h. Conditioned culture medium from HT-1080 fibrosarcoma cells was used as a standard for MMP-9 and MMP-2 activities. A dose dependant decrease of MMP-9 activity was observed in U-251 conditioned culture media after treating the cells with various concentrations of virus carrying TFPI-2 cDNA. MMP-9 activity was totally absent at 200 MOI indicating the secretion of the enzyme was completely inhibited.

Figure 8

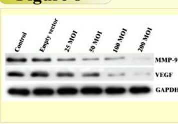

Figure 8. Western blot analysis for matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) in the cell lysate of U-251 cells after transfection with a recombinant adeno-associated virus carrying human TFPI-2 cDNA at concentrations of 25, 50, 100 and 200 MOI. An empty vector was used as a vector control at a concentration of 100 MOI. The membranes were reprobed and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content to demonstrate that similar amounts of protein had been loaded in each lane. A dose dependant decrease was observed in MMP-9 and VEGF protein levels after treating the cells with various concentrations of virus carrying TFPI-2 cDNA.

Figure 9

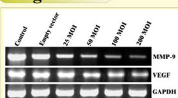

Figure 9. Semiquantitative RT-PCR analysis for the expression of MMP-9 and VEGF in U-251 cells. The cells were transfected with a recombinant adeno-associated virus carrying human TFPI-2 cDNA at concentrations of 25, 50, 100 and 200 MOI. An empty vector was used as a vector control at a concentration of 100 MOI. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. A dose dependant decrease was observed in the expression of MMP-9 and VEGF m-RNA after treating the U-251 cells with various concentrations of virus carrying TFPI-2 cDNA.

Conclusions

- Tissue factor pathway inhibitor-2 (TFPI-2) expression is lost during the progression of glioblastomas.
- The expression and activity of TFPI-2 is absent in the U-251 glioblastoma cell line.
- Restoration of TFPI-2 expression in U-251 cells resulted in decreased cell invasion and angiogenesis.
- Expression of TFPI-2 in U-251 cells resulted in the decreased expression and activity of MMP-9.
- Restoration of TFPI-2 in U-251 cells reduced the expression of VEGF both at the m-RNA and protein levels.
- Over expression of TFPI-2 in U-251 cells inhibited the formation microvasculature *in vivo* as demonstrated by the dorsal skin fold assay.

This poster is presented at the 97th Annual meeting of the American Association for Cancer Research held at Washington, DC, April 1-5, 2006