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Restoration of TFPI-2 in a Human Glioblastoma Cell Line Triggers Caspase Mediated Pathway and Apoptosis

Joseph George[1], Christopher S. Gondi[1], Meena Gujrati[2], Dzung H. Dinh[3] and Jasti S. Rao[1,3]

Departments of Cancer Biology and Pharmacology[1], Pathology[2] and Neurosurgery[3], University of Illinois College of Medicine, Peoria, IL 61656

Abstract

The induction of apoptotic pathways in cancer cells offers a novel and potentially useful approach to improve patient responses to conventional chemotherapy. Tissue factor pathway inhibitor-2 (TFPI-2) is a protease inhibitor that is abundant in the extracellular matrix (ECM) and highly expressed in non-invasive cells, but absent or undetectable in highly invasive human glioblastoma cells. Using a recombinant adeno-associated viral vector carrying human TFPI-2 cDNA (rAAV-TFPI-2), we stably expressed TFPI-2 in U-251 cells, a highly invasive human glioblastoma cell line. Our previous studies demonstrated that restoration of TFPI-2 in glioblastomas effectively prevents cell proliferation, angiogenesis and tumor invasion. In the present study, we determined whether TFPI-2 restoration could induce apoptosis through the caspase mediated signaling pathway. The results of caspase 9 and caspase 3 activity assays showed increased activity, which indicates enhanced apoptosis. Immunofluorescence for cleaved caspase 9 and 3 depicted increased expression and co-localization of both molecules. Western blot analysis demonstrated increased transcriptional activities of FasL, TNF-α, BAX, FADD and TRADD, as well as elevated levels of cleaved caspases and PARP. Semiquantitative RT-PCR depicted increased expression of TNF-α and FasL and the related death domains, TRADD and FADD. Taken together, these results demonstrate that restoration of TFPI-2 activates both intrinsic and extrinsic caspase-mediated, pro-apoptotic signaling pathways and induces apoptosis in U-251 cells. Furthermore, our study suggests that rAAV-mediated gene expression offers a novel and potential tool for cancer gene therapy.

Introduction

Glioblastomas are highly invasive and aggressive primary brain tumors associated with a dismal prognosis. The median survival of patients with glioblastoma treated with surgery, radiotherapy and chemotherapy is from 10 to 22 months. Limits in the efficacy of current treatment modalities call for the development of novel therapeutic approaches targeting the specific biological features of 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 P_1 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 the extracellular matrix (ECM). TFPI-2 exhibits strong inhibitory activity towards a broad spectrum of proteinases, including trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein and the factor VIIa-tissue factor complex. Recent studies have shown that TFPI-2 expression plays 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 in the induction of apoptotic pathways in glioblastomas.

Apoptosis, the programmed cell death, is critical for the development and maintenance of healthy tissues. There are two alternative pathways that initiate apoptosis: one is mediated by death receptors on the cell surface and the other is mediated by mitochondria.

The pathophysiological roles of the apoptotic signaling pathway have recently been identified in several human tumors including glioblastomas. In the present investigation, we selected an established glioblastoma cell line, U251 where TFPI-2 expression is totally absent due to the aberrant hypermethylation of TFPI-2 promoter CpG islands. We restored TFPI-2 protein levels in U251 cells through an adeno-associated viral vector carrying TFPI-2 gene and evaluated the effect of restored TFPI-2 on the signaling of cell surface death receptors as well as mitochondrial-mediated, pro-apoptotic pathways.

Figure 1

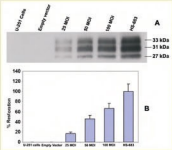

Figure 1. (A) Western blot analysis for the expression of tissue factor pathway inhibitor-2 (TFPI-2) in the extracellular matrix (ECM) of U251 glioblastoma cells. U251 cells were transfected with recombinant adeno-associated viral vectors engineered to express human TFPI-2 at concentrations of 25, 50 and 100 MOI and the levels of TFPI-2 in the ECM of U-251 cells were determined by western blotting. TFPI-2 protein from the ECM extracts of Hs 683 cells was used as a positive control. **(B) Quantitative evaluation of the percentage restoration of TFPI-2 protein levels in U-251 cells.** The Western blots were quantified using Image-Pro Discovery software to assess the percentage restoration of TFPI-2 protein levels in U-251 cells (Mean ± SD; N=6).

Figure 1 demonstrates that U251 cells transfected with the recombinant adeno-associated virus carrying human TFPI-2 cDNA successfully restored TFPI-2 protein levels in U251 cells in a dose dependent manner.

Figure 2

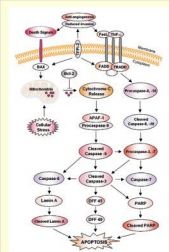

Figure 2. Mechanistic aspects of the caspase-mediated apoptotic pathway and the role of TFPI-2. Highly efficient, replication-deficient adenoviral vectors expressing TFPI-2 cDNA infect U251 cells through receptor-mediated endocytosis, traffic to the nucleus and use the host DNA machinery to transcribe and secrete TFPI-2. Recombinant TFPI-2 binds the ECM where it initiates phenotypic effects on cells. TFPI-2 restoration in U251 cells is associated with decreased angiogenesis, reduced cell migration, and invasion and initiation of apoptosis. The induction of apoptosis occurs through modification of death receptor/death ligand activity of the cell surface resulting in the activation of caspase-mediated apoptotic pathway.

Figure 3

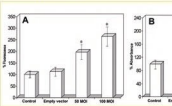

Figure 3. (A) Caspase 9 fluorometric protease activity assay of rAAV-TFPI-2-transfected U251 cells. Data represents 4 independent experiments (*p<0.001). **(B) Caspase 3 colorimetric protease activity assay of rAAV-TFPI-2-transfected U251 cells.** Data are representative of 4 independent experiments (*p<0.001).

Figure 3 demonstrates significant increase of caspases 9 and 3 in U251 cells transfected with rAAV-TFPI-2 compared to controls.

Figure 4

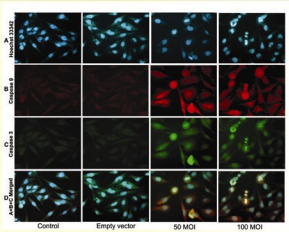

Figure 4. Double immunofluorescent staining for caspase 9 and caspase 3 expression. U251 cells were cultured on 8-well chamber slides at 2X10^5 cells per well and transfected with rAAV-TFPI-2 at concentrations of 50 and 100 MOI and EV at 100 MOI. The cells were incubated with appropriate primary and secondary antibodies and stained.

Figure 4 demonstrates marked increases in the activity of both caspases 9 and 3 after TFPI-2 restoration.

Figure 5

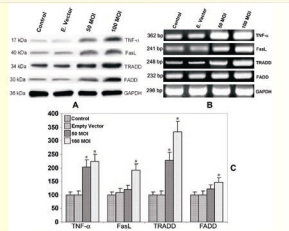

Figure 5. (A) Western blot analysis for the expression of TNF-α, FasL, FADD and TRADD in rAAV-TFPI-2-transfected U251 cells. Cells were transfected with 50 and 100 MOI of rAAV-TFPI-2 and EV at 100 MOI and the levels of TNF-α, FasL, FADD and TRADD by western blotting. The membranes were reprobed for GAPDH content to demonstrate that similar amounts of protein were loaded in each lane. **(B) Semiquantitative RT-PCR analysis for the expression of TNF-α, FasL, TRADD and FADD mRNA in rAAV-TFPI-2-transfected U251 cells using gene specific primers. (C) Real-Time RT-PCR analysis using SYBR green and gene specific primers for the quantitative evaluation of TNF-α, FasL, TRADD and FADD mRNA expression.** The data are mean S.D. of 6 samples (*p<0.001).

Figure 5 demonstrates a significant increase in the protein and mRNA levels of TNF-α, FasL, TRADD and FADD after restoration of TFPI-2 in U251 cells.

Figure 6

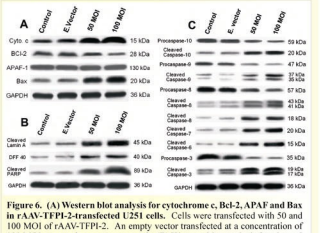

Figure 6. (A) Western blot analysis for cytochrome c, Bcl-2, APAF and Bax in rAAV-TFPI-2-transfected U251 cells. Cells were transfected with 50 and 100 MOI of rAAV-TFPI-2. An empty vector transfected at a concentration of 100 MOI was used as a vector control. **(B) Western blot analysis for cleaved lamin A, DFF 40 and cleaved PARP in rAAV-TFPI-2-transfected U251 cells. (C) Western blot analysis for caspases 10, 9, 8, 7, 6 and 3 in the cell lysates of rAAV-TFPI-2-transfected U251 cells.** In all samples, the nitrocellulose membranes were reprobed and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content to demonstrate that similar amounts of protein were loaded in each lane.

Figure 6 demonstrates that increased levels of Bax, released cytochrome c, activated caspases, cleaved lamin, cleaved PARP and DFF40 clearly indicate enhanced apoptosis after TFPI-2 restoration.

Conclusions

- Tissue factor pathway inhibitor-2 (TFPI-2) expression is lost during the progression of gliomas.
- TFPI-2 expression is absent in the highly invasive U251 glioblastoma cell line.
- Functional TFPI-2 protein is restored in U251 cells through adeno-associated viral vectors carrying TFPI-2 cDNA.
- Restoration of TFPI-2 in U251 cells resulted in increased caspase 9 and caspase 3 activities.
- Double immunofluorescence for caspase 9 and caspase 3 demonstrated increased staining and co-localization of both molecules as well as apoptosis after restoration of TFPI-2.
- Western blot and PCR analysis demonstrated increased expression of death domains after restoration of TFPI-2.
- TFPI-2 restoration in U251 cells resulted in increased levels of active caspases, cleaved PARP and DFF40 indicating enhanced apoptosis.

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