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Knockdown of TFPI-2 promotes migration and invasion of glioma cells

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ABSTRACT

Glioblastoma is the most malignant primary brain tumor. Due to its highly promigratory and proinvasive properties, standard therapy including surgery, chemotherapy and radiation fails in eradicating this highly aggressive type of cancer. Here, we evaluated the role of TFPI-2, a Kunitz-type serine protease inhibitor, which has been previously described as a tumor suppressor gene in several types of cancer, including glioma. TFPI-2 expression was absent in five of nine investigated high-grade glioma cell lines. Lentiviral knockdown of TFPI-2 in two of the TFPI-2-expressing cell lines (MZ-18 and Hs 638) was associated with pronounced changes in the cellular behavior: glioma cell proliferation, migration and invasion were significantly increased in TFPI-2 knockdown cells in comparison to empty vector-transfected control cells. Since TFPI-2 might exert its tumor suppressor function by inhibiting MMPs, we subsequently analyzed the effects of specific MMP inhibitors on cell invasion of TFPI-2 KD cells vs. control cells. The data obtained from these experiments suggest that the anti-invasive properties of TFPI-2 are associated with inhibition of MMP-1 and MMP-2, while inhibition of MMP-9 seems to play a minor role in this context. Our findings underscore the important role of TFPI-2 as a tumor suppressor gene and indicate that TFPI-2 may be a useful diagnostic marker for the aggressive phenotype of glial tumors.

Despite modern therapeutic regimens, patients suffering from malignant glioma still have a dismal prognosis. Histopathological characterization of gliomas allows classification into different subgroups (grade I–IV) [15]. The success of surgical resection and radiotherapy is hampered by the fact that malignant glioma cells deeply invade the adjacent brain tissue [7] which represents one of the major obstacles for therapy. Tumor invasion is a complex, multistep process and the mechanisms resulting in degradation of the ECM and tumor cell migration and invasion are not yet completely understood.

Tissue factor pathway inhibitor-2 (TFPI-2), an endogenous inhibitor of the tissue factor/factor VIIa complex (TF/FVIIa), is a ubiquitously expressed Kunitz-type serine protease inhibitor which also antagonizes the activity of other proteases such as plasmin, trypsin, chymotrypsin and cathepsin G. Its expression has been described to be inversely related to the degree of malignancy in several tumor entities. Methylation of the TFPI-2 promoter CpG islands has been proposed as a mechanism resulting in enhanced invasiveness and tumor growth [13,16].

The ECM is an important player in glioma cell invasion as it closely interacts with tumor cells along their way into the adjacent brain parenchyma. Glioma cells display a very unique and extensive repertoire of mechanisms effective in degrading the ECM resulting in fast and deep invasion of the tissue [3]. MMPs are critically involved in glioma cell invasion [18] and high expression of MMP-2 and MMP-9 in glioma has been previously demonstrated [25].

In this study, we further scrutinized the tumor suppressor function of TFPI-2 in glioma. To this end, we employed two reciprocal experimental models: (1) glioma cells with high TFPI-2 expression and a consecutive knockdown of TFPI-2 as well as (2) glioma cells with undetectable TFPI-2 expression and application of exogenous, recombinant TFPI-2. The data obtained by both approaches clearly reveals a crucial function of TFPI-2 in limiting glioma cell migration, invasion and proliferation.

Human glioma cell lines U87, U251, U343, U373, MZ-18, MZ-54, MZ-256 and MZ-304 and Hs 683 were cultivated in DMEM (Invitrogen, Karlsruhe, Germany da überall sonst auch nach der stadt steht) containing 10% FCS (Invitrogen), 100 U/ml Penicillin (Invitrogen), 100 mg/ml Streptomycin (Invitrogen) and 4 mM L-Glutamine (Invitrogen) and maintained in a cell incubator in a 5% carbon dioxide atmosphere at 37 °C in a relative humidity of 95%. Recombinant TFPI-2 (R&D Systems, Wiesbaden, Germany) was added at a concentration of 120 nM or 600 nM. To evaluate the effect of a TFPI-2 knockdown on MMP involvement we employed the following MMP-inhibitors: MMP-1 inhibitor (GM 1489, Calbiochem, Cat# 364200, K_i = 0.2 nM) was used at 1 nM

Abbreviations: TFPI-2, tissue factor pathway inhibitor-2; MMP, matrix metallo proteinase.

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concentration, MMP-2 inhibitor (MMP-2 Inhibitor I, Calbiochem, Cat# 444244, K_i = 1.7 μM) was employed at 5 μM concentration and MMP-9 inhibitor (MMP-9 Inhibitor I, Calbiochem, Cat# 444278, IC50 = 5 nM) was used at 10 nM concentration.

Western blotting was performed as described elsewhere [6]. For immunodetection, antibodies against TFPI-2 (1:200, Santa Cruz), MMP-1 (1:1000, R&D Systems), MMP-2 (1:1000, Abcam, Cambridge), MMP-9 (1:1000, New England Biolabs, Frankfurt, Germany) and GAPDH (1:20,000, Darmstadt, Germany) were used.

For assessing the proliferation of glioma cells we used MTT assays as published before [6].

For RT-PCR analysis RNA extraction was performed in two subsequent steps: After cultivation, cells were washed twice with PBS, trypsinated, centrifugated and again washed again; oder?. Cells were then lysed and RNA was extracted by using the Quiashredder and the RNeasy Midi Kit (Quiagen, Hilden) according to the manufacturer's instructions and stored at −21 °C. Total RNA concentration was measured by UV-photometry. Four microgram of total RNA from cell lysates was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen). 1 μl of the cDNA was used as template for the following PCR. The following primers were used for identification in our experiments: TFPI-2 (sense: 5′-AAAGCTTCGCTTTGCCAGTA-3′, antisense: 5′-ACGACCCCAAGAAATGAGTG-3′) 22 cycles; GAPDH (sense: 5′-CCTGACCTGCCGTCTAGAAA-3′, antisense: 5′-TTACTCCTTGGAGGCCATGT-3′) 20 cycles.

RNA was prepared as described above. After reverse transcription, transcripts were recovered by quantitative real-time PCR in a StepOnePlus Real-Time PCR system and TaqMan gene expression assays (Applied Biosystems, Darmstadt, Germany) in a 20 μl reaction. Gene expression assay Hs00197918_M1 (Applied Biosystems) was used to analyze TFPI-2 expression levels. Transcripts were normalized to TATA box binding protein as a housekeeping control gene. Analysis of expression changes was performed by $2^{-\Delta\Delta Ct}$ method.

To knock down TFPI-2, we employed lentiviral particles containing five different small hairpin RNAs (shRNAs) specific for TFPI-2 (SHVRS-NM_006528, Sigma–Aldrich) for transduction. Non-Targeting control transduction particles (SHC001V, Deisenhofen, Germany) were used as a negative, non-silencing control. New medium was added together with hexadimethrine bromide (Sigma–Aldrich) to a final concentration of 8 mg/l. Lentiviral particles were added to a MOI of 10 and incubated for 24 h followed by a medium change. Puromycin (Calbiochem) was added to a final concentration of 500 μg/l. For the subsequent experiments, only cultures transduced with one of the lentiviral sequences (SHVRSC-TRCN0000072725) were used.

Glioma cell migration and cell invasion were analyzed in scratch-migration-assays and modified Boyden-chamber invasion assays, respectively [23].

For cell death analysis supernatant was removed, cells were washed twice with PBS and trypsinated. Cells were resuspended in PBS and added to the supernatant that was kept before. Cell suspensions were centrifugated and resuspended in FACS HEPES buffer and stained with Annexin V and Propidium Iodide for 20 min. Flow cytomety was performed on a FACS Canto, BD Biosciences, Heidelberg, Germany and was followed by analysis using FACS Diva. Cells stained positive for Annexin V and Propidium Iodide were considered dead.All statistical analyses were performed using SPSS 16.0 software (SPSS GmbH Software; Munich, Germany). Data were compared by one-way ANOVA followed by Tukey's test. *P*-values smaller than 0.05 were considered to be statistically significant. Data are presented as means ± SEM.

For our initial expression studies, we employed a panel of eight grade III to IV high-grade glioma cell lines (U87, U251, U343, U373, MZ-18, MZ-54, MZ-206, MZ-304) and one oligodendroglioma cell line (Hs 683). There was undetectable TFPI-2 mRNA and protein expression in five of the cell lines (U251, U343, U373, MZ-54 and MZ-304) (Fig. 1A), suggesting silencing of the TFPI-2 promoter in these cells. Since it was previously reported that mRNA expression of TFPI-2 may be modulated by hypoxia and VEGF, we also investigated whether cultivation under hypoxic conditions affected expression of TFPI-2. There were no discernible differences in any of the investigated glioma cell lines, however (data not shown). To quantify TFPI-2 expression, we also performed quantitative RT-PCR with all RNAs prepared. TFPI-2 expression was shown to be highest in glioma cell line Hs 683 with detectable expression in cell lines U87, MZ-18 and MZ-256 (Fig. 1B).

To further investigate the tumor-suppressing role of TFPI-2 in our glioma cells, we performed a lentiviral-mediated stable knockdown of TFPI-2 in one high-grade astrocytoma (MZ-18) and one oligodendroglioma cell line (Hs 683) (Fig. 1C) both of which abundantly expressing TFPI-2 (Fig. 1A and B). A panel of five different TFPI-2-targeting sequences was transduced by lentiviral shRNA transfer. RT-PCR revealed major differences in the efficiency of TFPI-2 silencing by five different shRNA sequences (data not shown) with sequence 3 causing a complete knockdown of TFPI-2 expression as detected by RT-PCR and Western blotting (Fig. 1C). Of note, previous findings have suggested that overexpression of TFPI-2 can induce activation of caspases and apoptotic cell death in glioblastoma cells [5,24]. However, the knockdown of TFPI-2 did not change the amount of basal cell death (i.e. in the absence of additional death stimuli) in MZ-18 and Hs 683 cells in our experiments (data not shown).

Next, we analyzed the effects of the TFPI-2 knockdown on cell proliferation and migration in both cell lines. Knockdown of TFPI-2 led to an increased proliferation rate after 48 and 72 h, as analyzed by MTT assay (Fig. 1D).

To evaluate potential TFPI-2-dependent changes in cell migration, we performed scratch-migration-assays. Silencing of TFPI-2 evoked an induction of cell migration in cell lines MZ-18 and Hs-683 (Fig. 1E). In addition to the observed effects on cell migration, we also analyzed the potential role of TFPI-2 in preventing glioma cell invasion. To this end, we compared the effects of the TFPI-2 knockdown on the invasive behavior of MZ-18 and Hs 683 cells to their respective controls (Fig. 1F). In comparison to the high-grade astrocytoma cell line MZ-18, Hs 683 cells displayed a lower invasive behavior. Knockdown of TFPI-2 lead to a markedly increased invasive capacity, which was detectable in both cell lines. Hypoxia limited cellular migration and invasion, but could not abrogate the potentiating effects of the TFPI-2 knockdown (data not shown).

Conversely, addition of recombinant TFPI-2 to cell lines U251 and MZ-54 which do not express TFPI-2 led to a decrease in cell proliferation (Fig. 2A). Accordingly, addition of recombinant TFPI-2 to MZ-54 cells led to reduced cellular migration (Fig. 2B, left panel). Similar to its effects on proliferation and migration, recombinant TFPI-2 led to a significant, dose-dependent decrease in cellular invasion in MZ-54 cells (Fig. 2B, right panel).

To further analyze the molecular mechanisms of TFPI-2-mediated inhibition of cell invasion, we investigated the role of MMPs in this context. Analysis of MMP-1, MMP-2 and MMP-9 protein levels revealed a relatively uniform expression of MMPs in all cell lines except U373, indicating that loss of TFPI-2 expression had no major influence on MMP protein expression in the TFPI-2-deficient cells (Fig. 3A). Next, we investigated the effect of three different, specific MMP inhibitors (Fig. 3B). Inhibition of MMP-1 and MMP-2 was able to reduce cell invasion of MZ-18 and Hs 683 TFPI-2 KD cells to a level comparable to that observed in MZ-18 and Hs 683 control cells, respectively, whereas the MMP-9 inhibitor had no major inhibiting effect in TFPI-2 KD cells (Fig. 3B). Of note, treatment with MMP-1, MMP-2 and MMP-9 inhibitors at the concentrations used in this study did not result in any induction of