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

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A B S T R A C T
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.

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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, multiprocess 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) supplemented with 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, Ki = 0.2 nM) was used at 1 nM
according to the manufacturer's instructions and stored at 
-20 °C. Cells were then lysed and RNA was extracted by using PBS, trypsinated, centrifuged and again washed; subsequent steps: After cultivation, cells were washed twice with PBS, trypsinated, centrifuged and again washed; and finally, RNA was extracted using the Quiashredder and the RNeasy Midi Kit (Quiagen, Hilden, Germany). Cells were then lysed and RNA was extracted by using PBS, trypsinated, centrifuged and again washed

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, centrifuged and again washed again; and finally, RNA was extracted by using the Quiashredder and the RNeasy Midi Kit (Quiagen, Hilden, Germany)

Quantification of TFPI-2 expression was 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

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).
Fig. 1. TFPI-2 knockdown impedes glioma cell proliferation, migration and invasion. (A) Semiquantitative RT-PCR and Western blot analysis of TFPI-2 expression. The indicated glioma cell lines were cultivated for 24 h and mRNA expression levels of TFPI-2 and GAPDH were determined (upper panel). Protein expression of TFPI-2 was visualized by Western blotting (lower panel). (B) qPCR analysis of TFPI-2 expression levels in the indicated cell lines. Expression of TFPI-2 was normalized to cell line U87. (C) Lentiviral-mediated stable knockdown of TFPI-2 in glioma cell lines MZ-18 (left panel) and Hs 683 (right panel). Either an empty control vector (Ø-vec), or a vector containing an shRNA sequence directed against TFPI-2 were transduced. Cells were lysed and TFPI-2 mRNA and protein expression levels were determined. GAPDH served as a control. (D) MZ-18 or Hs 683 control cells (Ø-vec) and cells treated with a vector against TFPI-2 (TFPI-2 KD) were cultured for the indicated time and used for MTT analysis. (E) Cell count quantification of migrated cells. MZ-18 and Hs 683 control cells (Ø-vec) and cells treated with a vector against TFPI-2 (TFPI-2 KD) used for a migration assay as described above. Data are means ± SEM from n = 3 independent cultures. (F) Cells were added to modified Boyden-chambers at equal concentrations for invasion studies. After 24 h of incubation invaded cells with TFPI-2 knockdown were stained and counted. Cells treated with an empty control vector (Ø-vec) were used as a control. Data are means ± SEM from n = 10 high power fields. *P < 0.05 compared to controls.
glioma cell death in control and TFPI-2 knockdown cells of both cell lines analyzed (Fig. 3C).

Previous studies have proposed a role for TFPI-2 tumor growth, angiogenesis and invasion in other types of cancer [12,27] and it has been shown that TFPI-2 expression inversely correlates with glioma progression [21,16]. Previous findings have also demonstrated a potential role of TFPI-2 in regulating cell invasion of glial tumors [21,17]. In line with these observations, our studies revealed elevated glioma cell migration, invasion and proliferation after knockdown of TFPI-2, thereby underscoring the important tumor suppressor function of TFPI-2 in these types of tumors.

TFPI-2 is a protease inhibitor consisting of three Kunitz-type proteinase inhibitor domains [2] and strongly associated with the extra-cellular matrix (ECM). Other groups have demonstrated the presence of TFPI-2 in the ECM surrounding fibroblasts [20], endothelial cells [9] and also glioma cells [16]. TFPI-2 can inhibit various proteases including plasmin, trypsin, chymotrypsin, cathepsin G, plasma kallikrein and the TF/Factor VII complex [1,11,12]. Overactivation of proteolytic enzymes in gliomas is of great clinical significance as glioma cells hamper the success of surgery by excessive and deep infiltration of the adjacent brain tissue [13].

TFPI-2 has described as a broad inhibitor of ECM and MMPs [14,16]. Several studies have previously addressed the inhibitory effects of TFPI-2 on MMPs, established key mediators in tumor cell invasion [22]. TFPI-2 can inhibit MMP-1, MMP-2 and MMP-9 [8], but it is controversial whether this inhibition is carried out in a direct or an indirect manner. Since proteolytic activation of pro-MMPs requires the activity of serine proteases such as trypsin and plasmin, TFPI-2 may also mediate its inhibitory effects on MMP activity by inactivating these enzymes. In one study, TFPI-2 failed to coprecipitate with MMP-1, MMP-2 and MMP-9 [4]. Interestingly, our data suggest that the anti-invasive properties of TFPI-2 are associated with inhibition of MMP-1 and MMP-2 in glioma cells, while inhibition of MMP-9 seems to play a minor role in this context. In addition to enzymatic inhibition, TFPI-2 may also modulate the expression levels of MMPs. In one study, knockdown of TFPI-2 did not affect MMP-2 or MMP-9 expression, but was shown to increase MMP-1.

Fig. 2. Glioma cell migration and invasion is limited by TFPI-2. (A) Cell lines U 251 and MZ-54 with undetectable expression of TFPI-2 (con) and cells treated with recombinant TFPI-2 (120 nM) were cultured for the indicated time in a humidified incubator. After cultivation, MTT-metabolization was measured. Data are means ± SEM from n = 16 independent cultures. Data are normalized to the respective controls. * P < 0.05 compared to controls. # P < 0.05 compared to the indicated treatment. (B) Cell count quantification of migrated and invaded MZ-54 cells. After cultivation for 24 h MZ-54 cells were prepared for migration and invasion studies as described above and subsequently treated with recombinant TFPI-2 at the indicated concentration or not. Data are means ± SEM from n = 3 independent cultures (left panel). Data are means ± SEM from n = 10 high power fields (right panel). * P < 0.05 compared to controls. (C) Exemplary images (20×) of migrated and invaded MZ-54 cells. White bars indicate the dimensions of the scratch. As treatment, MZ-54 cells were incubated with recombinant TFPI-2 (120 nM). Untreated cells were used as a control.
Fig. 3. Role of TFPI-2 in inhibition of MMP-1, MMP-2 and MMP-9. (A) Expression analysis of MMP-1, MMP-2 and MMP-9 in all glioma cell lines analyzed. The indicated glioma cell lines were cultivated for 24 h and MMP expression was visualized by Western blotting. (B) MZ-18 and Hs 683 cells were treated with either an empty control vector (Ø-vec) or with a vector specific for TFPI-2 (TFPI-2 KD). Cells were incubated together with the indicated MMP-Inhibitors (MMP-1, 1 nM; MMP-2, 5 μM; MMP-9 10 nM) in modified Boyden chambers. Cells treated with DMSO were used as a control. Data are presented as means from $n=10$ microscopic fields. *$P<0.05$ compared to control cells.

expression in an in vitro lung cancer model[10]. However, we found no major expression changes of MMP1, MMP2 and MMP9 in TFPI-2-proficient vs. TFPI-2-deficient glioma cells, suggesting that the anti-invasive properties of TFPI-2 are largely mediated by inhibiting the enzymatic activity of MMPs.

Ectopic overexpression of TFPI-2 in glioma cell lines expressing little to none endogenous TFPI-2 also was shown to induce apoptotic glioma cell death[5,24]. In the cell lines used in our study the TFPI-2 knockdown had no impact on basal cell death in the cultures. Nevertheless, TFPI-2 deficiency might result in a higher resistance against chemotherapeutic agents and apoptosis, thereby further enhancing tumor malignancy.

In conclusion, our data emphasize the important tumor suppressor function TFPI-2 in glial tumors and shed new light on the complex mechanisms underlying enhanced glioma cell migration and invasion during tumor progression.
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