LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Tissue factor mediates the HGF/Met-induced anti-apoptotic pathway in DAOY medulloblastoma cells

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Abstract The classical treatment scheme for medulloblastoma (MB) is based on a tri-therapy approach consisting of surgical tumor resection, craniospinal axis radiation and chemotherapy. With current treatments relying mainly on non-specific cytotoxic therapy, a better understanding of the mechanisms underlying resistance to these treatments is important in order to improve their effectiveness. In this study, we report that stimulation of DAOY with HGF resulted in the protection of these cells against etoposide-induced apoptosis, this anti-apoptotic effect being correlated with an increase in the expression of tissue factor (TF), the initiator of the extrinsic pathway of coagulation. HGF-mediated protection from apoptosis was abolished by a c-Met inhibitor as well as by siRNA-mediated reduction of TF levels, implying a central role of Met-dependent induction of TF expression in this process. Accordingly, stimulation of DAOY with FVIIa, the physiological ligand of TF, also resulted in a significant protection from etoposide-mediated cytotoxicity. Overall, our results suggest the participation of the haemostatic system to drug resistance in MB and may thus provide novel therapeutic approaches for the treatment of these tumors.

Introduction

Medulloblastoma (MB) is the most common malignant tumor of the central nervous system (CNS) in children and occurs bimodally, with peak incidences between 3 and 4 years and between 8 and 9 years of age [1]. These neuroectodermal tumors account for almost 20% of all intracranial tumors in children, and for 40% of all childhood posterior fossa tumors [2].

For many years now, conventional therapies for MB have consisted of surgical resection, craniospinal axis radiation and chemotherapy. However, this treatment scheme relies mainly on conventional cytotoxic therapies and its efficacy remains limited, mainly due to its lack of specificity [1]. In order to improve the efficiency of these therapies, several studies have been directed towards elucidation of the molecular pathways involved in MB, leading to the identification of a number of receptor tyrosine kinases (RTK) as key components involved in MB progression [3–8].

Among these, the scatter factor/hepatocyte growth factor:Met (SF/HGF:Met) pathway has emerged as an important contributor to human neoplasia [9], and Met

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expression in MB is associated with poor prognosis [10, 11]. The HGF/Met pathway is implicated in numerous pro-tumorigenic actions such as cell cycle progression [12], cancer cell migration, invasion and metastasis [13, 14], tumor angiogenesis [15] and protection from apoptosis [16].

Interestingly, the *MET* oncogene also drives a genetic program linking cancer to haemostasis, principally by promoting PAI-1 and Cox-2 protein expression [17]. Supporting an involvement of the coagulation system in Met function, we also recently identified the HGF/Met pathway as being responsible for tissue factor (TF) expression in MB [18]. TF, a class 2 cytokine receptor, is a 47 kDa transmembrane glycoprotein which was originally identi-fied as the main trigger of the extrinsic pathway of blood coagulation [19]. TF could mediate its cellular actions via its extracellular catalytic domain or through its short intracellular cytoplasmic tail [19].

Beside its haemostatic function, TF plays many non-haemostatic functions in human neoplasia. For example, TF plays a crucial role in cancer cell migration, via both extracellular and intracellular domain-induced signaling, thus promoting cancer invasion and metastasis processes [20]. Moreover, TF regulates a complex cellular circuitry promoting tumor angiogenesis through signaling by its cytoplasmic tail [21]. Finally, like Met, TF and its ligand (FVIIa) are known to prevent apoptosis in cancer cells, thus allowing tumor expansion [22].

In this work, we observed that stimulation of DAOY with HGF leads to a Met-dependent up-regulation of TF expression levels and that this event is correlated with increased resistance of these cells to apoptosis induced by the chemotherapeutic drug etoposide. Therefore, TF expression in MB promotes tumor cell survival, thus low-ering chances of therapeutic success.

Materials and methods

Materials

Cell culture media were obtained from Wisant (St-Bruno, QC, Canada) and serum were purchased from Hyclone Laboratories (Logan, UT). Recombinant hepatocyte growth factor (HGF) was purchased from R&D Systems (Minne-apolis, MN). FVIIa was kindly provided by Dr. Georges-Étienne Rivard (Service d'Hématologie-Oncologie, Hôpital Sainte-Justine, Montréal, QC, Canada). c-MET specific inhibitor (SU11274) was obtained from EMD Chemicals (Gibbstown, NJ). All products for electrophoresis and wes-tern blotting were purchased from Bio-Rad (Hercules, CA). Anti-TF antibody was obtained from American Diagnostica (Stamford, CT) while anti-p53 rabbit polyclonal antibody

came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and anti-phospho-p53 monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-Erk1/2, anti-phospho-Erk1/2, anti-Akt, anti-phospho-Akt, anti-p38 and anti-phospho-p38 rabbit monoclonal antibodies were obtained from Cell Signalling Technology (Beverly, MA). Anti-Vimentin mouse monoclonal antibody was pur-chased from Santa Cruz Biotechnology Inc. Anti-Bcl-2 rabbit polyclonal antibody was obtained from Upstate (Charlottesville, VA). Transfection reagent Lipofect-amine^{TM} 2000 was purchased from Invitrogen (Burlington, ON, Canada). Acetyl-Asp-Glu-Val-Asp-7-AFC (Ac-DEVD-AFC), a specific substrate for caspase-3, was pur-chased from BioSource International (Camarillo, CA).

Cell culture

The medulloblastoma cell line DAOY was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C under a humidified atmosphere con-taining 5% CO_2. DAOY were maintained in Eagle's modified minimum essential medium (EMEM) supplemented with 10% (v/v) Bovine Calf Serum (BCS), 4 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin.

Cell stimulation with HGF and membrane isolation

DAOY cells were grown to confluence in 100 mm Petri dishes and starved for 48 h in serum-free media. In the time-course experiment, cells were stimulated for 6 h at 37°C with 50 ng/ml HGF. In experiments using SU11274, cells were treated for 1 h with the inhibitor (5 μM) prior to HGF stimulation. Cells were then washed with 4 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM sodium fluoride) (buffer A). Cells were scraped with 2 ml of buffer A. Cells were resuspended in 500 μl of buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM sodium fluoride) (buffer B) and homogenized using a Polytron twice for 30 s at speed 4. Cells were then cen-trifuged at 4,000 rpm for 5 min at 4°C and the resulting supernatants were centrifuged again at 55,000 rpm for 60 min at 4°C. Pellets were resuspended in 50 μl of buffer B. Protein concentrations were determined by the bi-cinchoninic acid method (Pierce).

Western blotting procedures

Identical amounts of protein from each sample were pre-pared in Laemmli sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.00125% bromophenol blue), boiled for 5 min and resolved by SDS-PAGE. Proteins were transferred onto

polyvinylidene difluoride membranes (PVDF) and blocked overnight at 4°C with TBST buffer (20 mM Tris–HCl, pH 7.5, 147 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin (BSA) or 5% milk. Membranes were incubated with the specific primary antibody for 1 h at room temperature. Immunoreactive bands were revealed following a 1 h incubation with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat antibodies, and the signals were visualized using an ECL detection system.

siRNA transfection method

Custom-made siRNA (hTF167i) targeting TF (target sequences are sense, r(GCG CUU CAG GCA CUA CAA A)dTdT and antisense, r(UUU GUA GUG CCU GAA GCG C)dTdT) [23] as well as a predesigned siRNA against this gene (SI00031066) were purchased from Qiagen. Transfections were done in serum- and antibiotics-free EMEM media. Meanwhile, siRNA TF or control siRNA (at 50 nM each) were mixed with 30 µl of Lipofectamine™ 2000 reagent and incubated for 20 min at room temperature. After this incubation, the mix was added to the cells for 6 h at 37°C. After this period, cell media were replaced with media containing 10% BCS as described in the previous section.

Fluorometric caspase-3 assay

Confluent cells were made quiescent by 24 h starvation in a medium containing 0.5% serum and cells were then stimulated (or not) with HGF (50 ng/ml) for 6 h. After this stimulation, cells were exposed to etoposide (50 µM) for 3 h. Cells were collected, washed in cold PBS and lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4°C. Lysates were clarified by centrifugation at $16,000 \times g$ for 20 min. Caspase activities were determined by incubation with 50 µM fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-AFC (Ac-DEVD-AFC, caspase-3-specific) in assay buffer (50 mM HEPES–NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, and 1 mM EDTA) on 96-well plates. The release of AFC ($\{\lambda\}$ex = 400 nm, $\{\lambda\}$em = 505 nm) was monitored for 30 min at 37°C on a SpectraMAX Gemini fluorescence plate reader (Molecular Devices). Caspase-3 activities were expressed as rfu/s/µg of protein used in the assay.

Staining of apoptotic cells

DAOY were grown on glass coverslips, starved and incubated or not with HGF prior to treatment with etoposide, as described above. After the treatment, the coverslips were washed twice with PBS, fixed for 60 min in 3.7% paraformaldehyde/PBS at room temperature, washed again twice in PBS and then permeabilized in 0.2% Triton X-100/PBS for 5 min at 4°C. Permeabilized cells were washed in PBS and the TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostic), following the manufacturer's protocol.

Cell survival

DAOY were plated at 25,000 cells per well and allowed to attach for 8 h at 37°C. Cells were then serum-starved for 32 h and FVIIa was added for another 6 h. Etoposide (0, 50 or 100 µM) was added to cells for 8 h and cell survival was quantified by cleavage of WST-1 in formazan by spectrophotometry at 450 nm.

Statistical analysis

The data are presented as means ± SEMs and statistical analyses were performed with Student's *t*-test where one group was compared with the control group.

Results

The HGF/Met pathway protects medulloblastoma cells (DAOY) against etoposide-induced apoptosis

DAOY cells treated with etoposide (50 µM) for 3 h showed characteristics of apoptosis, including DNA fragmentation and increased caspase-3 activity (Fig. 1a). However, when DAOY were stimulated with different concentrations of HGF (0–50 ng/ml) for 6 h prior to etoposide treatment, the cells showed a dose-dependent protection against the drug, as reflected by a marked reduction in caspase-3 activation and DNA fragmentation (Fig. 1a). In agreement with our previous observation that activation of the HGF/Met pathway resulted in a marked increase in TF expression in DAOY [18], we observed that the anti-apoptotic effect of HGF correlated with increased TF expression in these cells (Fig. 1b). To further explore the molecular mechanisms underlying HGF/Met anti-apoptotic activity, we monitored several proteins that play important roles in the apoptotic process. As shown in Fig. 1b, HGF had no effect on the expression levels of the antiapoptotic proteins Bcl-2 and survivin, but significantly inhibited (30%) the phosphorylation of p53 on Ser15 at higher concentrations (arrow). Interestingly, SU11274, a specific inhibitor of Met, completely blocked HGF-dependent TF expression and also reversed the protective effect of HGF by rendering DAOY cells sensitive to etoposide (Fig. 1c). These data indicate that the HGF anti-apoptotic function is
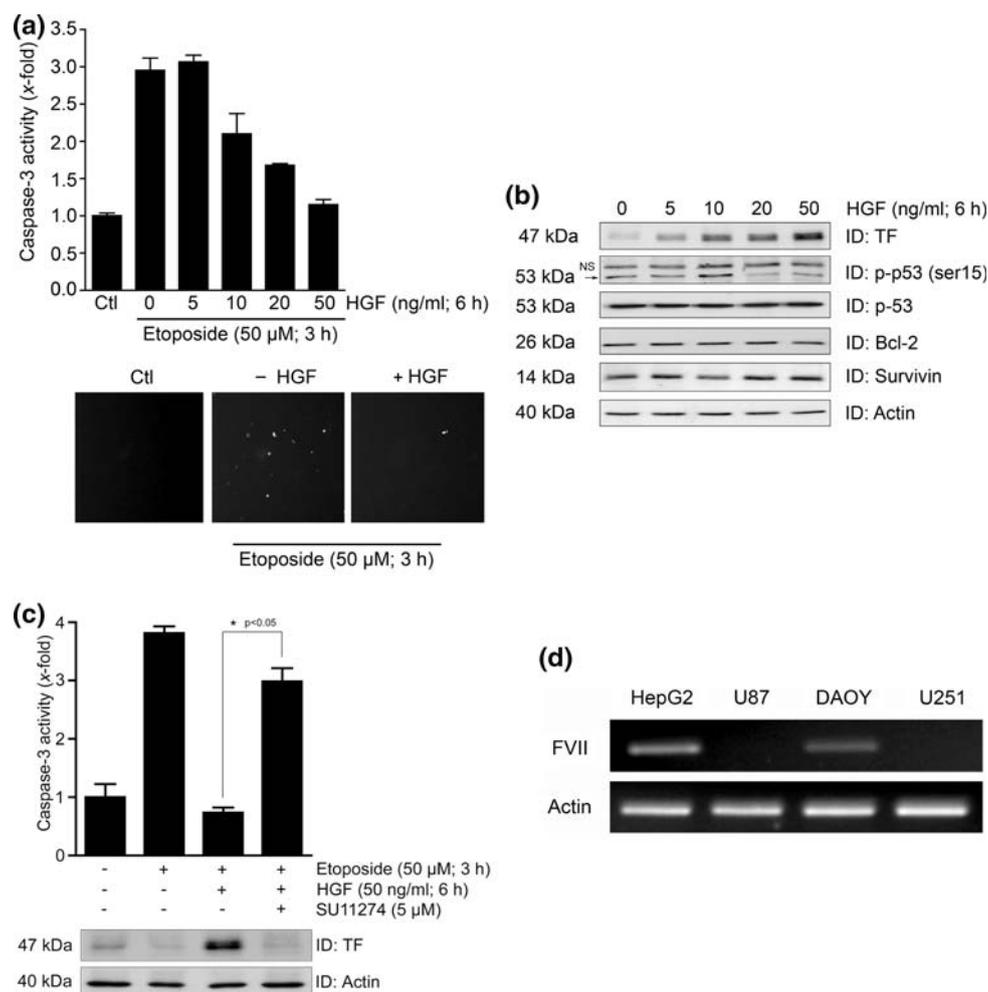
Fig. 1 Correlation between HGF-induced apoptosis protection and TF expression in DAOY. Confluent DAOY in 60-mm dishes were serum-starved in 0% serum for 48 h. DAOY were then stimulated with various HGF concentrations ranging from 0 to 50 ng/ml for 6 h. After stimulation cells were treated with etoposide (50 μM) for 3 h. **a** *Upper panel*: DAOY were harvested and lysed as described in "Materials and methods" section and cell lysates were used for the fluorometric caspase-3 assay. *Lower panel*: DAOY grown on coverslips were either left untreated or treated for 6 h with 50 ng/ml HGF and then incubated with 50 μM etoposide for 3 h. Fragmented DNA was detected by the TUNEL assay. **b** Equal amounts of protein from cell lysates were subjected to electrophoresis. TF and other apoptosis-related protein expression levels were visualized by western blot, using specific antibodies. *NS*: Non specific **c** DAOY in 60-mm dishes were serum starved in 0% serum for 48 h. Cells were pre-treated (or not) for 1 h with the Met specific inhibitor SU11274 (5 μM). After pre-treatment DAOY were stimulated (or not) with HGF (50 ng/ml) for 6 h and then etoposide (50 μM) was added for another 3 h. Cell lysates were used in the fluorometric caspase-3 assay and for western blot analysis. **d** One microgram of total RNA isolated from HepG2, DAOY, U87 and U251 cell lines was amplified by one-step RT-PCR using specific primers annealing to human FVII, or actin as a control

correlated with an increase in TF expression, suggesting a potential role for this protein in the survival response induced by HGF/Met. Since biological activity of TF requires the interaction of this protein with its natural ligand FVIIa, this suggests that formation of this complex may be involved in the cytoprotective effect of TF. Intriguingly, however, TF-mediated cytoprotection was observed in the absence of serum (a source of FVII), raising the interesting possibility that DAOY endogenously produce this factor. In this respect, RT-PCR analysis indicated that these cells indeed express FVII mRNA

whereas other brain tumor cell lines such as U87 and U251 showed no detectable levels of this transcript (Fig. 1d).

Effect of TF silencing on HGF-induced apoptosis protection in DAOY

In order to explore in more detail the link between TF expression and HGF-induced protection from apoptosis, we used siRNA technology to inhibit TF expression in DAOY. As shown in Fig. 2a, cells transfected with a control siRNA and stimulated with 50 ng/ml HGF for 6 h were resistant to

etoposide-induced caspase-3 activity. By contrast, cells transfected with the TF siRNA were not protected from apoptosis despite incubation of the cells with HGF prior to etoposide treatment.

In order to confirm this effect, we next monitored the impact of TF silencing on caspase-3 and vimentin cleavage, two hallmarks of apoptosis. In order to exclude non specific silencing of the TF gene, an additional siRNA was included in the experiment. Immunoblotting of the cell lysates showed a high HGF-dependent expression of TF in cells transfected with the control siRNA whereas TF levels were markedly reduced by both siRNAs targeting this gene (Fig. 2b). The etoposide-induced increase in caspase-3 activity observed in Fig. 2a is corroborated by the cleavage of this enzyme, as reflected by the appearance of 17 and 15 kDa fragments, and this activation of caspase-3 was inhibited by HGF under control siRNA conditions. In cells treated with the TF siRNAs, however, etoposide alone induced the cleavage of caspase-3 and HGF did not inhibit this activation, as shown by the unchanged quantity of the fragments. Activation of caspase-3 by etoposide could also be visualized by the cleavage of vimentin, a well-known

substrate of caspase-3, and again this process was reduced by preincubation with HGF. However, when TF expression was suppressed, HGF did not prevent the cleavage of vimentin. Overall, these results link the HGF anti-apoptotic activity to the expression of TF in DAOY, this process being associated with the inhibition of a caspase-3 dependent mechanism.

### TF:FVIIa increase cell survival upon etoposide treatment

In order to further document the role of TF in the protection of DAOY from etoposide-induced cell death, we next examined the influence of FVIIa, the physiological ligand of TF, on this process. In agreement with many reports showing that the interaction of TF with FVIIa activates an anti-apoptotic pathway in various cancer cells [24, 25], we observed that addition of FVIIa to DAOY resulted in the activation of several pathways involved in cell survival, including Erk-1/2, p38 MAPK and AKT (Fig. 3a). Interestingly, we also observed that FVIIa induced a complete inhibition of the phosphorylation of serine 15 on p53, as
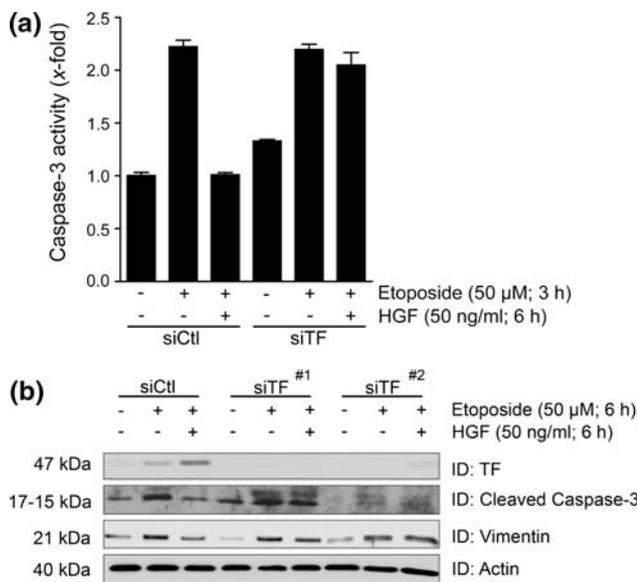
Fig. 2 TF mediates the HGF-induced apoptosis protection in DAOY. **a** DAOY were transfected with control or TF siRNAs as described in "Materials and methods" section and allowed to recover for 24 h before being made quiescent by serum-starvation in 0.5% serum for another 24 h. Cells were then stimulated (or not) with HGF (50 ng/ml) for 6 h before being treated (or not) with etoposide (50 μM) for an additional 3 h. DAOY were harvested and lysed as described in "Materials and methods" and cell lysates were used for the fluorometric caspase-3 assay. **b** Cells were transfected with control and two distinct TF siRNAs and subjected to HGF and etoposide treatments as described above. Equal amounts of protein from cell lysates were subjected to electrophoresis and western blotting. Levels of TF, cleaved caspase-3 and cleaved vimentin were detected using specific antibodies
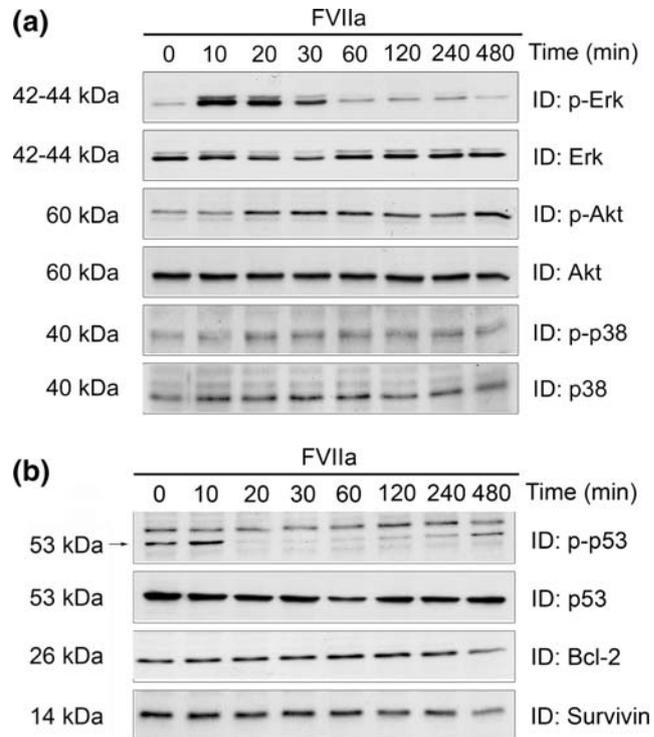
Fig. 3 Effect of FVIIa on DAOY signal transduction processes. Confluent and serum-starved DAOY were stimulated from 0 to 8 h with FVIIa (50 nM) and after cell lysis, equal amounts of protein were subjected to electrophoresis to monitor variations in the extent of phosphorylation of Erk-1/2, p38 and AKT (**a**) as well as on the levels of markers of different apoptotic pathways (**b**)
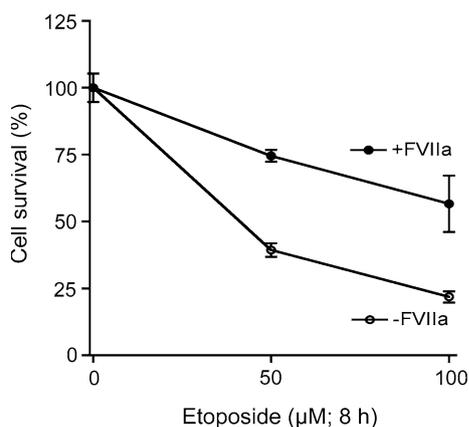
Fig. 4 FVIIa renders DAOY more resistant to etoposide treatment. DAOY were plated in 96-well plates and allowed to attach for 8 h before serum starvation in 0% serum for 24 h. FVIIa was then added (or not) for 6 h and cells were treated with different concentrations of etoposide ranging from 0 to 100 μM for 8 h. Cell survival was quantified as described in "Materials and methods" section

previously observed following stimulation of the cells with HGF (Fig. 1b).

We next conducted in vitro cytotoxicity experiments to determine whether the effect of FVIIa on intracellular anti-apoptotic pathways was correlated with increased cell survival. DAOY, which express detectable basal levels of TF, were grown in the presence or absence of FVIIa and were then incubated with different concentrations of etoposide (0–100 μM). As shown in Fig. 4, we observed that in the presence of FVIIa, 57% of DAOY survive even at high etoposide concentrations (100 μM), while in the absence of FVIIa less than 20% of cells remain viable after this treatment. These results suggest that, in a tumor environment where the TF:FVIIa complex is active, cancer cells are likely to be more resistant to chemotherapeutic drugs. Since we have previously shown that MB tumors expressing high levels of Met also express high levels of TF [18], these findings may help to explain how these tumors are more resistant to chemotherapy.

Discussion

In the present work, we observed that HGF exerts a cyto-protective effect in the MB DAOY cell line, as reflected by the c-Met-dependent increase in the resistance of these cells to etoposide-induced apoptosis. Such an involvement of the HGF/Met pathway in the protection of tumor cells from apoptotic death has been previously described for glioma and epithelial carcinomas [16, 26] and thus suggest that this pathway may represent a common anti-apoptotic mechanism used by cancer cells to circumvent cytotoxic drug-induced cell death.

One major finding of this study is that the cytoprotective effect of HGF was correlated with a marked increase in TF expression, suggesting that this process participate to the resistance of DAOY to etoposide. Such a close relationship between increased TF levels and HGF-mediated anti-apoptotic activity is strengthened by the observation that reduction of TF expression by RNA interference restored the sensitivity of DAOY to etoposide. Moreover, the inhibition of HGF-mediated TF expression using a kinase inhibitor specific to c-Met was correlated with an increase in etoposide-mediated apoptosis, again suggesting the participation of TF to the cytoprotective effect of HGF. To the best of our knowledge, this is the first report establishing a link between the anti-apoptotic activity of the HGF/Met signaling axis and TF expression.

The association between TF expression and malignancy is well established and this protein is now recognized to play an important role in the progression of cancer [27–30]. In addition to its activation of a variety of intracellular signaling pathways that lead to stimulate tumor cell migration [20] and angiogenesis [21], and thereby contribute to cancer metastasis [31], additional studies have shown that TF expression may also protect tumor cells from apoptosis, as reflected by impaired cell survival and apoptosis following downregulation of TF levels [32]. Since increased TF expression is a widespread feature of tumor cells [33] and that high HGF levels are associated with tumorigenicity and malignant progression of several types of cancers, including MB [10, 11], the close relationship existing between these two pathways on cell survival is thus likely to significantly contribute to tumor progression.

The mechanisms underlying the TF-dependent cyto-protective effect of HGF remain to be established. In agreement with previous studies showing that the binding of FVIIa to TF is necessary for activation of signaling pathways downstream of TF [34], we observed that addition of FVIIa to DAOY resulted in a marked protection of these cells from etoposide-mediated cell death, suggesting that the cytoprotective effect requires formation of a TF:FVIIa complex. Although FVII is synthesized in the liver and released into the bloodstream under physiological conditions, we observed that DAOY synthesize FVIIa endogenously, as reported for some cancer cell lines [35], further suggesting that a FVII/TF complex is involved in the observed cytoprotective effect.

TF induces the activation of several anti-apoptotic pathways in various tumor cell types. For instance, in breast cancer cells, TF:FVIIa inhibited apoptosis by upregulating survivin through the Erk-1/2 MAPK and AKT pathways [24] while in neuroblastoma, TF:FVIIa interaction induced Bcl-2 expression via the JAK/STAT5 signaling pathway [25]. Since we observed that neither survivin nor Bcl-2 were

affected by FVIIa or HGF stimulation of DAOY cells, it is likely that other cell survival pathways are activated by these ligands in this cell line. In this respect, the TF:FVIIa complex is known to signal directly by cleaving the protease activated receptor PAR-2 in several tumors [36]. Since activation of PAR receptors induces neuroprotection [37], the potential involvement of this pathway deserves future investigation.

We also observed that stimulation of DAOY with HGF or FVIIa inhibits p53 phosphorylation on serine 15, a residue that is normally phosphorylated upon DNA damage induced by UV radiation, chemical agents or chemotherapeutic drugs [38]. Phosphorylation at Ser15 activates p53 by diminishing its affinity to MDM2, a negative regulator of p53, therefore allowing p53 translocation to the nucleus in order to achieve cell cycle arrest or to trigger apoptosis [38]. This effect of HGF on p53 is interesting since other investigators have shown that MB overexpress the p53-inactivating oncogene *WIP1/PPM1D* which encodes *Wild-type p53-induced phosphatase-1*, a negative regulator of p53 [39]. Based on these observations, it is thus tempting to speculate that the anti-apoptotic effect of HGF could involve an alteration of the p53 phosphorylation status.

In a clinical context, our results suggest that tumors expressing high levels of both Met and TF are more likely to be resistant to chemotherapy. These observations, as well as other studies [40], thus suggest that the combination of existing anticoagulant drugs and chemotherapy could improve chemotherapeutic treatment. For example, Warfarin, the most commonly used anticoagulant, specifically targets the synthesis of vitamin K clotting factors such as FVII. Therefore, pre-treatment with low-dose Warfarin before initiating chemotherapy protocols would likely minimize FVII levels in circulation and, at the same time, could sensitize tumors to chemotherapeutic drugs [41]. This strategy has already been tested for aggressive metastatic breast cancer and has proven to be efficient [42].

The hyper-activation of the haemostatic system is more than merely a consequence of clinical cancer and recent work has shown that it plays an important role in the strategy used by tumors to promote their growth. For this reason, the coagulation system as a whole has been recently gaining momentum as a promising target for the treatment of various cancers, including brain tumors [43]. Further studies aimed at the elucidation of the mechanisms underlying the participation of the haemostatic system in the drug resistance of MB may provide interesting novel therapeutic approaches for the treatment of pediatric brain tumors.

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