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Necrosis Induction in Glioblastoma Cells Reveals a New ''Bioswitch'' Function for the MT1-MMP/G6PT Signaling Axis in proMMP-2 Activation versus Cell Death Decision[1]

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Abstract

Cytoskeleton disorganization is an early step in the activation process of matrix metalloproteinase 2 (MMP-2) by membrane type 1 MMP (MT1-MMP) but is also associated with endoplasmic reticulum (ER) dysfunction and subsequent cell death. Given evidence that the ER-embedded glucose-6-phosphate transporter (G6PT) regulates glioblastoma cell survival and that MT1-MMP is a key enzyme in the cancer cell invasive phenotype, we explored the molecular link between G6PT and MT1-MMP. Cytoskeleton-disrupting agents such as concanavalin A (ConA) and cytochalasin D triggered proMMP-2 activation and cell death in U87 glioma cells. ConA decreased G6PT gene expression, an event that was also observed in cells overexpressing the full-length recombinant MT1-MMP protein. Overexpression of a membrane-bound catalytically active but cytoplasmic domain–deleted MT1-MMP was unable to down-regulate G6PT gene expression or to trigger necrosis. Gene silencing of MT1-MMP with small interfering RNA prevented proMMP-2 activation and induced G6PT gene expression. ConA inhibited Akt phosphorylation, whereas overexpression of recombinant G6PT rescued the cells from ConA-induced proMMP-2 activation and increased Akt phosphorylation. Altogether, new functions of MT1-MMP in cell death signaling may be linked to those of G6PT. Our study indicates a molecular signaling axis regulating the invasive phenotype of brain tumor cells and highlights a new ''bioswitch'' function for G6PT in cell survival.

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Introduction

The endoplasmic reticulum (ER) is a membrane-bound organelle present in all eukaryotic cells. Recently, ER stress signaling has been linked to disease states involving insulin resistance, disordered lipid metabolism, and hypoxia tolerance in tumor progression [1,2]. In addition, the ER is a multifunctional metabolic compartment that controls entry and release of calcium, sterol biosynthesis, apoptosis, and the release of arachidonic acid [3,4]. Despite its complex organization, the ER is a continuous membrane compartment whose architecture depends on microtubule dynamics [5]. The ER is primarily known as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. Recent evidence suggests that the microtubulin cytoskeleton and the centrosomes (the microtubulin cytoskeleton–organizing centers) are essential for the trafficking and internalization of 'the membrane-bound matrix metalloproteinase MT1-MMP [6], involved in brain tumor cell invasion, extracellular matrix (ECM) degradation and cell–ECM interaction [7]. Interestingly, altered expression, maturation and trafficking of MT1-MMP to the plasma membrane were observed in diabetic states [8,9], a condition known to upregulate the expression of an ER-embedded protein, the glucose-6-phosphate transporter (G6PT) [10]. G6PT expression was shown to be down-regulated by MT1-MMP in bone marrow–derived stromal cells, where it was suggested to provide a molecular link between proMMP-2 activation and chemotaxis processes in cell mobilization [11].

Several factors are required for optimum protein folding, including ATP, Ca^{2+}, and an oxidizing environment that will allow disulfide-bond formation [12]. Because of this specialized environment requirement, the stresses that perturb cellular energy levels, redox state, or Ca^{2+} concentration can often result in the intracellular accumulation of unfolded protein, which is called ER stress response. Recently, we have provided evidence that

Abbreviations: ConA, concanavalin A; CytoD, cytochalasin D; ECM, extracellular matrix; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P transporter; MMP, matrix metalloproteinase; PI, propidium iodide; siRNA, small interfering RNA

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G6PT regulated U87 glioma cell chemotaxis [13] and survival [14]. Tumor cells often show evidence of constitutive ER stress, possibly due to hypoxia and glucose depletion [15]. In fact, the ATP-depleting agents and ER stress inducers 2-deoxyglucose and 5-thioglucose have been shown to inhibit MMP-2 secretion from U87 glioma cells [13], a process known to contribute to tumor development [16]. G6PT is thought to have a role in sequestering intracellular Ca^{2+} within the ER through an ATP-mediated process [17]. Because manipulating the ER stress response of tumor cells is a promising therapeutic strategy [15] and because various anticancer drugs have been shown to induce ER stress and to affect the invasive or metabolic control of cancer cells [18,19], we explored the potential molecular link between MT1-MMP and G6PT functions within the ER that could potentially regulate the brain tumor cell invasive phenotype.

Results

Differential Induction of Cell Necrosis and Cell Apoptosis by Cytoskeleton-Disrupting Agents

Concanavalin A (ConA) and cytochalasin D (CytoD) have been shown to disrupt the cytoskeleton architecture [20]. After cell staining with annexin V/propidium iodide (PI), flow cytometry was used to assess the extent of cell death induced by both agents. From the side scatter (SSC)/forward scatter (FSC) plots, the changes in cell "morphology" features that each agent induced are clearly visible (Figure 1*A*). When annexin V/PI cell staining was performed, ConA treatment resulted in a marked increase in necrosis (Figure 1*A*, *bottom*, *upper left quadrant*), whereas CytoD triggered late apoptosis (Figure 1*A*, *bottom*, *upper right quadrant*). Cell viability and total cell death were quantified (Figure 1*B*). Furthermore, cell necrosis and cell apoptosis (early and late) (Figure 1*C*) were also separately quantified to show the differential induction of necrosis by ConA and induction of apoptosis by CytoD.

Recombinant MT1-MMP and Cytoskeleton-Disrupting Agents Induce proMMP-2 Activation

Latent proMMP-2 activation into its active MMP-2 form has been correlated with cell death [21,22]. Accordingly, we have previously shown that the cytoskeleton-disrupting agents ConA and CytoD triggered proMMP-2 activation in U87 glioma cells [23]. Using fluorescent microscopy, we validated overexpression of full-length wild-type (wt) recombinant MT1-MMP or the membrane-bound cytoplasmic-deleted

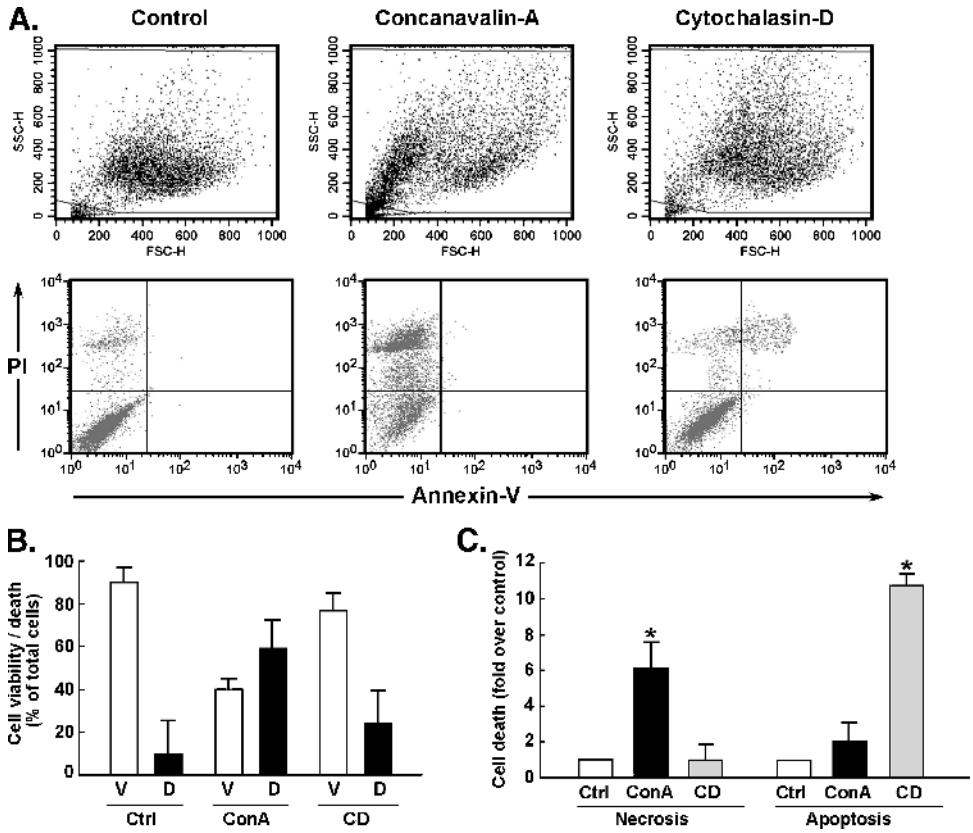

Figure 1. *Differential induction of cell necrosis and cell apoptosis by cytoskeleton-disrupting agents. U87 glioblastoma cells were cultured as described in the Materials and Methods section until they reached ~ 75% to 90% confluence. They were then serum-starved for 24 hours before the addition of 10 μg/ml ConA or 1 μmol/l CytoD (CD). Incubation was continued for another 24 hours. (A) Flow cytometry was then used to either assess light-scattering properties associated with changes in cell morphology (top panels) or cell death from annexin V/PI-stained cells (bottom panels). (B) Cell viability (white columns) was assessed as the percentage of total cells present in the lower left quadrants. Cell death (black columns) represents the combined cells present in necrosis, early/late apoptosis (black columns). (C) The respective cell death proportions attributable to either necrosis or apoptosis (early and late) are shown. Data are the averages ± SEM of four independent experiments. Statistical significance is represented by (*).*

recombinant MT1-MMP both fused to green fluorescent protein (GFP) (Figure 2*A*) and consequent proMMP-2 activation demonstrated by gelatin zymography (Figure 2*B*). Transfections of cells with cDNA encoding GFP alone did not affect the ability of ConA or CytoD to induce proMMP-2 activation (data not shown). These results suggest that, similar to cytoskeleton-disrupting agents ConA and CytoD, MT1-MMP–mediated proMMP-2 activation is also potentially linked to the control of cell survival in U87 glioma cells.

ConA-Mediated Activation of proMMP-2 Requires MT1-MMP

To assess the involvement of MT1-MMP in the proMMP-2 activation process and the necrotic effects of ConA, we specifically downregulated MT1-MMP gene expression using a specific MT1-MMP gene silencing strategy [24]. Cells were transfected with mismatched (Mock) or MT1-MMP–targeted small interfering RNA (siRNA) duplexes, as described in the Materials and Methods section, and then treated with increasing concentrations of ConA. Total RNA was isolated and reverse transcription–polymerase chain reaction (RT-PCR) confirmed that MT1-MMP gene downregulation was successfully achieved (Figure 3*A*). Conditioned media from these conditions were also isolated to assess the MT1-MMP–mediated proMMP-2 activation by ConA. Gelatin zymography clearly showed significantly decreased proMMP-2 activation in the cells in which MT1-MMP gene expression had been knocked down (Figure 3*B*). This was quantified by scanning densitometry, showing close to 90% inhibition (data not shown). This clearly suggests that the ConA-mediated events that we previously observed involve MT1-MMP in proMMP-2 activation and are likely involved in cell death.

MT1-MMP Cytoplasmic Domain Is Responsible for Cell Death Signaling

Although extracellular catalytic inhibition of MT1-MMP only partially reversed its ability to trigger cell death [11], the implication of MT1-MMP in signaling intracellular cell death processes was thus evaluated. Transient transfection using cDNA plasmids encoding either the full-length or the membrane-bound cytoplasmic-deleted recombinant forms of MT1-MMP was performed in U87 glioma cells. Annexin V/PI staining was then performed and cell death (necrosis and apoptosis) was assessed by flow cytometry (Figure 4*A*). Overexpression of native MT1-MMP significantly triggered cell necrosis by more than 10 times, whereas cell apoptosis was also induced approximately twofold (Figure 4*B*, *gray bars*). Although still catalytically active at the cell surface (Figure 2*B*), the deletion of the MT1-MMP cytoplasmic domain significantly abrogated the induction of both cell

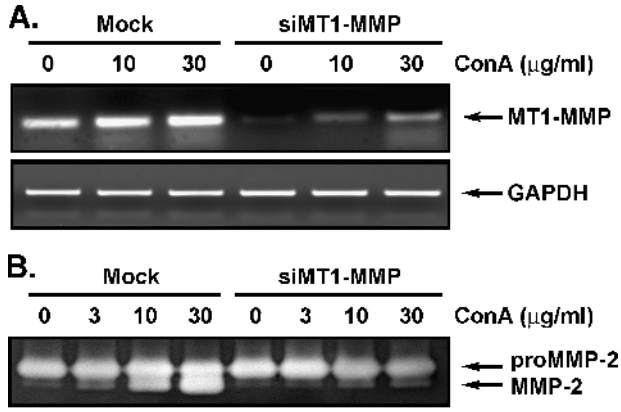

Figure 3. *ConA-mediated activation is an MT1-MMP–mediated event. Because proMMP-2 activation is thought to proceed through an MT1-MMP–mediated event, U87 glioblastoma cells were transfected with siRNA against MT1-MMP or mismatched siRNA (Mock) for 48 hours, as described in the Materials and Methods section, before treatment with increasing concentrations of ConA. (A) Total RNA was isolated and MT1-MMP gene expression was assessed by RT-PCR as described in the Materials and Methods section. The gene expression level of GAPDH was used as the internal control. (B) Gelatin zymography was performed to assess the extent of proMMP-2 activation in the conditioned media of serum-starved Mock and siMT1-MMP cells treated with increasing concentrations of ConA.*

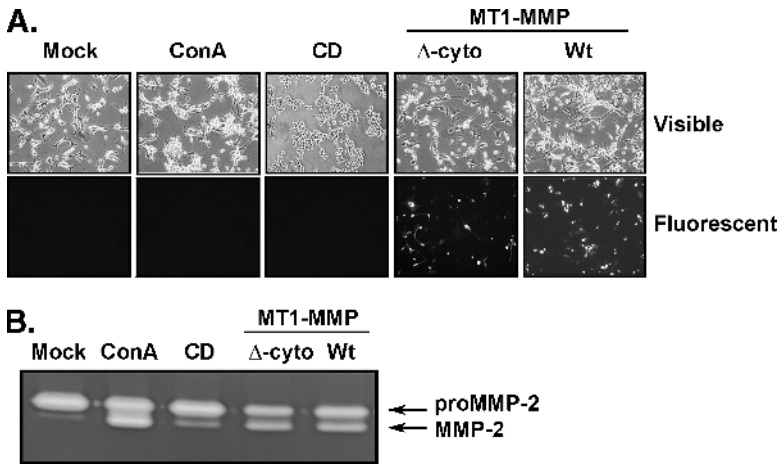

Figure 2. *Recombinant MT1-MMP and cytoskeleton-disrupting agents induce proMMP-2 activation. U87 glioblastoma cells were treated as described in the legend to Figure 1. Transfections of cDNA plasmids encoding full-length Wt-MT1-MMP and cytoplasmic domain–truncated MT1-MMP both fused to GFP were carried out as described in the Materials and Methods section. (A) Changes in cell morphology are shown through phase-contrast microphotography (visible light), whereas transfection efficiency was validated by fluorescence microscopy. (B) Gelatin zymography was carried out to assess the extent of proMMP-2 activation levels, as described in the Materials and Methods section, using conditioned media isolated from each of the serum-starved cell conditions.*

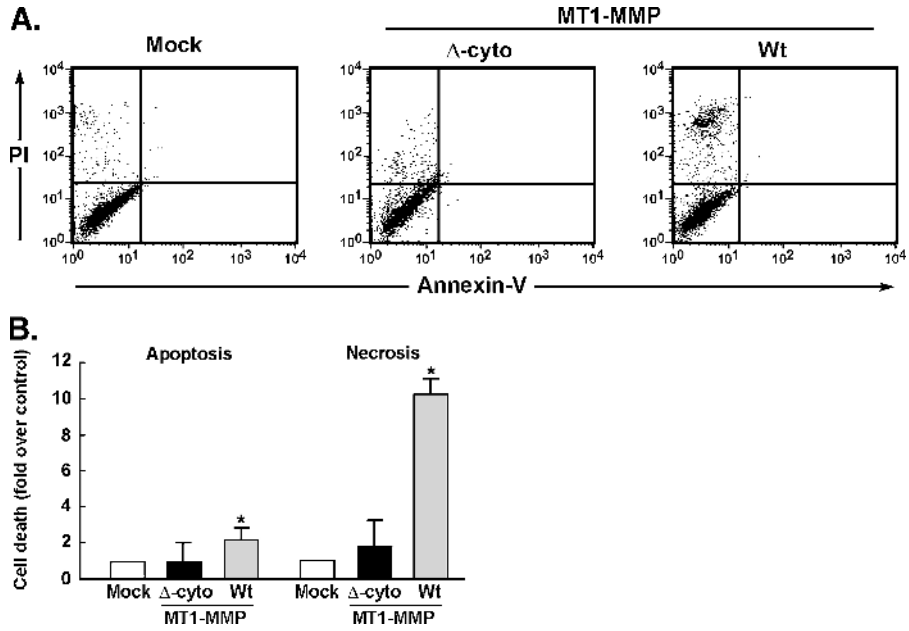

Figure 4. *MT1-MMP overexpression triggers cell death and is signaled through its intracellular cytoplasmic domain. U87 glioblastoma cells were transfected with cDNA encoding full-length Wt-MT1-MMP or its cytoplasmic domain–truncated form as described in the Materials and Methods section. (A) Flow cytometry was used to assess the extent of cell death in annexin V/PI-stained cells. (B) Quantification of cell death caused by either combined early and late apoptosis (upper and lower right quadrants) or necrosis (upper left quadrants) was performed for cells transfected with cytoplasmic domain–truncated MT1-MMP (Δ-cyto) or full-length Wt-MT1-MMP.*

necrosis and apoptosis (Figure 4*B*, *black bars*). These observations suggest that active MMP-2 is not responsible for cell death and that some MT1-MMP–mediated intracellular signaling is a prerequisite for the control of cell survival.

MT1-MMP Overexpression and ConA Treatment Downregulate G6PT Gene Expression

To investigate the intracellular events involved in MT1-MMP– and ConA-mediated cell death, we examined the prosurvival microsomal glucose-6-phosphate translocase (G6PT) as a potential link. Cytoskeleton disruption is often linked to ER stress [25,26], and silencing of G6PT, a microsomal resident protein, has recently been shown to induce cell death in U87 glioma cells [14]. We thus isolated total RNA from ConA-treated cells and from MT1-MMP–transfected cells because cell necrosis was a common event in both conditions. RT-PCR was performed as described in the Materials and Methods section and we found that G6PT gene expression was significantly reduced in ConA-treated and in the MT1-MMP–transfected cells (Figure 5*A*). Interestingly, in agreement with its inability to trigger cell death, deletion of MT1-MMP's cytoplasmic domain was also ineffective in reducing G6PT gene expression. Gene expression of glucose-6-phosphatase (G6Pase)-β, the only other component of the G6Pase system that was expressed in U87 cells [13] and of GAPDH remained unaffected and can be considered as unaffected internal controls (Figure 5*A*). ConA treatment and MT1-MMP overexpression resulted, as expected, in an increase in MT1-MMP transcript levels. Altogether, this demonstrates that necrosis-inducing

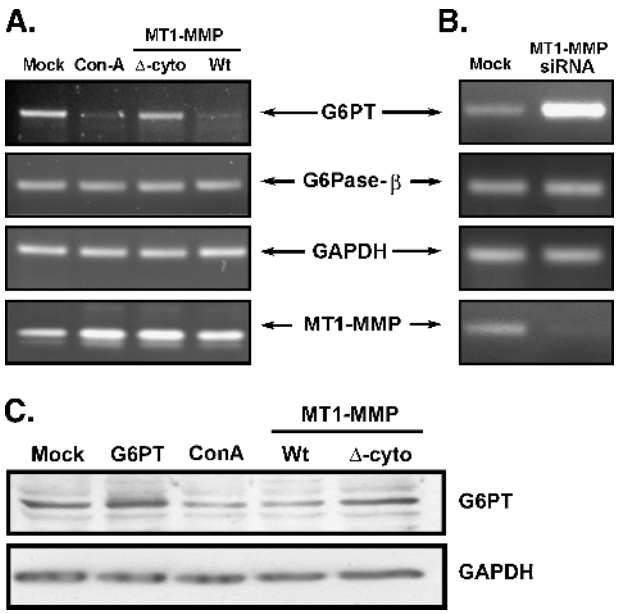

Figure 5. *Both MT1-MMP overexpression and ConA treatment downregulate G6PT gene and protein expression. (A) Total RNA was isolated from untreated (Mock), ConA-treated, or U87 glioblastoma cells transfected with either the cytoplasmic domain–truncated MT1-MMP (Δ-cyto) or full-length Wt-MT1-MMP cDNA. RT-PCR was performed to assess the changes in G6PT, G6Pase-β, GAPDH, or MT1-MMP gene expression in each condition. (B) MT1-MMP gene expression was specifically downregulated in U87 glioblastoma cells transfected with siMT1-MMP but not in mismatched siRNA-transfected cells (Mock) as described in the Materials and Methods section. Total RNA and RT-PCR were performed as in (A). (C) Cell lysates were isolated from U87 cells transfected with cDNA encoding G6PT, Wt-MT1-MMP, or Δ-cyto MT1-MMP, or treated with ConA. Western blotting and immunodetection was performed with anti-G6PT and anti-GAPDH antibodies.*

conditions, such as those triggered by ConA or overexpression of recombinant MT1-MMP, are molecularly linked to the prosurvival functions of G6PT. Interestingly, when MT1-MMP gene expression was silenced, the expression of G6PT increased significantly in comparison to the mismatched siRNA-transfected cells (Mock), suggesting that MT1-MMP exerted a repressive effect on G6PT gene regulation (Figure 5*B*). Modulation of G6PT gene expression was further confirmed at the protein level. We showed that ConA treatment or Wt-MT1-MMP overexpression downregulated G6PT protein expression (Figure 5*C*). Thus, our results show that G6PT gene regulation is signaled by the intracellular MT1-MMP cytoplasmic domain.

G6PT Overexpression Antagonizes the ConA-Mediated Lethal Effect

To characterize the molecular mechanism linking MT1-MMP to G6PT, we next assessed whether constitutively expressed recombinant G6PT could overcome the lethal effect of ConA. MT1-MMP synthesis by a mechanism that involves phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR was recently highlighted [27] and, thus, Akt phosphorylation state was explored. U87 cells were transiently transfected with cDNA encoding G6PT and then treated with the cell death inducer ConA. Immunodetection of total and phosphorylated Akt was performed on Mock and G6PT-transfected cell lysates. We observed that ConA decreased the basal levels of phosphorylated Akt by up to 50%, although not of total Akt protein expression, in Mock-transfected cells (Figure 6*A*). Transient overexpression of recombinant G6PT completely reversed the effects of ConA on Akt phosphorylation (Figure 6*B*). Despite being more associative than mechanistic, these observations confirm the G6PT prosurvival activity [14] and show that targeting PI3K/Akt signaling by ConA induces apoptosis [28]. Finally, we showed by zymography that G6PT overexpression significantly antagonized ConA-mediated proMMP-2 activation (Figure 6*C*), an effect that may involve inhibiting MT1-MMP functions.

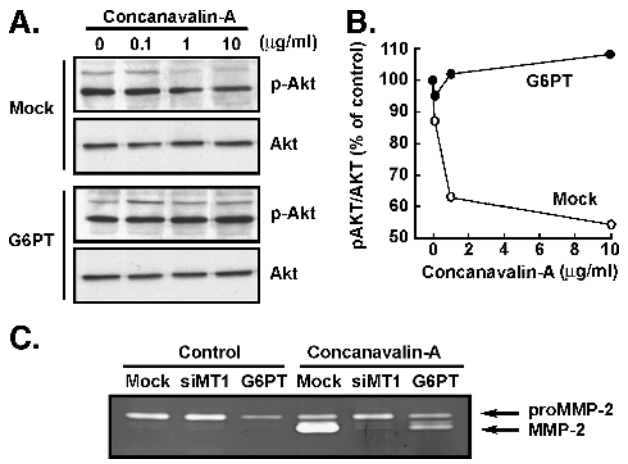

Figure 6. *G6PT overexpression antagonizes the ConA-mediated lethal effect. (A) Mock or G6PT-transfected U87 cells were treated with increasing ConA concentrations and cell lysates were used to immunodetect the levels of total or phosphorylated Akt (p-Akt). (B) Scanning densitometry was used to quantify the Akt immunoreactive bands, and results were expressed as the ratio of p-Akt over Akt. (C) Conditioned media isolated from serum-starved Mock, siMT1-MMP (siMT1), or G6PT-transfected U87 cells were used to perform gelatin zymography.*

Discussion

Gliomas remain a great challenge in oncology today as they account for more than 50% of all brain tumors and are by far the most common primary brain tumors in adults [29]. More importantly, the mechanisms involved in the resistance of migrating glioblastoma cells to chemotherapy or to radiation-induced cell death have long been recognized [30] and still receive much attention in order to optimize future cellular targets for the treatment of glioblastomas [31]. A relationship between cell migration and apoptosis was highlighted by the observations that resistance to apoptosis is closely linked to tumorigenesis, but that paradoxically migrating tumor cells can also still be induced to die by nonapoptotic mechanisms such as necrosis [32]. In fact, tissue necrosis is a characteristic feature of malignant gliomas, in particular glioblastoma, and is most likely the consequence of rapidly increasing tumor mass that is inadequately oxygenated by the preexisting vasculature [33]. Because tumor cells respond to hypoxic stress by upregulating a variety of genes involved in glucose uptake, glycolysis, and angiogenesis, all essential to maintaining nutrient availability and intracellular ATP levels [34], the intracellular metabolic compartments regulating cell survival and invasiveness are of particular interest. Besides mitochondria and lysosomes, the ER is very important in this respect, as it is now recognized as an important sensor of cellular stress and plays a key role in the release and activation of death factors such as cathepsins, calpains, and other proteases through intracellular calcium flux [35]. For instance, migrating glioblastoma cells have recently been shown to overexpress death-associated protein-3 [36]. Therefore, new routes should be investigated as possible issues to combat apoptotic-resistant migrating glioblastoma cells.

Given the ER localization of G6PT and the crucial role that the ER plays as a metabolic compartment, we suggest that G6PT is a key mediator in the regulation of cancer cell survival and ECM degradation signaling. In fact, regulation of G6PT expression may function as a ''bioswitch'' (Figure 7*A*) enabling cells to promote either migration or cell death processes. Switching from one state to another may occur in response to external stimuli, such as hypoxia, or as a result of intracellular metabolic changes [37]. Intracellular regulation of Ca^{2+} flux and cytosolic ATP and G6P levels are among the parameters that G6PT may modulate in the transformed proliferating cells. As such, metabolic profiling of cell growth and death in cancer is already used to identify the changes in glucose utilization for macromolecule synthesis in cancer [38,39]. Among the several brain tumor–derived cell lines tested, G6PT expression was found the highest in the highly infiltrating and angiogenic U87 glioma cells [13]. This potentially suggests that metabolic adaptive capacity, in part through G6PT, may regulate the invasive phenotype of aggressive cancer cells. Documenting the pleiotropic roles

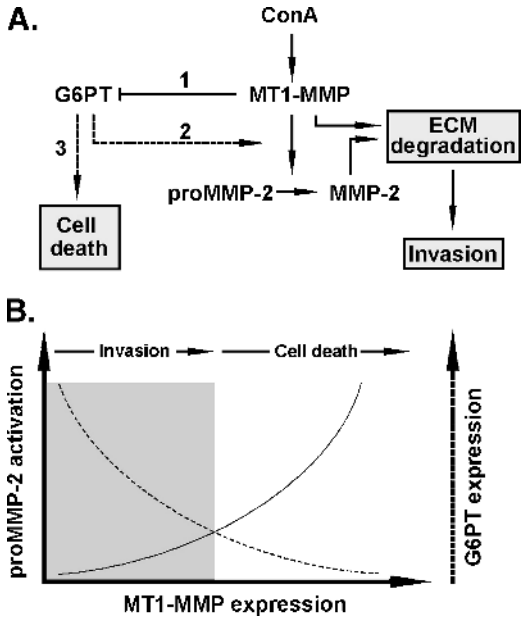

Figure 7. *G6PT as a bioswitch intermediate in the regulation of proMMP-2 activation and cell death signaling. (A) Summarized scheme of the events that lead to MT1-MMP–mediated signaling in proMMP-2 activation and in cell death. ConA upregulates MT1-MMP gene and protein expression, which in turn downregulates G6PT expression (1). Low levels of G6PT release the inhibitory effect on MT1-MMP–mediated proMMP-2 activation (2), which altogether leads to ECM degradation and cell invasion. When a specific balance is reached between MT1-MMP and G6PT expression (i.e., high MT1-MMP expression, low G6PT expression), cell death signaling is then activated (3). (B) Our data show that MT1-MMP expression leads to both proMMP-2 activation (solid line) and to concomitant downregulation of G6PT expression (dashed line). Conceptually, we suggest that a balance between the early invasive processes that are initiated by the increased MT1-MMP expression correlate with proMMP-2 activation to hydrolyze the ECM and promote cell migration (left, shaded area). Concurrently, G6PT expression decreases until it reaches a threshold where its inhibitory effect on proMMP-2 activation is released, which then leads to cell death (right area). The intersection between the G6PT expression curve and that of the proMMP-2 activation is the bioswitch that reflects the balance between cell invasion and cell death signaling.*

of G6PT in cancer cells will thus help optimize or design new antitumor therapies.

We previously showed that inhibiting G6PT function by chlorogenic acid or by ATP-depleting agents such as 2-deoxyglucose in brain tumor–derived cells did not directly affect MT1-MMP catalytic function but still resulted in decreased invasiveness [13]. Moreover, cancer cells frequently display high rates of aerobic glycolysis compared with their nontransformed counterparts, and the possible applications of 2-deoxyglucose in anticancer therapies further support the theory that inhibiting G6PT function in cancer cells could decrease tumor progression. Some evidence also suggests that ER stress-inducing agents are useful as cancer agents and that excessive ER stress leads to apoptosis. These agents include glycosylation inhibitors (e.g., tunicamycin and 2-deoxyglucose), agents that deplete ER Ca^{2+} (e.g., the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin and various ionophores) and agents that induce reductive stress (dithiotreitol and β-mercaptoethanol) [40].

We also found that cytoskeleton remodeling is among the first events in a cascade of activation that leads to MT1-MMP–mediated downregulation of brain cancer cell survival, in part through PI3K/Akt-mediated plasma membrane to nucleus signaling. Interestingly, part of the switch between cell migration and cell death comes under the control of the PTEN/Akt/PI3K/mTOR pathway [30]. Survival through PI3K/Akt signaling is complex [41] and the activity of the PI3K/Akt pathway is, in fact, often upregulated in brain tumors as a result of excessive stimulation by growth factor receptors and Ras [42]. Moreover, glioblastomas frequently carry mutations in the *PTEN* tumor suppressor gene, whose tumor suppressor properties are closely related to its inhibitory effect on the PI3K-dependent activation of Akt signaling [43]. The activation of the PI3K pathway is significantly associated with increasing tumor grade, lower levels of apoptosis, and an adverse clinical outcome in the case of human gliomas [44]. All these factors indicate that aberrant PI3K/Akt signaling means that both cell proliferation in glioma cells and cell migration have become abnormal. A number of publications have already reported that an aberrantly activated PI3K/Akt pathway renders tumor cells resistant to cytotoxic insults, including those related to anticancer drugs [45,46]. In light of these results and because G6PT was able to reverse the cytotoxic effects of ConA, it is tempting to suggest that functional targeting of G6PT such as by the use of chlorogenic acid or by its analogs, the most potent G6PT inhibitors, could augment the effectiveness of chemotherapy on glioma cells.

Maintenance of cytoarchitecture is required for cell survival because its perturbation by CytoD- or ConA-mediated MT1-MMP mechanisms diminished cell survival and were correlated to proMMP-2 activation [21,22] (this study). In fact, silencing of the MT1-MMP gene prevented ConA from activating proMMP-2. Moreover, we showed that the intracellular domain of MT1-MMP is an absolute requirement for transducing the intracellular signaling that leads to cell death. Although the exact identity of the amino acid residues from the MT1-MMP intracellular domain remains to be addressed, speculations about the Tyr573, Cys574, and Val582 have been put forward as important for MT1-MMP signaling [47,48]. Similarly, a caspase-dependent mechanism has recently been associated with MT1-MMP function in endothelial cell morphogenic differentiation [49]. This suggests that MT1-MMP acts as a potential cell death sensor/effector that signals ECM degradation processes to be activated. Interestingly, hypoxia increased levels of MT1-MMP and the MT1-MMP transcription factor regulator Egr-1 in bone marrow–derived stromal cells [50], a condition that led to cell death [51]. Moreover, ConA was found ineffective in activating proMMP-2 or inhibiting G6PT gene expression in bone marrow stromal cells isolated from Egr-$1^{-/-}$ mouse [11].

The fact that G6PT overexpression inhibited ConA-induced proMMP-2 activation, but not cell death, further suggests that complex differential regulation takes place and highlights the pleiotropic intracellular functions of G6PT. Moreover, this observation also provides insight into the cellular event chronology, confirming that MT1-MMP–mediated activity and signaling are among the first steps that inhibit G6PT expression, ultimately leading to cell death.

Interestingly, our data are consistent with some of the abnormal polymorphonuclear neutrophil phenotypes observed in glycogen storage disease type 1b, a clinical condition where the G6PT gene and/or protein activity is defective [52,53]. In fact, it has been hypothesized that G6PT might function as a G6P receptor/sensor [53] or that it could favor calcium sequestration in the ER lumen [17]. Finally, although no effects in response to MT1-MMP or cytoskeleton disruption were observed on the ER-embedded G6Pase-β, recent evidence regarding G6Pase-β involvement in cell survival was demonstrated in neutrophils, as disruption of the G6Pase-β gene expression also led to cell death, an event suggestive of a vital interaction between G6PT and G6Pase-β [54].

In summary, we highlight new functions of MT1-MMP in cell death signaling that may potentially be linked to those of the ER-embedded functions of G6PT. In fact, we believe that this signaling axis may not be exclusive to one cell line, but rather may regulate cell mobilization processes through metabolic and/or cell survival control such as similarly demonstrated for bone marrow–derived stromal cells [11]. Our study further shows a molecular axis linking the invasive phenotype of brain tumor cells to their potential metabolic control by G6PT and supports the notion of an MT1-MMP/G6PT bioswitch (Figure 7*B*) that could regulate glucose homeostasis and thus restrain cancer cell proliferation, inhibit ECM degradation, or induce cell death. By revealing tumor-specific metabolic shifts in tumor cells, metabolic profiling studies will further enable drug developers to identify the metabolic steps that control cell proliferation, thus aiding the identification of new anticancer targets and screening of lead compounds for antiproliferative metabolic effects.

Materials and Methods

Materials

SDS and BSA were purchased from Sigma (Oakville, ON, Canada). Cell culture medium was obtained from Life Technologies (Burlington, ON, Canada). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, Canada). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). The polyclonal antibodies against Akt and phospho-Akt were purchased from Cell Signaling (Danvers, MA). All other reagents were from Sigma-Aldrich Canada.

Cell Culture and Transfection Method

The U87 glioblastoma cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in Eagle's minimum essential medium (MEM) containing 10% (vol/vol) FBS (HyClone Laboratories, Logan, UT) and 2 mmol/l glutamine at 37°C under a humidified atmosphere containing 5% carbon dioxide. U87 glioblastoma cells were transiently transfected with the cDNA constructs encoding either the membrane-bound cytoplasmic domain–truncated MT1-MMP (Δ-cyto) where the last 20 amino acid residues were deleted, or the full-length Wt-MT1-MMP fused to GFP [55], or with 20 nmol/l siRNA (see below) using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). The occurrence of MT1-MMP–specific gene knockdown was evaluated by semiquantitative RT-PCR and validated by assessing MT1-MMP–mediated proMMP-2 activation. Mock transfections of U87 cultures with pcDNA (3.1+) or cDNA encoding GFP expression vectors alone were used as controls.

RNA Interference

RNA interference experiments were performed using Lipofectamine 2000. An siRNA against MT1-MMP (siMT1-MMP) and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield, IN) and annealed to form duplexes. The sequence of the siMT1-MMP used in this study is as follows: 5′-CCA--CCAGAAGCUGAAGGUAGAAdTdT-3′ (sense) and 5′-UUCUACCUUCAGCUUCUGGdTdT-3′ (antisense) [24]. The diminution of MT1-MMP expression, as assessed by RT-PCR, ranged routinely from 75% to 90% (not shown).

Total RNA Isolation and RT-PCR Analysis

Total RNA was extracted from cultured U87 cells using TRIzol reagent (Invitrogen). One microgram of total RNA was used for first-strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen). Primers for G6PT, G6Pase-β, MT1-MMP, and GAPDH were all derived from human sequences and validated [13]. PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification and the products were resolved on 1.8% agarose gels containing 1 μg/ml ethidium bromide.

Gelatin Zymography

Gelatin zymography was used to assess the extent of proMMP-2 activation in conditioned media. Briefly, a 20-μl aliquot of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled water. Gels were further incubated at 37°C for 20 hours in 20 mmol/l NaCl, 5 mmol/l $CaCl_2$, 0.02% Brij-35, 50 mmol/l Tris–HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid, 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting Procedures

Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes, which were then blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mmol/l NaCl, 20 mmol/l Tris–HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1:1000 dilution) in TBST containing 3% BSA and 0.02% NaN_3, followed by a 1-hour incubation with horseradish peroxidase–conjugated anti–rabbit IgG (1:2500 dilution) in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by

enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC, Canada).

Analysis of Cell Death by Flow Cytometry

Cell death was assessed by flow cytometry as described previously [14]. Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with PBS. Then, 10^5 cells were pelleted and suspended in 200 μl of buffer solution and stained with annexin V–fluorescein isothiocyanate and PI according to the manufacturer's protocol (BD Biosciences, Mississauga, ON, Canada). The cells were diluted by adding 300 μl of buffer solution and processed for data acquisition and analysis on a (Becton Dickinson, San Jose, CA) FACS Calibur flow cytometer using CellQuest Pro software. The *x*- and *y*-axes indicate the fluorescence of annexin V and PI, respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is well known to translocate to the outer surface of the plasma membrane, which still remains physically intact. As annexin V binds to phosphatidylserine but not to PI, and the dye is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin V positive/PI negative). Cells in late apoptosis are stained with annexin V and PI (annexin V positive/PI positive). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin V negative/PI positive).

Statistical Data Analysis

Data are representative of three or more independent experiments. Statistical significance was assessed by Student's unpaired *t* test and probability values less than .05 were considered significant; an asterisk (*) identifies such significance in each figure.

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