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MT1-MMP Down-regulates the Glucose 6-Phosphate Transporter Expression in Marrow Stromal Cells

*A MOLECULAR LINK BETWEEN PRO-MMP-2 ACTIVATION, CHEMOTAXIS, AND CELL SURVIVAL**

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Bone marrow-derived stromal cells (BMSC) are avidly recruited by experimental vascularizing tumors, which implies that they must respond to tumor-derived growth factor cues. In fact, BMSC chemotaxis and cell survival are regulated, in part, by the membrane type-1 matrix metalloproteinase (MT1-MMP), an MMP also involved in pro-MMP-2 activation and in degradation of the extracellular matrix (ECM). Given that impaired chemotaxis was recently observed in bone marrow cells isolated from a glucose 6-phosphate transporter-deficient ($G6PT^{-/-}$) mouse model, we sought to investigate the potential MT1-MMP/G6PT signaling axis in BMSC. We show that MT1-MMP-mediated activation of pro-MMP-2 by concanavalin A (ConA) correlated with an increase in the sub-G_1 cell cycle phase as well as with cell necrosis, indicative of a decrease in BMSC survival. BMSC isolated from Egr-$1^{-/-}$ mouse or MT1-MMP gene silencing in BMSC with small interfering RNA (siMT1-MMP) antagonized both the ConA-mediated activation of pro-MMP-2 and the induction of cell necrosis. Overexpression of recombinant full-length MT1-MMP triggered necrosis and this was signaled through the cytoplasmic domain of MT1-MMP. ConA inhibited both the gene and protein expression of G6PT, while overexpression of recombinant G6PT inhibited MT1-MMP-mediated pro-MMP-2 activation but could not rescue BMSC from ConA-induced cell necrosis. Cell chemotaxis in response to the tumorigenic growth factor sphingosine 1-phosphate was significantly abrogated in siMT1-MMP BMSC and in chlorogenic acid-treated BMSC. Altogether, we provide evidence for an MT1-MMP/G6PT signaling axis that regulates BMSC survival, ECM degradation, and mobilization. This may lead to optimized clinical applications that use BMSC as a platform for the systemic delivery of therapeutic or anti-cancer recombinant proteins *in vivo*.

Recent advances in the understanding of stem cell mobilization, cell-matrix interaction, and biodistribution have enabled the development of new therapeutic strategies (1, 2). Although locally transplanted bone marrow-derived stromal cells (BMSC)[2] have already been used clinically (3–5), less invasive routes of BMSC transplantation have become the focus of recent attention (6–8). In fact, several clinical applications now use intravenous administration of genetically engineered BMSC either as an ideal vehicle for gene transfer or as a platform for the systemic delivery of therapeutic recombinant proteins *in vivo* (6, 9, 10). This implies that these circulating, systemically infused cells must respond to serum-derived cues that direct their ultimate biodistribution. The molecular players regulating cellular mobilization, chemotaxis, and cell survival of BMSC have received little attention.

Among the mediators known to exert potent cellular chemotactic effects, sphingosine 1-phosphate (S1P) is one of the most important bioactive lysophospholipids secreted in blood plasma either upon platelet activation (11) or from brain tumor-derived glioma cells (12). In fact, we have demonstrated that BMSC chemotaxis was very strong in response to S1P (13) and required reorganization of the actin cytoskeleton and remodeling of the extracellular matrix (ECM) through a complex, cooperative signal transduction network involving cell surface matrix metalloproteinase (MMP) activity (14). Currently, the molecular characterization and the nature of that MMP, regulating both BMSC chemotaxis and interaction with the ECM protein microenvironment, remain poorly understood. Recently, we highlighted functional cross-talk between the membrane type-1 MMP (MT1-MMP) and the S1P receptor EDG-1-mediated signaling in BMSC chemotaxis (13). Interestingly, aside from its classical role in ECM proteolysis, MT1-MMP is also involved in transducing crucial intracellular signaling that may control several processes related to BMSC mobilization and cell survival (13–16).

Given that impaired chemotaxis was recently observed in bone marrow cells isolated from a microsomal glucose 6-phos-

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[2] The abbreviations used are: BMSC, bone marrow-derived stromal cell(s); ConA, concanavalin A; ECM, extracellular matrix; G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P transporter; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; PI, propidium iodine; siRNA, small interfering RNA; S1P, sphingosine 1-phosphate; PBS, phosphate-buffered saline; Wt, wild type; GFP, green fluorescent protein.

phate transporter-deficient ($G6PT^{-/-}$) mouse model (17, 18) and that G6PT was demonstrated to regulate cell migration (19), we hypothesized whether MT1-MMP and G6PT might share some common molecular and cellular impact on the regulation of BMSC chemotaxis and ECM degradation. Since its discovery, G6PT has been shown to integrate and regulate many metabolic functions such as glycemia, lipidmia, uricemia, and lactic acidemia (20). More importantly, its activity cannot be substituted as G6PT deficiencies lead to glycogen storage disease type Ib characterized, not only by disturbed glucose homeostatsis but by severe myeloid dysfunctions (20). Lack of G6PT function alters, for instance, neutrophil chemotaxis and calcium flux (21), and its expression inhibition decreases cell survival (19, 22, 23).

Recent evidence has provided new functions for MT1-MMP in modulating cell survival through as yet unidentified intracellular processes (13, 24). The originality of our research relies upon the combined evidence that cell surface MMP signaling is essential for BMSC chemotaxis (14) and that G6PT may function as an intracellular *"bio-switch"* in cell death *versus* cell mobilization in BMSC (19). We decided to investigate the potential molecular and functional link that may exist between MT1-MMP and G6PT functions.

EXPERIMENTAL PROCEDURES

Materials—SDS, bovine serum albumin (BSA), Hoechst 33258, and propidium iodine were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cell culture media was obtained from Invitrogen (Burlington, Ontario, Canada). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). The ECL reagents were from Amersham Biosciences (Baie d'Urfé, Québec, Canada). Micro bicinchoninic acid protein assay reagents were from Pierce. The polyclonal antibody against MT1-MMP was from Chemicon (AB815), while the polyclonal antibody against G6PT was generated against the ^{5}GYGYYRTVIFSAMFGGY^{21} peptide derived from the human G6PT primary sequence (GenBank™ accession number AAD19898) (25) at the Biotechnology Research Institute (Montreal, Québec, Canada). All other reagents were from Sigma-Aldrich Canada.

Cell Culture—BMSC were isolated from the whole femur and tibia bone marrow of either C57BL/6 female mice or from a colony of $Egr\text{-}1^{-/-}$ (null) C57BL/6 female mice (gift of Dr. Lorraine E. Chalifour, Lady Davis Institute for Medical Research) (26). Cells were cultured and characterized as described previously (14). Analysis by flow cytometry performed at passage 14 revealed that BMSC expressed CD44, yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3), and Tie2 (angiopoietin receptor) (data not shown).

Chemotactic Cell Migration Assay—BMSC, which had been mock-transfected or transfected with siMT1-MMP, were trypsinized and seeded at 10^5 cells/filter on 0.15% gelatin/PBS-precoated Transwells (Corning/Costar; Acton, MA; 8-μm pore size) assembled in 24-well Boyden chambers, which were filled with 600 μl of Dulbecco's modified Eagle's medium supplemented or not with 1 μM S1P or of growth factor-enriched medium isolated from serum-starved U87 glioblastoma cells in the lower compartment. Cell migration was allowed to proceed for 6 h at 37 °C in 5% CO_2. Non-migrating cells that remained on the upper side of the Transwell filters were carefully removed with cotton swabs. Cells that had migrated to the lower side of the filters were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet, 20% MeOH and counted. The migration was quantified by analyzing at least ten random fields per filter for each independent experiment using Northern Eclipse 6.0 software (Empix Imaging Inc., Mississauga, Ontario, Canada) for computer-assisted imaging.

RNA Interference—RNA interference experiments were performed using Lipofectamine 2000. A small interfering RNA against MT1-MMP (siMT1-MMP) and mismatch small interfering RNA (siRNA) were synthesized by EZBiolab Inc. (Westfield, IN) and annealed to form duplexes. The sequence of the siMT1-MMP used in this study is conserved between human (GenBank™ accession number NM_004995) and murine (GenBank™ accession number NM_008608) genes and is as follows: 5′-CCAGAAGCUGAAGGUAGAAdTdT-3′ (sense) and 5′-UUCUACCUUCAGCUUCUGGdTdT-3′ (antisense) (27). Knockdown of MT1-MMP expression, as assessed by RT-PCR, ranged routinely from 75 to 90% efficiency (data not shown).

Total RNA Isolation and Reverse Transcriptase (RT)-PCR Analysis—Total RNA was extracted from cultured BMSC using TRIzol reagent. 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). Primer sequences for MT1-MMP, G6PT, glucose-6-phosphatase (G6Pase)-β, and glyceraldehyde-3-phosphate dehydrogenase have been validated and published elsewhere (19). PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification, and the products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Gelatin Zymography—Gelatin zymography was used to assess the extent of pro-MMP-2 activation in the culture medium as described previously (19). 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 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/2,500 dilution) in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences).

Analysis of Cell Cycle by Flow Cytometry—Distribution of BMSC throughout the cell cycle was assessed by flow cytometry (28). Serum-fasting preparation was performed prior to analysis, and therefore the cell populations were synchronous. Cells were harvested by gentle scraping, pelleted by centrifugation, washed with ice-cold PBS/EDTA (5 mM), then resuspended in 1 volume of PBS/EDTA and fixed with 100% ethanol overnight.

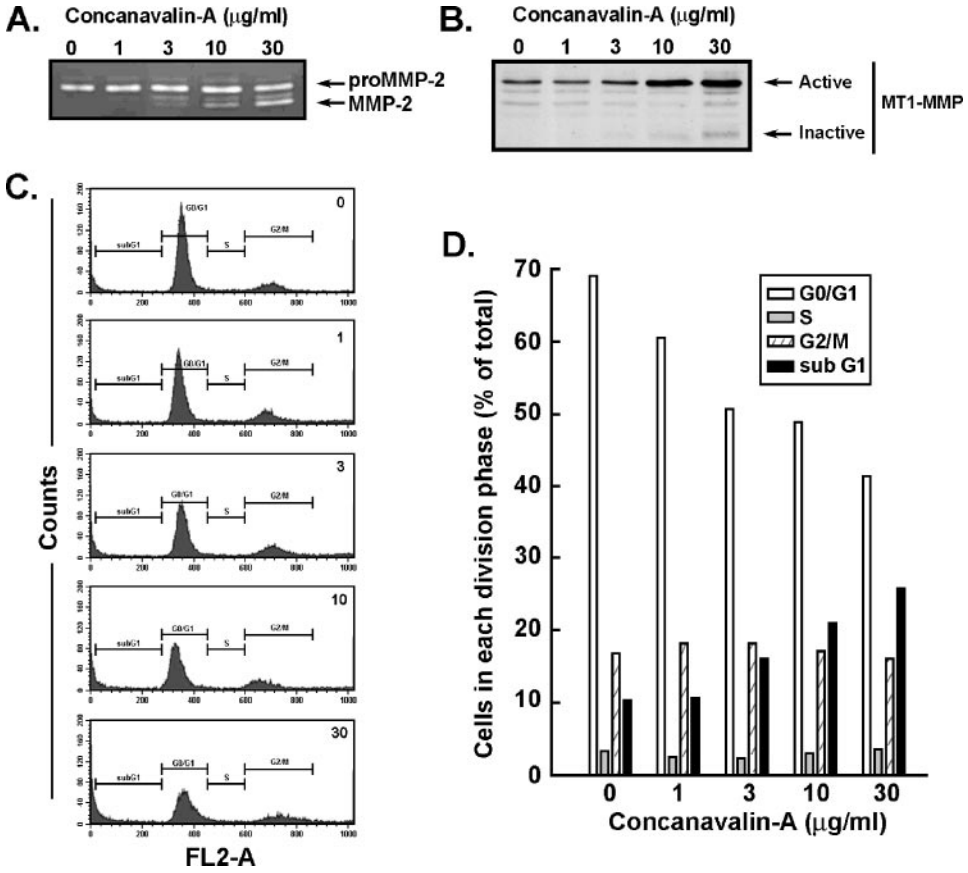

FIGURE 1. **MT1-MMP-mediated ativation of latent pro-MMP-2 by concanavalin A correlates with a decrease in G_0/G_1 and an increase in sub-G_1 cell cycle phases.** BMSC were serum-starved for 24 h then treated with various concentrations of concanavalin A for 18 h. *A*, conditioned medium was harvested, and 20 μl was used to assess pro-MMP-2 activation using gelatin zymography. *B*, BMSC were treated as above and cell lysates used to perform immunodetection of the MT1-MMP proteolytic form (active ~60 kDa, inactive ~43 kDa). *C*, cells were treated as above and cell cycle was analyzed by flow cytometry as described under "Experimental Procedures." *D*, a representative experiment showing cells present in each division phase where the number of cells was quantified and expressed as a percentage of total cells.

Three volumes of staining solution, containing propidium iodine (PI, 50 μg/ml) and DNase-free RNase (20 μg/ml), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a BD Biosciences FACS Calibur flow cytometer equipped with CellQuest Pro software. In particular, the characteristics of cell distribution in the sub-G_1 region were studied on the DNA histogram.

Analysis of Apoptosis by Flow Cytometry—Cells floating in the supernatant and adherent cells were harvested by trypsin solution and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with PBS. Then, 2×10^5 cells were pelleted and suspended in 200 μl of buffer solution and stained with annexin-V-fluorescein isothiocyanate and PI as described by the manufacturer (BD Biosciences). The cells were diluted by adding 300 μl of buffer solution and processed for data acquisition and analysis on a BD Biosciences FACS Calibur flow cytometer using CellQuest Pro software. The *x* and *y* axes indicated the fluorescence levels 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. Cell death was monitored in the early stages of apoptosis (annexin-V^+/PI^-), in late apoptosis (annexin-V^+/PI^+), and in necrotic cells (annexin-V^-/PI^+). The percentage of apoptotic and necrotic cells was also evaluated by fluorescence microscopy using Hoechst 33258/PI cell staining (29).

Statistical Data Analysis—Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t* test and was used to compare the Ilomastat effect to vehicle-treated cells. Probability values of less than 0.05 were considered significant, and an *asterisk* identifies such significance in Figs. 2 and 4.

RESULTS

MT1-MMP-mediated Activation of Latent Pro-MMP-2 by Concanavalin A Correlates with a Decrease in G_0/G_1 and an Increase in Sub-G_1 Cell Cycle Phases—Lectins such as concanavalin A (ConA) and wheat germ agglutinin are known to regulate MT1-MMP-mediated pro-MMP-2 and to induce cell death (30, 31). ConA, in particular, is further well documented to promote MT1-MMP trafficking from a storage pool to the cell surface (32) and is, thus, routinely used to assess the MT1-MMP-mediated processes involved in pro-MMP-2 activation (13, 16). A dose-response to ConA was performed in BMSC, and pro-MMP-2 activation was assessed by zymography. Latent pro-MMP-2 was increasingly processed to its active MMP-2 form (Fig. 1*A*) and, concomitantly, MT1-MMP proteolytic processing to its inactive 43-kDa form was also induced (Fig. 1*B*). Furin inhibition with the synthetic furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone confirmed the nature of the active MT1-MMP build-up (data not shown) as reported previously (33). Because pro-MMP-2 activation is thought to interfere with cell survival and proliferation (34, 35), we also sought to investigate the effect of ConA on BMSC cell cycle progression. Cells were treated with various doses of ConA, staining was performed with PI, and flow cytometry was used to assess the proportion of cells found in the G_0/G_1, S, G_2/M, and sub-G_1 cell cycle phases (Fig. 1*C*). ConA induced a dose-dependent decrease in G_0/G_1 that was concomitantly observed with an increase in sub-G_1 (Fig. 1*D*). While the S and G_2/M cell cycle phases remained unaffected, these observations suggest that ConA triggers some cell death process.

Ilomastat Inhibits Concanavalin A-induced Pro-MMP-2 Activation and Cell Necrosis/Late Apoptosis—In light of our previous observation that ConA potentially decreased BMSC

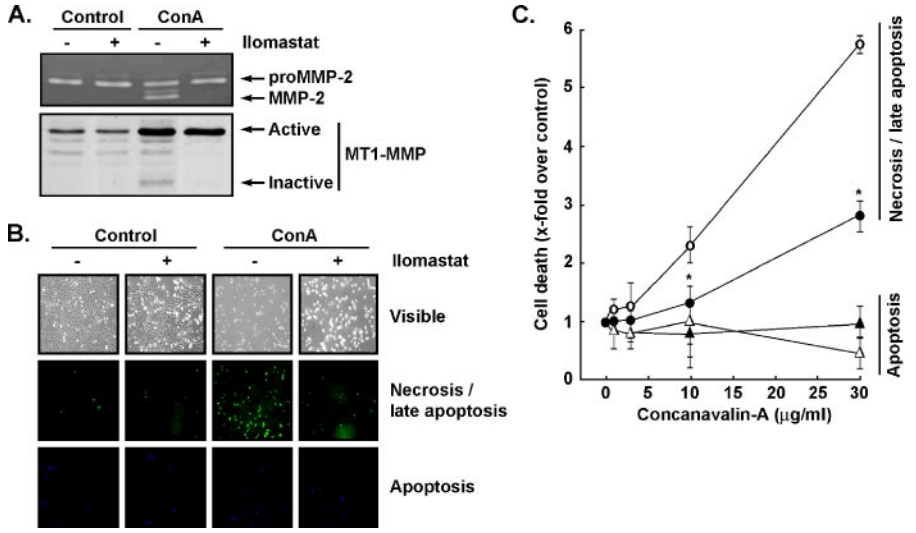

FIGURE 2. **Ilomastat inhibits concanavalin A-induced pro-MMP-2 activation and cell necrosis.** BMSC were serum-starved for 24 h then treated with 30 μg/ml of concanavalin A in the presence or absence of 10 μM Ilomastat for 18 h. *A*, conditioned medium was harvested, and 20 μl was used to assess pro-MMP-2 activation using gelatin zymography (*upper panel*). The corresponding cell lysates were used to immunodetect MT1-MMP proteolytic processing (*lower panel*). *B*, BMSC were cultured and treated as described above. Hoechst (apoptosis) and propidium iodine (necrosis/late apoptosis) staining was then performed and visualized using fluorescence microscopy. *C*, BMSC were treated with increasing concentrations of concanavalin A as in Fig. 1 in the presence (*black symbols*) or absence (*open symbols*) of Ilomastat. Cell apoptosis (*triangles*) and necrosis (*circles*) were analyzed by flow cytometry after double staining with annexin-V and propidium iodine as described under "Experimental Procedures." Probability values of less than 0.05 were considered significant, and an *asterisk* identifies such significance in Ilomastat-treated cells against untreated control.

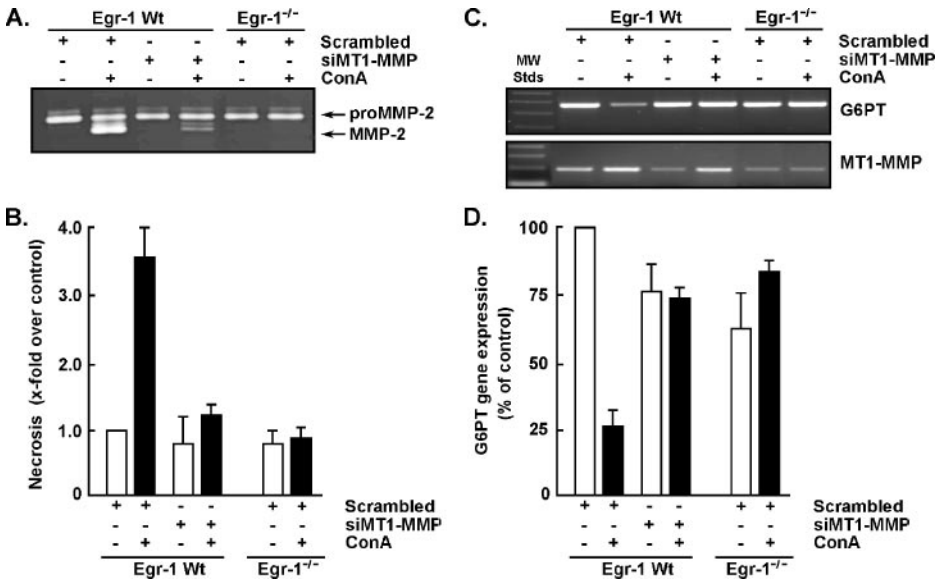

FIGURE 3. **Gene silencing of MT1-MMP diminishes concanavalin A ability to induce cell necrosis.** BMSC (*Egr-1 Wt*) transfected with either scrambled mismatched siRNA oligonucleotids (*Scrambled*) or with the siRNA against MT1-MMP (*siMT1-MMP*) or BMSC isolated from Egr-$1^{-/-}$ mouse were serum-starved in the presence or absence of 30 μg/ml concanavalin A. *A*, conditioned medium was harvested, and 20 μl was used to assess pro-MMP-2 activation using gelatin zymography. *B*, cell death was assessed in BMSC treated as above and then stained with annexin-V and propidium iodine as described under "Experimental Procedures." *C*, total RNA was isolated and RT-PCR used to assess the gene expression levels of G6PT and MT1-MMP. *D*, semiquantitative evaluation of G6PT gene expression was performed using scanning densitometry and values expressed as percent of untreated mock-transfected cells.

staining was not changed between all the conditions tested (Fig. 2*B*, *lower blue panels*). In contrast, necrosis/late apoptosis staining of the cells with PI showed increased cell death in ConA-treated cells, which was significantly reduced in the presence of Ilomastat (Fig. 2*B*, *middle green panels*). When cell death was assessed through annexin-V/PI staining and analyzed by flow cytometry, we observed that only cell necrosis (Fig. 2*C*, *open circles*) was induced by ConA treatment, while cell apoptosis was unaffected (Fig. 2*C*, *open triangles*). Interestingly, only partial inhibition (~65%) by Ilomastat was observed when cells were treated with both ConA and Ilomastat (Fig. 2*C*, *closed circles*), while the combined treatment did not affect cell apoptosis (Fig. 2*C*, *closed triangles*). The differential inhibitory efficacies of Ilomastat on pro-MMP-2 activation and cell necrosis/late apoptosis suggests that inhibiting the catalytic function of cell surface MT1-MMP is not sufficient to completely inhibit ConA-induced cell necrosis/late apoptosis.

Gene Silencing of MT1-MMP Diminishes Concanavalin A Ability to Induce Cell Necrosis/Late Apoptosis—To further investigate the role of MT1-MMP in ConA-mediated activation of pro-MMP-2 and in cell necrosis/late apoptosis, we specifically down-regulated MT1-MMP gene expression using siRNA or isolated BMSC from early growth response-1-deficient (Egr-$1^{-/-}$) mice, Egr-1 being the transcription factor that tightly regulates MT1-MMP gene expression. Egr-1 protein expression in BMSC was validated (36) and cells assumed to have two normal alleles of *Egr-1* wild type (Wt) (*Egr-1*$^{+/+}$) gene. These were either transfected with scrambled nucleotide siRNA or with siMT1-MMP and compared with BMSC isolated from Egr-$1^{-/-}$ mice. Upon treatment with ConA, pro-MMP-2 was significantly activated in Egr-1 Wt BMSC, while a significant reduction in pro-MMP-2 activation was noticed in Egr-$1^{-/-}$ BMSC and in BMSC in which MT1-MMP expression was silenced (Fig. 3*A*). When these same sets of cells were assessed for ConA-induced cell death, ConA was

survival, we next assessed whether cell apoptosis or necrosis was involved. First, cells were treated (or not) with ConA in the presence or absence of Ilomastat, a broad range MMP inhibitor. Gelatin zymography shows that Ilomastat efficiently inhibited both pro-MMP-2 activation and MT1-MMP proteolytic activation by ConA (Fig. 2*A*). Apoptosis as revealed by Hoechst

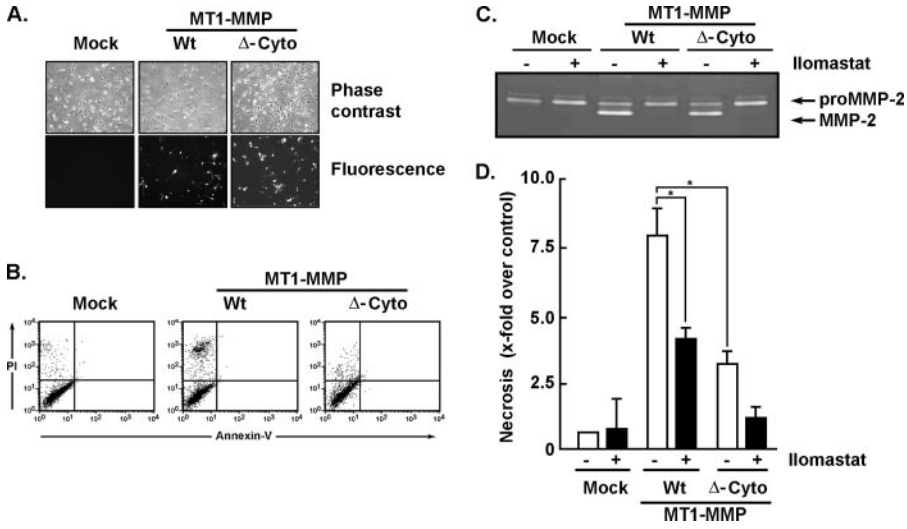

FIGURE 4. **Induction of necrosis by MT1-MMP requires intracellular signaling through its intact intracellular domain.** BMSC were transiently transfected with empty plasmid cDNA or cDNA encoding the full-length (*Wt*) or cytoplasmic truncated (Δ-*Cyto*) GFP-fused MT1-MMP. *A*, representative pictures showing transfection efficiency and expression of the GFP-MT1-MMP recombinant proteins. *B*, because of the staining with annexin-V-fluorescein isothiocyanate, BMSC were transfected with plasmids cDNA encoding the non-GFP recombinant Wt and Δ-Cyto MT1-MMP. BMSC were then serum-starved for 18 h in the presence or absence of 10 μM Ilomastat and conditioned medium harvested to assess pro-MMP-2 activation using gelatin zymography. *C* and *D*, cell death was then assessed in transfected BMSC and quantified in the presence (*black bars*) or absence (*white bars*) (D) of Ilomastat through staining with annexin-V and propidium iodine as described under "Experimental Procedures."

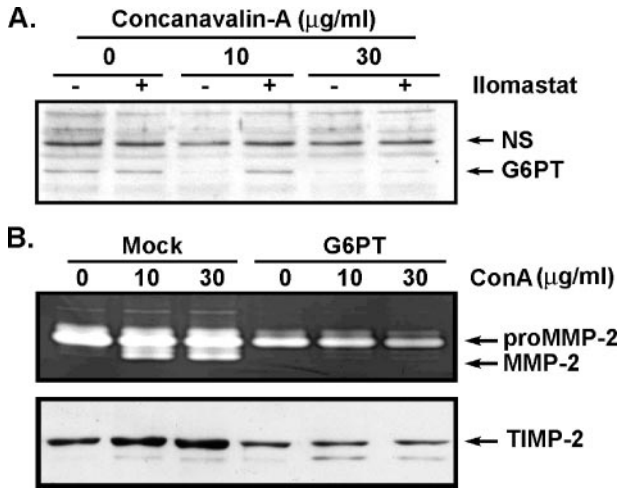

FIGURE 5. **Concanavalin A inhibits G6PT gene and protein expression.** BMSC were treated as in Fig. 1 in the presence of various doses of concanavalin A in the presence or absence of 10 μM Ilomastat. *A*, cell lysates were used to immunodetect the microsomal G6PT at ~43 kDa (*NS*: nonspecific immunoreactive protein). *B*, BMSC were transfected with empty plasmid cDNA (*Mock*) or a cDNA encoding G6PT and were treated as above in the presence or absence of 10 μM Ilomastat, and conditioned media were harvested and used to assess pro-MMP-2 activation using gelatin zymography.

unable to trigger cell necrosis in siMT1-MMP-transfected cells and in Egr-1$^{-/-}$ BMSC (Fig. 3*B*). Next, total RNA was isolated from cells under the same conditions and assessed for G6PT and MT1-MMP gene expression. We observed that ConA, as expected, induced MT1-MMP gene expression in mock and still was able to induce the remaining low endogenous MT1-MMP gene expression in siMT1-MMP-transfected cells (Fig. 3*C*). However, while ConA down-regulated glucose 6-phosphate transporter (G6PT) gene expression in mock-transfected cells, it had no inhibitory effect on the G6PT mRNA level in BMSC transfected with siMT1-MMP or isolated from Egr-1$^{-/-}$ BMSC (Fig. 3*D*). These results suggest that MT1-MMP and its transcription factor regulator Egr-1 are required in the G6PT gene expression regulation.

Induction of Cell Death by MT1-MMP Requires an Intact Intracellular Domain—To test the hypothesis that alternate domains in MT1-MMP are involved in signaling cell death in BMSC, we transiently transfected cells with cDNAs encoding recombinant forms of a GFP-fused, full-length Wt MT1-MMP as well as of a GFP-fused cytoplasmic-truncated MT1-MMP (Δ-Cyto) (37). Overexpression of the recombinant fluorescent forms of MT1-MMP confirmed that BMSC are efficiently transfectable (Fig. 4*A*). Interestingly, when annexin-V/PI staining of the cells was performed, a significant (~8.5-fold increase) proportion of the cells overexpressing the Wt MT1-MMP shifted into necrosis, while overexpression of the Δ-Cyto MT1-MMP only partially (~3-fold increase) triggered cells into necrosis (Fig. 4*B*). Functionality of the membrane-bound, recombinant MT1-MMP forms was also confirmed by their capacity to activate latent pro-MMP-2 into MMP-2 as shown by gelatin zymography, and this catalytic function was inhibited by Ilomastat (Fig. 4*C*). Notably, when cell necrosis was assessed in the MT1-MMP transfected cells, Ilomastat, as it did in ConA-treated cells, partially inhibited the Wt MT1-MMP-induced cell death and completely antagonized the slight increase in cell necrosis that was due to the overexpression of the Δ-Cyto MT1-MMP. Collectively, this shows that the catalytic function of MT1-MMP is partially responsible for the induction of cell death but that the intracellular domain of MT1-MMP is the most crucial player in signaling intracellular events that diminish cell survival.

Concanavalin A Inhibits G6PT Gene and Protein Expression—In light of the documented prosurvival functions of G6PT (22, 23) and because of the potential common roles that G6PT and MT1-MMP possess in regulating cell chemotaxis, we assessed whether MT1-MMP mediated events also affected G6PT expression. BMSC were treated with concentrations of ConA known to trigger cell necrosis and G6PT protein expression was immunodetected in cell lysates. We observed that ConA at 10–30 μg/ml significantly inhibited G6PT expression, while Ilomastat prevented that decrease (Fig. 5*A*), suggesting that G6PT potentially regulates some ConA-induced MT1-MMP-mediated events. To further assess the impact of G6PT, conditioned medium was harvested from mock-transfected or G6PT-transfected cells that were treated (or not) with ConA. We observed that ConA induced an MT1-MMP-mediated activation of pro-MMP-2 and concomitant increase in extracellu-

lar TIMP-2 levels (Fig. 5*B*) in agreement with previous reports (38). Cells overexpressing G6PT completely inhibited pro-MMP-2 activation by lowering extracellular TIMP-2 secretion (Fig. 5*B*) and possibly preventing an optimal MT1-MMP·TIMP-2·pro-MMP-2 ternary complex. Unexpectedly, G6PT overexpression in BMSC could not prevent cell necrosis (data not shown).

MT1-MMP Gene Silencing and G6PT Inhibition Abrogates BMSC Chemotaxis in Response to Sphingosine 1-Phosphate—To assess the effects of MT1-MMP and G6PT on BMSC chemotaxis, we specifically down-regulated either MT1-MMP or G6PT gene expression and evaluated BMSC response to either S1P or to tumor growth factor-enriched media (U-87 CM). We found that BMSC efficiently responded to S1P or to U-87 CM chemoattraction as demonstrated by their increased migration (Fig. 6). However, MT1-MMP gene silencing abrogated the BMSC response to S1P, while only chlorogenic acid-treated cells but not MT1-MMP silencing abrogated the BMSC migration in response to U-87 CM. Collectively, this shows that MT1-MMP and G6PT functions are essential for BMSC chemotaxis. The lack of G6PT expression seems to further affect the effects of the multiple growth factors secreted by brain tumor cells.

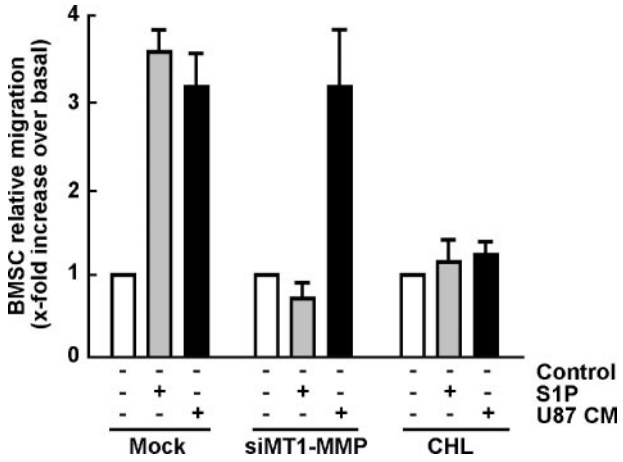

FIGURE 6. **MT1-MMP gene silencing and G6PT inhibition abrogates BMSC chemotaxis in response to sphingosine 1-phosphate.** BMSC were transfected with scrambled mismatched siRNA oligonucleotids (*Mock*) or with siMT1-MMP or treated with 100 μM chlorogenic acid, then seeded (20,000 cells/filter) on top of gelatin-coated modified Boyden chambers and left to migrate for 18 h in response to brain tumor-derived growth factors from U-87 glioma cells (*U-87 CM*, *black bars*) or for 48 h in response to 1 μM S1P (*gray bars*).

DISCUSSION

Investigation of the molecular players involved in BMSC mobilization has led to improved usage of these cells in therapeutic oncology (39, 40). Accordingly, BMSC migration capacity and their chemotactic response to circulating tumor-derived growth factors has led to the demonstration that BMSC can efficiently migrate toward experimental gliomas, making these cells a promising candidate for cellular carrier systems of anti-glioma therapy (41, 42). Among the circulating chemotactic mediators, chemokines play key roles in hematopoietic stem cell trafficking (43), while circulating lipid mediators such as S1P have emerged as one of the most potent *in vitro* BMSC chemotactic agents (13, 14). Recent findings, in fact, demonstrated that S1P is a tumorigenic growth factor, likely produced by tumor cells themselves, which could be targeted by lipidomic based cancer therapeutics (44). In line with this evidence, an original MT1-MMP/S1P signaling axis has been recently proposed to regulate both chemotaxis in BMSC (13, 14) as well as angiogenic functions in endothelial cells (45). Our current study reveals that MT1-MMP activities in ECM degradation and BMSC mobilization also regulate cell survival, potentially under the metabolic control of microsomal G6PT.

Recently, a new and unprecedented function for G6PT as a potential regulator of cell chemotaxis was reported (19). This exciting new role for G6PT in regulating cell mobilization may also affect cell proliferation, cell cycle division, ECM degradation, and response to growth factors. The latter events have, interestingly, also been shown to involve MT1-MMP functions such as in cell migration (46) and in ERK and in RhoA/ROK signaling (14, 15, 47). More recently, we have revealed several of these MT1-MMP roles in regulating the angiogenic and chemotactic properties of BMSC in response to hypoxia (36) and to brain tumor-derived U-87 glioma cells *in vitro* and *in vivo* (48). Interestingly, hypoxia increased MT1-MMP and Egr-1 levels in BMSC (36), a condition that lead to cell death (49). Whether G6PT plays a role in these conditions is currently under investigation.

In our study, we show that maintenance of cytoarchitecture is required for cell survival, since its perturbation by cytochalasin-D- or ConA-mediated MT1-MMP mechanisms down-regulated cell survival and were associated with pro-MMP-2 activation (Refs. 34 and 35 and this study). In fact, silencing of the MT1-MMP gene or of the MT1-MMP transcription factor regulator Egr-1 expression prevented ConA from inducing cell death and from activating pro-MMP-2. Moreover, we show that inhibiting the catalytic function of MT1-MMP only partially reversed the lethal effect of MT1-MMP, while we show that the intracellular domain of MT1-MMP is, in contrast, an absolute prerequisite for transducing the intracellular signaling that leads to cell death. Whether this MT1-MMP cytoplasmic domain also regulates the MT1-MMP internalization rate (50) that could be responsible for cell death signaling remains to be investigated and may be extremely relevant in light of the data we provide.

A caspase-dependent mechanism has recently been associated with MT1-MMP function in endothelial cell morphogenic differentiation (24). Interestingly, MT2-MMP has recently been suggested to act as an anti-apoptotic factor (51), a conclusion that was reached from *in vitro* selected premalignant resistant clones. In contrast to the classical MT1-MMP functions, overexpression of the human MT2-MMP in COS-1 cells did not lead to pro-MMP-2 activation (52), suggesting an alternate and distinct role for MT2-MMP in comparison with MT1-MMP. In fact, an increase in the activation ratio of pro-MMP-2 correlated directly with the expression of MT1-MMP but not of MT2-MMP (53, 54). Finally, differential regulation in response to EGF was also observed between MT1-MMP and MT2-MMP further indicating a potential distinct function between these two membrane-anchored MMP (55). Altogether, the differential functions and the balance between MT1-MMP and MT2-

MMP expression possibly provide a tight regulation of the ECM degradation and cell survival processes. Our observations suggest that MT1-MMP may act as a potential cell death sensor/effector that signals ECM degradation processes to be activated.

The fact that G6PT overexpression inhibited ConA-induced pro-MMP-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 events chronology confirming that MT1-MMP-mediated activity and signaling are among the first steps that inhibit G6PT expression, which ultimately leads to cell death. Interestingly, our data are consistent with some of the abnormal polymorphonuclear neutrophil phenotype observed in glycogen storage disease type 1b, a clinical condition where the G6PT gene and/or protein activity is defective (23, 56). In fact, induction of cell apoptosis (23), and diminution of several processes such as respiratory burst, chemotaxis, phagocytosis and calcium signaling (57), were observed when G6PT functions were inhibited with analogs of chlorogenic acid, the most potent inhibitor of G6PT (58). Chlorogenic acid cellular targeting was recently found to induce apoptosis of chronic myelogenous leukemia cell lines and primary cells from chronic myelogenous leukemia patients (59). Alterations in several biochemical parameters, glucose phosphorylation, calcium mobilization, and hexose uptake and transport, have also been described as possible mechanisms through which the G6PT functional defects may be involved *in vivo*. More recently, G6PT involvement in chemotaxis was also observed in G6PT$^{-/-}$ mice (21). That study demonstrated that neutropenia is directly caused by the loss of G6PT activity and that chemotaxis and calcium flux were defective in G6PT$^{-/-}$ animals. Since cells such as polymorphonuclear neutrophil or BMSC have no detectable G6Pase activity or expression, G6PT must play a role different from that exerted in the liver where it is functionally coupled to the G6Pase enzyme. In fact, it has been hypothesized that G6PT might function as a G6P receptor/sensor (56) or that it could favor calcium sequestration in the endoplasmic reticulum lumen (60). A potential mechanism may involve Ca^{2+} influx regulation, which is thought to inhibit MT1-MMP proteolytic processing independent of ConA action (61). Since furin enzymes are calcium-dependent (62) and have been implicated in MT1-MMP activation, it can further be hypothesized that overexpression of G6PT leads to increased sequestration of an intramicrosomal pool of Ca^{2+}, which would not then be available for cytosolic enzyme regulation. Moreover, such mechanism may potentially hamper rapid ConA-induced trafficking of MT1-MMP to the plasma membrane, a prerequisite for cell surface proteolytic activity to occur (32). It thus becomes tempting to suggest that such intracellular metabolic control of Ca^{2+} pools, and possibly of glucose/G6P, may in part contribute to regulating BMSC chemotaxis and cell mobilization.

In conclusion, our study provides the first molecular link between the ECM degrading functions of MT1-MMP and the microsomal G6PT functions that could collectively regulate BMSC chemotaxis and cell survival. In line with our current results, high glucose concentration, a condition that is known to up-regulate the G6PT expression (63), was found to antagonize the ConA-induced activation of pro-MMP-2 (64). Investigation of the metabolic control of the response of BMSC to tumor-derived growth factors will not only increase our understanding of their mobilization in pathological conditions (65) but also contribute in understanding BMSC chemotactic mechanisms involved in tissue regeneration therapeutic modalities (66–68).

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