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Induction of autophagy biomarker BNIP3 requires a JAK2/STAT3 and MT1-MMP signaling interplay in Concanavalin-A-activated U87 glioblastoma cells

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ABSTRACT

Plant lectins have been considered as possible anti-tumor drugs because of their property to induce autophagic cell death. Given that expression of membrane type-1 matrix metalloproteinase (MT1-MMP) has been found to regulate expression of the autophagy biomarker Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), we sought to investigate possible signaling interplay mechanisms between MT1-MMP and BNIP3 in Concanavalin-A (ConA) lectin-activated U87 glioblastoma cells. ConA induced acidic vacuole organelle formation as well as BNIP3 and MT1-MMP gene and protein expressions, whereas only BNIP3 expression was dose-dependently inhibited by the JAK2 tyrosine kinase inhibitor AG490 suggesting a requirement for some STAT-mediated signaling. Gene silencing of MT1-MMP and of STAT3 abrogated ConA-induced STAT3 phosphorylation and BNIP3 expression. Correlative analysis shows that STAT3 signaling events occur downstream from MT1-MMP induction. Overexpression of a full length MT1-MMP recombinant protein led to increased BNIP3 gene and protein expressions. The cytoplasmic domain of MT1-MMP was also found necessary for transducing STAT3 phosphorylation. Among JAK1, JAK2, JAK3, and TYK2, only JAK2 gene silencing abrogated ConA's effects on MT1-MMP and BNIP3 gene and protein expressions. Our study elucidates how MT1-MMP signals autophagy, a process which could contribute to the chemoresistance phenotype in brain cancer cells.

1. Introduction

Distinct from apoptosis, autophagy is an evolutionarily conserved, multi-step lysosomal degradation process in which cells degrade long-lived proteins and damaged organelles [1]. Recently, high expression levels of the Bcl-2/E1B 19 kDa interacting protein 3 (BNIP3), a key regulator of cell death/autophagy and an effector of a necrosis-like atypical death program, were reported in glioblastoma multiforme (GBM) and found to confer a survival advantage to brain tumor cells [2]. As GBM is also characterized by regions of hypoxia and often by large necrotic areas, such continuous or excessive stress ultimately results in autophagy-induced cell death. Accordingly, under stress conditions BNIP3 can translocate from the nucleus to the cytoplasm where it disrupts mitochondrial functions and leads to cell death [3]. BNIP3 can also act as an inducer of autophagy in many cell types and under different environmental conditions including hypoxia [4–6]. BNIP3 can also suppress the mTOR signaling pathway to mediate autophagic cell death in glioma cells [7]. Contrarily, BNIP3 also increased cell survival under hypoxic stress by translocating to the nucleus of human glial cells [8,9]. Thus, current opinion regarding the role of autophagy in tumorigenesis is that it can be either a tumor suppressor or a tumor promoter depending on the tumor type and stage [10].

Chemotherapy and radiotherapy were recently reported to upregulate BNIP3 expression and to ultimately trigger apoptotic cell death in colorectal cancer cells [11]. Given the signaling crosstalk between apoptosis and autophagy [11b], therapies that could produce the induction of autophagy-dependent cell death in gliomas may therefore become of interest. Interestingly, plant lectins have been documented as causing autophagic cell death and, as such, have been considered as possible anti-tumor drugs [12]. Among these lectins, numerous studies have demonstrated that Concanavalin A (ConA), a Ca^{2+}/Mn^{2+}-dependent and mannose/glucose-binding legume lectin, can induce apoptotic cell death via the mitochondrial pathway in diverse types of cancer cells, including human melanoma A375 cells and human hepatocellular carcinoma HepG2 cells [13,14]. Recently, ConA was reported to up-regulate COX-2 and to down-regulate Akt expression via the IKK/NF-κB-dependent pathway in U87 glioblastoma cells [15]. Other studies have demonstrated that cell death can occur through BNIP-3-mediated mitochondrial autophagy. In agreement with this, ConA-

Abbreviations: BNIP3, BCL2/adenovirus E1B 19kDa protein-interacting protein 3; ConA, concanavalin-A; ECM, extracellular matrix; EGCG, (−)-epigallocatechin-gallate; GBM, glioblastoma multiforme; JAK, janus kinase; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; STAT, signal transducers and activators of transcription.

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treated U87 glioblastoma cells exhibited up-regulated expression of BNIP3, ATG3, ATG12 and ATG16-L1, and these inductions were reversed when expression of membrane type-1 matrix metalloproteinase (MT1-MMP), a plasma membrane-anchored matrix metalloproteinase, was silenced [16]. Interestingly, while ConA has been shown to trigger the phosphorylation of STAT3 in mesenchymal stromal cells, regulation of BNIP3 was also found to be linked to JAK/STAT signaling [17,18].

Among the subclass of matrix metalloproteinases anchored to the cell membrane by a transmembrane domain [19], MT1-MMP possesses a cytoplasmic domain that plays critical roles in transducing several intracellular signaling pathways [20]. Moreover, this cytoplasmic domain is important for MT1-MMP internalization and recycling processes at the plasma membrane where it is believed to localize in invadopodia [21,22]. Although a role for MT1-MMP has been suggested as a cell death sensor/effector through the regulation of endoplasmic reticulum stress in U87 glioblastoma cells [23], and in necrosis [24], the exact MT1-MMP-mediated signaling that leads to induction of autophagic proteins like BNIP3 [16] remains unknown. In the present study, we used the lectin ConA from *Canavalia ensiformis* to mimic biological lectin/carbohydrate interactions and to also induce MT1-MMP gene expression[25]. Furthermore, we investigated whether any MT1-MMP/JAK/STAT signaling interplay may be involved in the pro-autophagic actions of ConA in glioblastoma cells.

2. Material and methods

2.1. Materials

Sodium dodecylsulfate (SDS), Concanavalin A, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Cell culture media were obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The JAK family tyrosine kinase inhibitor Tofacitinib (CP-690550) was from Cedarlane (Burlington, ON) while AG490 was from Calbiochem (La Jolla, CA). Anti-STAT3 (79D7) and anti-phospho-STAT3 (Tyr705) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). The polyclonal antibody against the MT1-MMP catalytic domain was from Chemicon (Temecula, CA). The monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Advanced Immunochemicals (Long Beach, CA). The polyclonal antibody against BNIP3 was from EMD Millipore (Billerica, MA). The polyclonal antibody against JAK2 was from New England Biolabs (Pickering, ON). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada.

2.2. Cell culture

The human U87 glioblastoma cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in Eagle's Minimum Essential Medium containing 10% (v/v) calf serum (HyClone Laboratories, Logan, UT), 1 mM sodium pyruvate, 100 units/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37 °C with 95% air and 5% CO_2.

2.3. Immunofluorescent microscopy

U87 cells were seeded on cover slips, transiently transfected with a siRNA against MT1-MMP or a siRNA control and then treated (or not) with 30 μg/mL Concanavalin-A for 24 h. Media were removed and cells were fixed in formalin phosphate buffer (Fisher Scientific, Ottawa, ON) for 20 min, permeabilized with Triton X-100 for 5 min and then blocked for 1 h in 1% BSA/PBS. Immunostaining was performed for 1 h with the anti-BNIP3 antibody 1:200 in 1% BSA/PBS, followed by 1:200 anti-rabbit-alexa Fluor 488 (Invitrogen, ON). A solution of 10 μg/mL DAPI diluted in PBS was used to stain the nuclei. Fluorescence was then examined by microscopy.

2.4. Gelatin zymography

Gelatin zymography was used to assess the extracellular levels of the latent proMMP-2 and active MMP-2 activities. Briefly, analiquot (20 μl) of the culture medium was subjected to SDS-polyacrylamide gel electrophoresis (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 h in 20 mM NaCl, 5 mM CaCl2, 0.02% Brij-35, 50 mM Tris–HCl buffer, and pH 7.6 and then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid and 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

2.5. 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% non-fat 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 (1/1000 dilution) in TBST containing 3% bovine serum albumin and 0.1% sodium azide, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfé, QC).

2.6. Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from cell monolayers using TriZol reagent (Life Technologies, CA). For cDNA synthesis, 2 μg of total RNA was reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using SsoFast EvaGreen Supermix (Bio-Rad). DNA amplification was carried out using a CFX connect Real-Time System (Bio-Rad) and product detection was performed by measuring binding of the fluorescent dye EvaGreen to double stranded DNA. The QuantiTect primer sets were provided by QIAGEN: MT1-MMP (Hs_Mmp14_1_SG QT00001533), BNIP3 (Hs_BNIP3_1_SG QT00024178), JAK1 (Hs_JAK1_1_SG QT00050225), JAK2 (Hs_JAK2_1_SG QT 00062650), JAK3 (Hs_JAK3_1_SG QT00078673), TYK2 (Hs_TYK2_1_SG QT00012978), GAPDH (Hs_GAPDH_2_SG QT01192646), β-actin (Hs_Actb_2_SG QT01680476) and PPIA (Hs_PPIA_4_SG QT01866137). The relative quantities of target gene mRNA compared against two internal controls chosen from GAPDH, β-actin or PPIA RNA, were measured by following a ΔCT method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and that of GAPDH and β-actin mRNAs were calculated by CFX manager Software version 2.1 (Bio-Rad) and the relative quantified value (RQV) was expressed as $2^{-\Delta C}{}_T$.

2.7. Transfection method and RNA interference

Cells were transiently transfected with 20 nM siRNA against MT1-MMP (Hs_MMP14_6 HP validated siRNA; QIAGEN, SI03648841), against STAT3 (Hs_STAT3_7 HP validated siRNA; QIAGEN, SI02662338), against JAK1 (Hs_JAK1_5 HP validated siRNA; QIAGEN, SI00605514), against JAK2 (Hs_JAK2_7 HP validated siRNA; QIAGEN, SI02659657), against JAK3 (Hs_JAK3_5 HP validated siRNA; QIAGEN, SI00604800), against TYK2

(Hs_TYK2_5 HP validated siRNA; QIAGEN, SI02223221) or scrambled sequences (AllStar Negative Control siRNA; QIAGEN, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen, CA). MT1-MMP-, JAK- and STAT3-specific gene knockdowns were evaluated by qRT-PCR as described above.

2.8. Detection of acidic vesicular organelles (AVO)

U87 cells were serum-starved in media containing specific treatments with Concanavalin-A alone or in combination with AG490 or Tofacitinib. Acridine orange (0.5 μg/mL; Sigma-Aldrich, ON) was added to each well and cells were incubated for 15 min at 37 °C in the dark. Fluorescence was then examined by microscopy.

2.9. Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk identifies such significance in the figures.

3. Results

3.1. Gene silencing of MT1-MMP inhibits BNIP3 induction by Concanavalin-A

Among the autophagy biomarkers screened, BNIP3 gene expression has been found to be significantly induced by ConA along with ATG3, ATG12, and ATG16L1 [16]. Although MT1-MMP was found to contribute to BNIP3 transcriptional regulation, no information is available regarding effects at the protein level. U87 glioblastoma cells were therefore transiently transfected with either specific siRNA to silence MT1-MMP gene expression (siMT1-MMP) or with a non-specific sequence (siScrambled). Immunofluorescent staining was performed to detect BNIP3 expression (Fig. 1A, upper panels), which was induced in ConA-treated cells (Fig. 1B) and repressed when MT1-MMP gene expression was silenced (Fig. 1A, lower panels; Fig. 1B). This was further confirmed by immunoblotting of the cell lysates where both MT1-MMP and BNIP3 protein expression was induced by ConA, a condition also known to trigger latent proMMP-2 processing into active MMP-2 (Fig. 1C, upper panel). Finally, qRT-PCR was also performed and confirmed the protein and immunostaining results described above as well as the efficient MT1-MMP gene silencing (Fig. 1D).

3.2. JAK2 inhibition antagonizes ConA-induced acidic vacuole formation and BNIP3 expression

Since ConA had recently been shown to trigger autophagy in part through a JAK/STAT signaling pathway in hepatoma cells [14] and in glioblastoma cells [16], we tested whether the JAK2 tyrosine kinase inhibitor AG490, as well as the JAK inhibitor Tofacitinib affected autophagy induction. We found that ConA-induced BNIP3 gene expression was dose-dependently inhibited by AG490, while ConA-induced MT1-MMP gene expression remained unaffected (Fig. 2A). In contrast, Tofacitinib was inefficient at downregulating either of the ConA-induced genes. When MT1-MMP-mediated proMMP-2 activation was assessed upon ConA treatment, neither of the JAK inhibitors tested inhibited the extracellular MT1-MMP catalytic functions (Fig. 2B). Finally, when cells were immunostained for BNIP3 or stained with Acridine Orange to monitor acidic vacuole organelle (AVO) formation, we observed that both AG490 and Tofacitinib efficiently antagonized ConA-induced AVO formation, while only AG490 abrogated ConA-induced BNIP3 expression (Fig. 2C). Altogether, these observations suggest that JAK2 inhibition abrogates ConA-induced BNIP3 transcriptional regulation but not that of MT1-MMP. Varying degrees of contribution from the various JAK family members seem to be involved in the overall autophagic process.

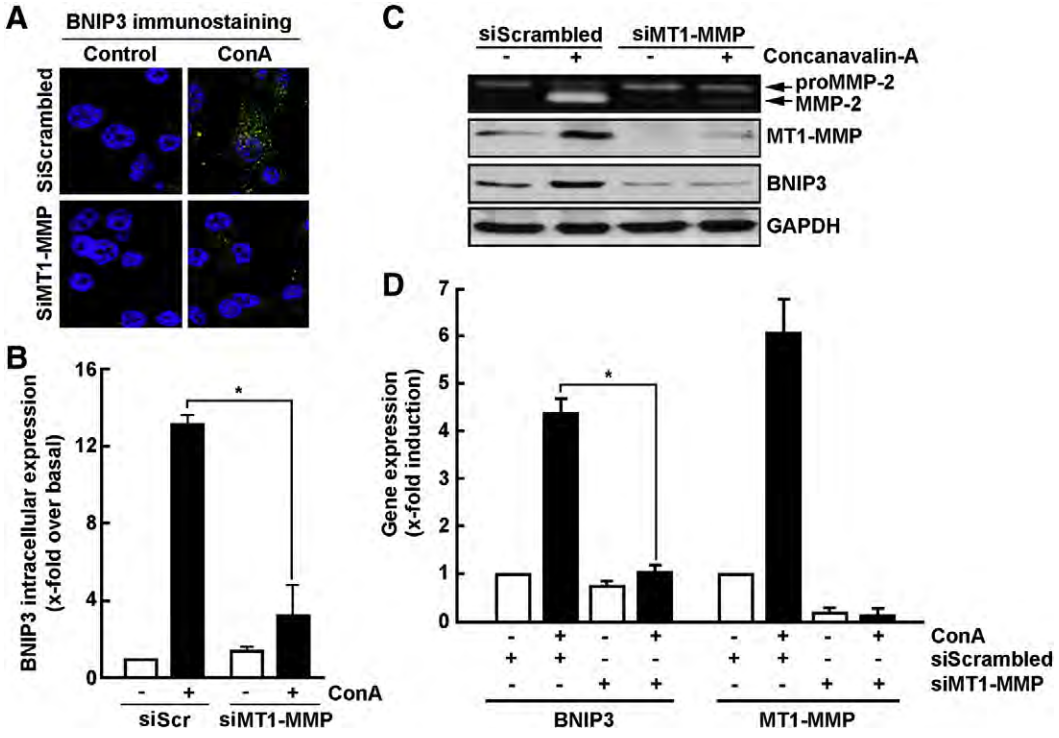

Fig. 1. Gene silencing of MT1-MMP inhibits BNIP3 induction by Concanavalin-A. Gene silencing was performed with either siScrambled (siScr) or SiMT1-MMP in U87 glioblastoma cells. Cells were then serum-starved in the presence or absence of 30 μg/mL Concanavalin-A (ConA) for 24 h. A) Immunostaining with anti-BNIP3 antibody and DAPI nuclear staining were performed as described in the Material and methods section, and B) BNIP3 intracellular fluorescence was measured by microscopy and quantified. C) Conditioned media were harvested in order to perform gelatin zymography (upper panel) and cell lysates isolated as described in the Material and methods section in order to perform MT1-MMP, BNIP3 and GAPDH immunodetection. D) Total RNA was extracted and qRT-PCR performed as described in the Material and methods section to assess MT1-MMP and BNIP3 gene expressions.

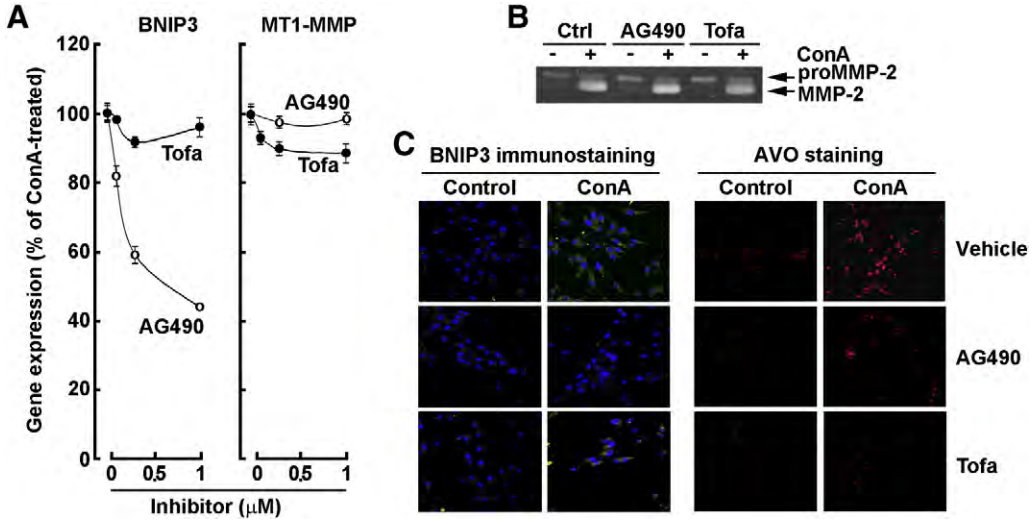

Fig. 2. JAK2 inhibition antagonizes Concanavalin-A-induced acidic vacuole formation and BNIP3 expression. A) Serum-starved U87 cells were treated for 30 min with various concentrations of AG490 or Tofacitinib (Tofa, CP-690550) and then treated with 30 μg/mL of ConA for 24 h. Total RNA isolation and qRT-PCR were performed as described in the Material and methods section to assess MT1-MMP and BNIP3 gene expressions. B) Serum-starved U87 were pretreated with vehicle or 1 μM AG490 or Tofacitinib (Tofa) for 30 min, and then treated with 30 μg/mL of ConA for 24 h. Conditioned media were isolated as described in the Material and methods section in order to assess proMMP-2 activation. C) Serum-starved U87 cells were treated as described in B). Immunostaining with anti-BNIP3 antibody and DAPI (nucleus) were performed as described in the Material and methods section, and phase contrast BNIP3 intracellular fluorescence was acquired by microscopy. Acidic vesicular organelles (AVO) staining was performed and fluorescence acquired by microscopy.

3.3. MT1-MMP expression is required to trigger STAT3 phosphorylation by ConA

Since BNIP3, but not MT1-MMP, gene expression was abrogated upon AG490 treatment and that inhibition of JAK2 led to decreased BNIP3 protein expression (Fig. 2), we used gene silencing strategies to downregulate either MT1-MMP (siMT1-MMP) or STAT3 (siSTAT3) expression. We found that, in siScrambled-transfected cells, increasing concentrations of ConA led to increased proMMP-2 activation, MT1-MMP and BNIP3 protein expression (Fig. 3A, upper panels). MT1-MMP silencing inhibited ConA-induced proMMP-2 activation as well as BNIP3 induction (Fig. 3A, middle panels). When STAT3 was silenced, proMMP-2 activation and MT1-MMP were unaffected, while ConA-induced BNIP3 expression was abrogated (Fig. 3A, lower panels). In order to evaluate the functional impact of MT1-MMP silencing on STAT3 activation, we monitored STAT3 phosphorylation (Fig. 3B), which was transiently induced by ConA, peaking at 4 h (Fig. 3C), and significantly abrogated when MT1-MMP expression was silenced (Fig. 3D). Densitometric analysis of the immunoblots allowed us to directly correlate the extent of STAT3 phosphorylation with the level of BNIP3 expression in siScrambled-transfected cells (Fig. 4A, open circles), while silencing of MT1-MMP abrogated both STAT3 phosphorylation and BNIP3 induction (Fig. 4A, closed circles). On the other hand, MT1-MMP expression also directly correlated with BNIP3 expression

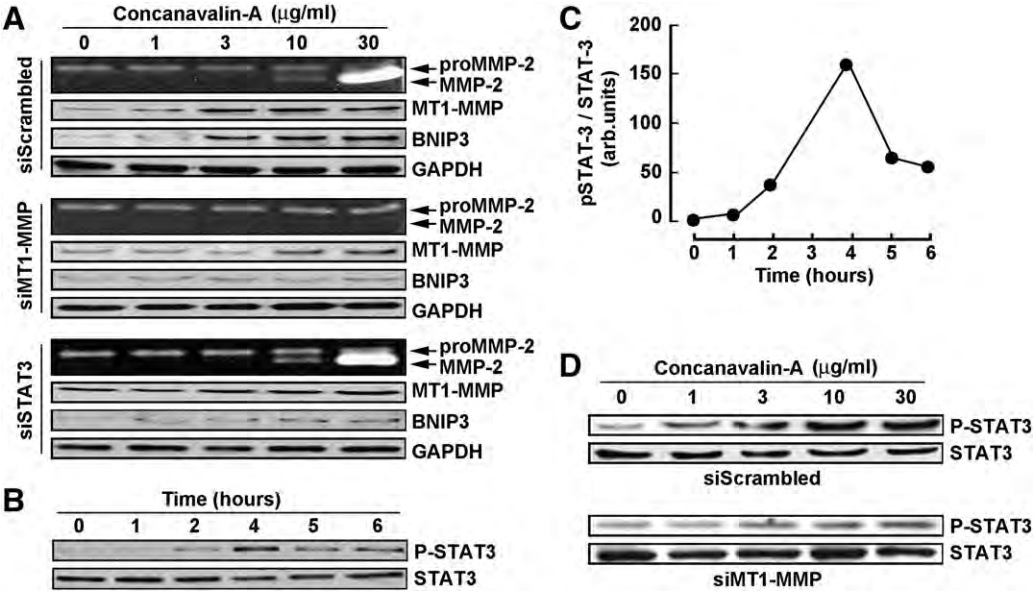

Fig. 3. MT1-MMP expression is required to trigger STAT3 phosphorylation by Concanavalin-A. A) U87 cells were transiently transfected with siScrambled, siMT1-MMP or siSTAT3 for 24 h. Serum-starved cells were then treated with various concentrations of Concanavalin-A. Conditioned media were isolated and proMMP-2 activation assessed by zymography as described in the Material and methods section. Cell lysates were prepared and used to perform MT1-MMP, BNIP3 and GAPDH immunodetection. B) Serum-starved U87 cells were treated with 30 μg/mL Concanavalin-A for up to 6 h. Cell lysates were tested to assess P-STAT3 and STAT3 expressions. Densitometric measurement was performed from a representative experiment and shows the P-STAT3/STAT3 ratio C). D) U87 cells were transiently transfected with siScrambled or siMT1-MMP for 24 h. Serum-starved cells were then treated with various concentrations of Concanavalin-A. Cell lysates were used to assess P-STAT3 and STAT3 expressions.

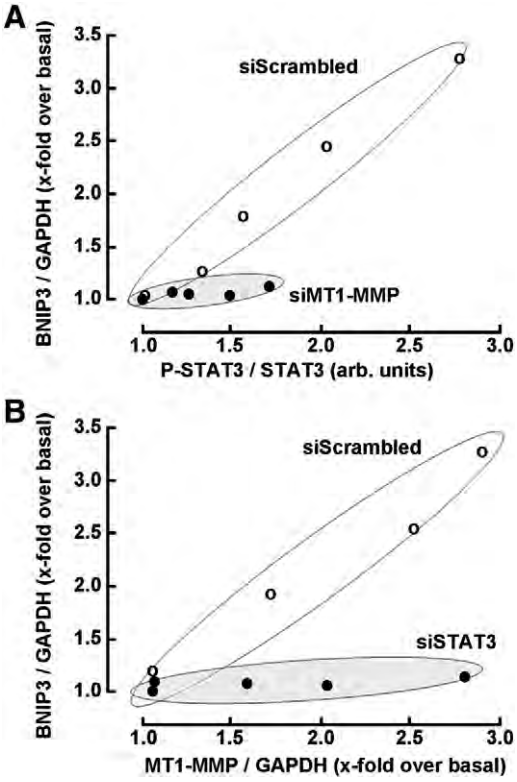

Fig. 4. Schematic representation of the correlation between MT1-MMP, BNIP3, and STAT3 phosphorylation status. Correlative analysis was performed with representative densitometric data extracted from Fig. 3. A) Correlative analysis between BNIP3/GAPDH and P-STAT3/STAT3 for each of the five Concanavalin-A concentrations used to treat siScrambled-transfected cells (white dots) and siMT1-MMP-transfected cells (black dots). B) Correlative analysis between BNIP3/GAPDH and MT1-MMP/GAPDH for each of the five Concanavalin-A concentrations used to treat siScrambled-transfected cells (white dots) and siSTAT3-transfected cells (black dots).

(Fig. 4B, open circles), while STAT3 silencing only abrogated BNIP3 induction by ConA but not the induction of MT1-MMP (Fig. 4B, closed circles).

3.4. The intracellular domain of MT1-MMP is required to trigger BNIP3 expression

Given the crucial role of MT1-MMP in the relay of ConA-mediated signaling, we next assessed the direct effect of recombinant MT1-MMP overexpression on BNIP3 expression. Cells were transiently transfected with cDNA plasmids encoding either the full length MT1-MMP (Wt) or the cytoplasmic-deleted MT1-MMP (ΔCyto) recombinant proteins. When immunostaining of BNIP3 was performed (Fig. 5A), we found that overexpression of Wt-MT1-MMP significantly triggered BNIP3 expression, whereas expression of the ΔCyto-MT1-MMP was ineffective (Fig. 5B). Overexpression and appropriate plasma membrane localization of both MT1-MMP recombinant forms were functionally confirmed by zymography since both were able to catalyze conversion of the proMMP-2 latent form into its active MMP-2 form (Fig. 5C). However, deletion of the MT1-MMP intracellular domain abrogated both BNIP3 expression and STAT3 phosphorylation (Fig. 5C). Extraction of total RNA followed by qRT-PCR analysis shows that the intracellular domain of MT1-MMP is required to trigger BNIP3 transcriptional regulation (Fig. 5D).

3.5. JAK2 is required in Concanavalin-A-mediated BNIP3 but not MT1-MMP transcriptional regulation

In order to assess their potential contributions to ConA-mediated STAT3 phosphorylation, we next used siRNA strategies to silence gene expression of the JAK family kinase members JAK1, JAK2, JAK3, and TYK2. Silencing was effectively achieved for all four genes as gene expression was reduced by 62–85% (Fig. 6A), without affecting the cells' capacity to activate proMMP-2 in response to ConA treatment (Fig. 6B). We found that BNIP3 gene expression was significantly reduced only under

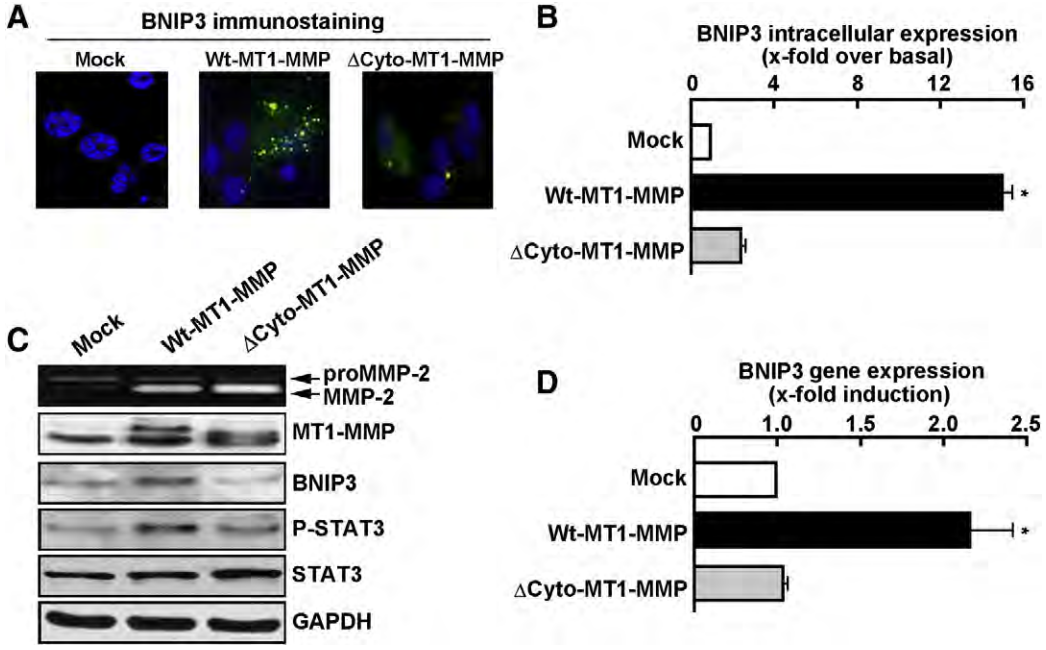

Fig. 5. The intracellular domain of MT1-MMP is required to trigger BNIP3 expression. A) U87 cells were transiently transfected with plasmid cDNAs encoding either a full length MT1-MMP recombinant protein (Wt-MT1-MMP), a cytoplasmic domain-deleted MT1-MMP recombinant protein (ΔCyto-MT1-MMP) or with an empty pcDNA vector (Mock). Immunostaining with anti-BNIP3 antibody and DAPI were performed as described in the Material and methods section, and BNIP3 intracellular fluorescence acquired by microscopy. B) Quantification of the intracellular expression of BNIP3 as observed in A). C) Cell lysates as well as conditioned media were isolated as described in the Material and methods section in order to respectively perform MT1-MMP, BNIP3, P-STAT3, STAT3 and GAPDH immunodetections or gelatin zymography to assess proMMP-2 activation status. D) Total RNA isolation and qRT-PCR were performed as described in the Material and methods section in order to assess BNIP3 gene expression.

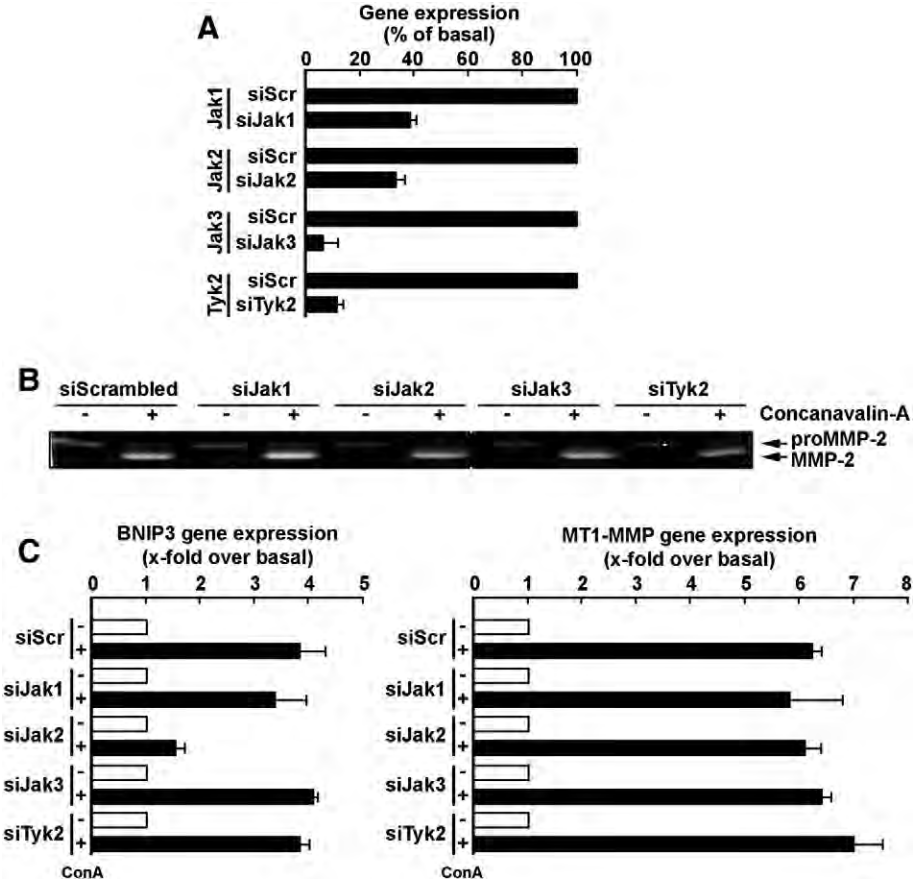

Fig. 6. JAK2 is required to regulate BNIP3 but not MT1-MMP transcription upon Concanavalin-A treatment. U87 cells were transiently transfect with siScrambled (siScr), siJAK1, siJAK2, siJAK3 or siTyk2 for 24 h. Serum-starved cells were then treated (or not) with 30 μg/mL Concanavalin-A (ConA). A) Total RNA was isolated, cDNA synthesized and qPCR performed as described in the Material and methods section to validate JAK1, JAK2, JAK3 and Tyk2 gene expression decreases. B) Conditioned media were isolated and gelatin zymography performed in order to assess proMMP-2 activation status. C) qRT-PCR was performed to assess BNIP3 and MT1-MMP gene expressions in vehicle or ConA-treated cells.

conditions where JAK2 was silenced (Fig. 6C, left panel) while MT1-MMP remained unaffected (Fig. 6C, right panel). Furthermore, when JAK2 gene expression was silenced, overexpression of the Wt-MT1-MMP recombinant protein was unable to trigger either STAT3 phosphorylation or BNIP3 expression (Fig. 7).

4. Discussion

Our current study documents the transducing functions of MT1-MMP in triggering JAK/STAT signaling, which in turn effectively regulates the transcriptional regulation and expression of the autophagy

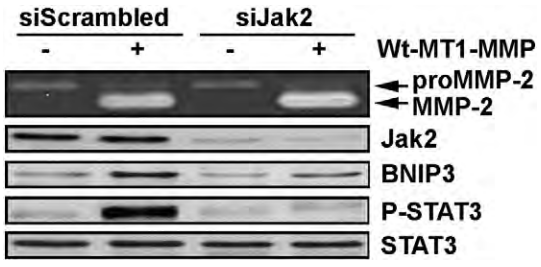

Fig. 7. MT1-MMP-mediated induction of BNIP3 and of STAT3 phosphorylation requires JAK2. U87 cells were transiently transfected with siScrambled or siJAK2 for 24 h. Then, cells were further transiently transfected with a cDNA plasmid encoding the full length Wt-MT1-MMP recombinant protein (Wt-MT1-MMP) or with an empty vector. Cell lysates as well as conditioned media were isolated in order to perform JAK2, BNIP3, P-STAT3 and STAT3 immunodetection as well as gelatin zymography to assess proMMP-2 activation status.

biomarker BNIP3 and which induces autophagic acidic vacuole formation in ConA-activated glioblastoma cells. JAK/STAT signaling is involved in numerous cancers and has proven to be an important pathway through which cancer cells survive, proliferate and increase their tumorigenicity [26]. Supporting this, inhibition of the JAK2/STAT3 signaling pathway was reported to impede the migratory and invasive potential of human glioblastoma cells [27], and to slow disease progression in orthotopic xenografts of human glioblastoma brain tumor stem cells [26]. In addition to the role it plays in angiogenesis and metastatic processes [28], the MT1-MMP-to-JAK/STAT signaling axis may therefore be viewed as a new autophagy regulator that could lead to survival of cancer cells (Fig. 8)[29,30].

The new signal transducing role ascribed to the intracellular domain of MT1-MMP, in contrast to its classical and well-documented extracellular hydrolytic functions, is thus receiving much attention. In act, numerous MT1-MMP cytoplasmic domain binding proteins have been identified and include the μ2 subunit of adapter protein 2, gC1qR, p130Cas, MTCBP-1 and the phosphorylated form of caveolin-1 [31–35]. The cytoplasmic domain of MT1-MMP has been shown to trigger multiple signaling pathways including the activation of ERK cascade [36], the induction of a RhoA/ROK [37] as well as the early signaling cascade that leads to endoplasmic reticulum stress [23] and proinflammatory phenotype [15]. The cytoplasmic domain of MT1-MMP was further demonstrated to ultimately promote invasion activity of MT1-MMP by preventing its internalization [31]. Unfortunately, it remains unknown whether any of the above-mentioned MT1-MMP intracellular binding intermediates are involved in autophagy regulation or in autophagy biomarker

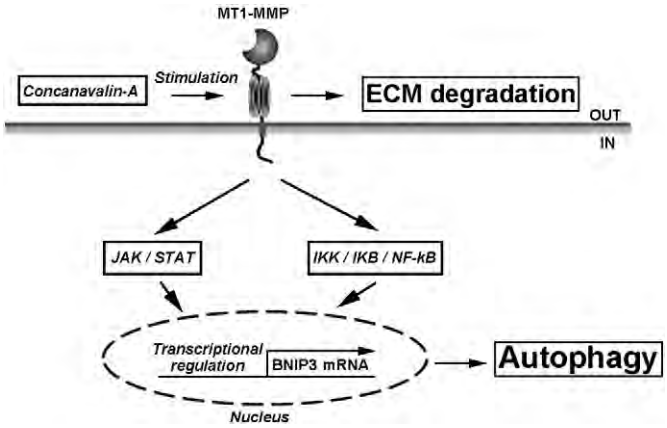

Fig. 8. A MT1-MMP-JAK-STAT signaling axis regulates BNIP3 expression. Summarized scheme showing the potential mechanism of action of Concanavalin-A-induced BNIP3 expression in U87 glioblastoma cells. MT1-MMP is a cell surface matrix metalloproteinase which, upon stimulation triggers JAK/STAT signaling (this study, 17) and other signaling pathways such as that involving IKK/IkB/NF-kB (15). Collectively, these signaling pathways may trigger autophagy and this is, in part, reflected by induction of BNIP3.

induction such as would seem to be the case for BNIP3 induction in this study.

BNIP3 is in fact an atypical representative of the Bcl-2 protein family and a regulator of non-apoptotic programmed cell death [38]. BNIP3 was demonstrated to interact with LC3 and to promote autophagy of both mitochondria and endoplasmic reticulum [39]. BNIP3 was also shown to participate in the activation of autophagy by mediating 6-thioguanine and 5-fluorouracil-induced autophagy [40]. As our current study correlates BNIP3 expression with the induction of AVO both by ConA and directly upon MT1-MMP overexpression, our data suggest that BNIP3 expression may serve as an autophagy biomarker consequent to MT1-MMP-mediated signaling in glioblastoma cells. To this end, we demonstrated that BNIP3 expression levels are correlated with a signaling cascade where MT1-MMP is a major contributor and which required phosphorylation of STAT3. Interestingly, increased JAK2/STAT3 signaling was also correlated with increased apoptosis through upregulation of BNIP3 gene expression [18]. Further support to our contention that MT1-MMP functions in BNIP3 expression was previously demonstrated when upregulation of BNIP3 and of autophagy-related gene members ATG3, ATG12 and ATG16-L1 expression in ConA-treated U87 cells was reversed when MT1-MMP gene expression was silenced [16]. Finally, the pharmacological JAK inhibitors AG490 and Tofacitinib enabled us to confirm the requirement for JAK2 in the induction of BNIP3. Whether any recruitment and/or interaction between JAK2 and MT1-MMP are required remains unknown. Structure–function analysis from this study minimally confirms the mandatory requirement for the cytoplasmic domain of MT1-MMP in the induction of BNIP3. Whether this structural requirement involves recruitment and/or interaction with specific intracellular intermediates is currently under investigation.

Recycling of cytosolic proteins and/or specific organelles is believed to be a means for cancer cells to escape apoptosis-mediated cell death in order to survive and continue their growth under stressful conditions [12]. Given its strong expression in glioblastoma cells, MT1-MMP was inferred to contribute, in part, to the acquisition of an invasive, radio- and chemoresistant phenotype of brain cancer cells [19]. It therefore becomes tempting to suggest that the MT1-MMP-mediated signaling that leads to autophagy enables those cancer cells to escape cell death. In contrast, MT1-MMP has been in turn shown to induce endothelial cell morphogenic differentiation by a caspase-dependent mechanism [41], and its recombinant overexpression in human osteoblastic SaOS-2 cells to induce apoptosis [42]. Furthermore, previous necrosis induction studies in glioblastoma cells also revealed a “bioswitch” function for a MT1-MMP/G6PT signaling axis [24]. Finally, ConA was found to increase the sub-G1 cell cycle population and to trigger cell death and this was thought to require MT1-MMP [43]. How the balance between MT1-MMP-mediated pro-apoptotic and pro-survival signaling is performed still remains to be further explored.

5. Conclusions

Among strategies developed to target MT1-MMP, the chemopreventive properties of diet-derived (−)-epigallocatechin-gallate (EGCG) were recently found to not only inhibit JAK/STAT signaling [44], but also alter MT1-MMP-mediated intracellular signaling through suppression of JAK/STAT [45]. In fact, EGCG has been well documented to target MT1-MMP-mediated functions in proMMP-2 activation [46], cell migration [47], CD44 cell surface shedding [37], as well as in the transcriptional control of proangiogenic cytokine expression [45]. Assuming MT1-MMP-mediated autophagy may be considered as an escape mechanism that would prevent cancer cell death, it is plausible that EGCG could efficiently inhibit MT1-MMP functions and sensitize cancer cells to pro-apoptotic radio- or chemotherapeutic modalities. In conclusion, the MT1-MMP-mediated signaling crosstalk between autophagy and cell death/survival has now to be considered in tailoring new pharmacological interventions aimed at reducing glioma growth using combined modalities or compounds with multiple functional targets.

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