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SPARC Upregulates MT1-MMP Expression, MMP-2 Activation, and the Secretion and Cleavage of Galectin-3 in U87MG Glioma Cells

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Abstract

Secreted protein acidic and rich in cysteine (SPARC) is highly expressed in human gliomas and promotes glioma invasion. We have shown by cDNA array analysis that SPARC upregulates membrane type 1-matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-2 (MMP-2) transcripts. To confirm these findings at the protein level and determine whether SPARC expression correlates with increased MMP activity, we used Western blot to assess the levels of MT1-MMP, and gelatin zymography to assess MMP-2 levels and activity. We also examined the expression, secretion, and cleavage of galectin-3, a target of MT1-MMP and MMP-2. Our data confirm that SPARC upregulates MT1-MMP levels and MMP-2 activity. There was also an increase in secreted galectin-3, as well as an increase in the proteolytically processed form of galectin-3. Previous studies have demonstrated that MT1-MMP, MMP-2 and galectin-3 are increased in gliomas. Our results suggest that their upregulation and activation may be a consequence of increased SPARC expression. These data provide a provisional mechanism whereby SPARC contributes to brain tumor invasion.

Keywords

SPARC; MMP-2; MT1-MMP; Galectin-3; glioma; invasion

Introduction

SPARC (also known as osteonectin and BM-40) is a 32-kDa secreted glycoprotein that has complex biological activities. As a matricellular protein, SPARC regulates cell-matrix interactions, but does not contribute significantly to the extracellular matrix (ECM) structure. SPARC is highly expressed in tissues that are undergoing morphogenesis, remodeling, and repair. The expression of SPARC modulates numerous cellular processes including, but not limited to, cell proliferation, adhesion, and spreading [4].

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High levels of SPARC have been correlated with increased expression of specific MMPs, which degrade components of the ECM and may promote cell migration, such as during tumor cell invasion. Indeed, SPARC has been associated with invasion in several different types of cancer [3,4]. SPARC has been shown to increase the expression and/or activation of MMP-1 (collagenase), MMP-3 (stromelysin), and MMP-9 in cultured rabbit synovial fibroblasts [32], MMP-2 in breast cancer cell lines [10], and with MMP-2, MMP-3, and MMP-9 in glioma [27].

Galectin-3 is a 31-kDa member of the family of β -galactoside binding lectins that modulates normal development [21], and biological processes involved in cancer, including tumor invasion and metastasis [6]. It is secreted into the ECM where it mediates cell-cell and cell-matrix interactions. Galectin-3 has a C-terminal carbohydrate binding domain (CBD) and an N-terminal domain important for galectin-3 dimerization [29]. The N-terminal domain is made up of Gly-X-Tyr repeats, which makes the protein susceptible to proteolysis by MMP-2, MMP-9 [22,31], and the MT1-MMP soluble fragment [31]. Thus, cleavage of galectin-3 is considered to be an indicator of MMP-2, MMP-9, and MT1-MMP activity [31]. The processing of galectin-3 results in the generation of 22-kDa and/or 27-kDa degradation products that include the CBD and maintain carbohydrate binding activity [20,29,31]. Thus, cleavage of galectin-3 may be an additional function of the MMPs to regulate galectin-3 activity [20].

We have demonstrated that SPARC is highly expressed in human brain tumors [24,26]. Using the non-invasive U87MG glioma cell line that is low in endogenous SPARC, we have shown that SPARC induces tumor invasion in vitro [12] and in vivo [28]. To identify genes involved in SPARC-induced invasion, we performed a cDNA array analysis [13]. These data indicated that SPARC upregulation promoted increased MMP-2 and MT1-MMP transcript abundance, 2.2 and 2.3 fold, respectively. To confirm these findings at the protein level and to determine whether SPARC contributes to increased MMP activity, we used immunoblot and gelatin zymography to assess the levels of latent and active MT1-MMP and MMP-2. We also examined galectin-3, a target of these proteinases.

Materials and methods

Derivation of the U87MG-SPARC-transfected clones was previously described [12]. The SPARC-transfected (A2b2 and A2bi) and control vector-transfected (B1b2 and C2a2) clones were maintained in DMEM plus 10% FBS, geneticin (400 μ g/ml), and puromycin (1 μ g/ml) and grown in Opti-MEM as indicated.

Gelatin zymography was used to determine the effect of SPARC expression on MMP-2 levels and species. SPARC- and control-transfected clones were plated (2×10^5 cells) for 8 h, washed twice with PBS, and grown in serum-free media for 48-72 h. Conditioned media were collected and centrifuged. Aliquots (30 μ l) of the conditioned media were subjected to 0.1% gelatin SDS-PAGE. The gels were incubated in renaturing buffer (2.5% Triton X-100) for 30 min, rinsed in water, and incubated at 37°C for 16 h in zymogram developing buffer (200 mM NaCl, 5 mM CaCl_2 , 0.02% Brij-35, 50 mM Tris-HCl, pH 7.5). Gels were stained with 0.5% Coomassie Brilliant Blue R-250 and de-stained in water containing 10% acetic acid and 30% methanol. A twin gel was stained with Coomassie Blue to show equal loading.

RT-PCR was performed to confirm the changes in MT1-MMP at the transcript level observed by cDNA array analysis. Cells (2×10^5) were plated for 3 days, and RNA was isolated using the Tri-reagent kit (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Intact RNA was verified by 1.1% formaldehyde gel electrophoresis, quantitated, and first strand cDNA synthesis and RT-PCR were performed as previously reported [13] using primers for MT1-MMP (forward 5'-ATAAACCCAAAAACCCACC-3'

and reverse 5'-ACACCCAATGCTTGTCTCC-3') and for GAPDH (forward 5'-CGTCTTCACCACCATGGAGA-3' and reverse 5'-CAGGGGTCTTACTCCTTGGA-3'). GAPDH was coamplified as a normalizing control.

For MT1-MMP analysis, cells (2×10^5) were plated for 18 h. Lysates were extracted using single detergent lysis buffer as described [29] and concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal concentrations were resolved by 10% SDS-PAGE [29]. Proteins were transferred to nitrocellulose membranes and immunoblot analysis was performed with MT1-MMP antibody # AB-815 (Chemicon International, Temecula, CA) or the anti-MT1-MMP cytoplasmic tail antibody (Triple Point Biologics, Forest Grove, OR) with detection using Super Signal West Femto Enhanced Chemiluminescence reagents (Pierce). Membranes were stripped and probed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL Western Blotting Detection Reagents (Amersham, Piscataway, NJ).

For galectin-3 studies, cells (4×10^5) were plated for 24 h, washed twice with Opti-MEM, and incubated in Opti-MEM for 2 days. Media were collected, treated with protease inhibitor cocktail (Calbiochem, San Diego, CA) to prevent further cleavage of galectin-3, and concentrated approximately 25-fold using Centricon Centrifugal Filters (Millipore, Bedford, MA). Cell lysates were extracted using single detergent lysis buffer as described previously [25]. Media and lysate samples were mixed with reducing Laemmli SDS sample buffer, boiled, and resolved by 10% SDS-PAGE, followed by transfer to Immobilon-P membranes (Millipore). Membranes were probed using the HL-31 polyclonal galectin-3 antibody (from Dr. Avraham Raz) that recognizes the full-length galectin-3 and its cleaved products. Protein was detected, and membranes were stripped and probed for actin detection as above. Densitometry analyses of the relative MMP-2, MT1-MMP, or galectin-3 levels in the SPARC-transfectants and control cells were performed using actin as a loading control as previously reported [13].

Recombinant human galectin-3 (from Dr. Avraham Raz) was incubated at 37°C with a recombinant catalytic domain of human MT1-MMP (Calbiochem) in a 1:10 molar ratio of MT1-MMP to galectin-3 in 50 mM Tris/HCl pH 7.5 buffer supplemented with 150 mM NaCl, 5 mM CaCl₂ and 0.02% Brij 35. At various times, an aliquot of the reaction containing ~40 ng of galectin-3 was collected and mixed with SDS-sample buffer. Samples were then resolved by reducing 12% SDS-PAGE followed by immunoblot analysis using the HL-31 polyclonal galectin-3 antibody as above. To determine the cleavage site of galectin-3 by MT1-MMP, galectin-3 was incubated for 1 h at 37°C with the catalytic domain of MT1-MMP as described above. Three µg of galectin-3 were then subjected to 12% reducing SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie Blue R-250. The protein band corresponding to the ~22-kDa degradation product was excised and sent to ProSeq (Boxford, MA) for N-terminal sequencing.

Results

In this report, we utilized zymography and immunoblot analyses of SPARC- and control-transfected cells to determine whether increases in transcript observed using cDNA array analysis correlated with increases in protein expression and/or activity. Immunoblot analysis (Fig. 1A) confirmed the increased expression of SPARC in the cell lysates and conditioned media of the SPARC-expressing clones relative to the vector-transfected clones. Zymography (Fig. 1B) indicated that increased SPARC expression was associated with increased MMP-2 activity in both the lysates and conditioned media. A twin Coomassie Blue-stained gel of the lysates indicated equal loading (data not shown).

We next examined the expression of MT1-MMP, which along with TIMP-2, is the physiological activator of MMP-2. Using RT-PCR, increased levels of MT1-MMP transcript were observed in SPARC- versus control-transfected cells (Fig. 2A). Using immunoblot analysis, MT1-MMP protein levels were also higher in the SPARC-transfected clones compared to controls. The protein was detected using antibodies that detect the cytoplasmic tail and the hinge region (Fig. 2A). Densitometry indicated that the SPARC-transfected cells had > 2-fold increase in MT1-MMP transcript (Fig. 2B) and protein (Fig. 2C) compared to control-transfected cells. No changes were observed in TIMP-2 levels (data not shown).

We previously reported that galectin-3 is cleaved by MMP-2 and MMP-9 at the Ala⁶²-Tyr⁶³ peptide bond generating a ~22-kDa fragment [20]. Using conditioned media containing a recombinant soluble ectodomain of MT1-MMP we have also reported an increase in galectin-3 processing, which indicated MT1-MMP activity [31]. However, the kinetics and cleavage site of MT1-MMP-dependent galectin-3 degradation were not reported. Here we examined the kinetics of galectin-3 degradation by MT1-MMP in a purified system and determined the N-terminal sequence of the cleaved product. To this end, recombinant galectin-3 was incubated with a recombinant catalytic domain of human MT1-MMP and aliquots were collected at various times for immunoblot analysis. MT1-MMP cleaved galectin-3 (31-kDa) to a ~22-kDa product in a time-dependent manner (Fig. 3). The ~22-kDa degradation product was readily detected after 10-min incubation, and after 2 h most of the galectin-3 was converted to the degradation product. N-terminal sequencing of the 22-kDa fragment revealed an N-terminus starting with Tyr⁶³ consistent with a cleavage at the Ala⁶²-Tyr⁶³ peptide bond. These results establish galectin-3 as an MT1-MMP substrate via a cleavage site that is also targeted by MMP-2 and MMP-9 [20].

Since galectin-3 is a target of MMP-2 and MT1-MMP, we examined protein levels of the 31-kDa and the proteolytically processed forms. Immunoblot (Fig. 4A) and densitometric (Fig. 4C) analyses of equally loaded lysates showed similar levels of galectin-3 in the SPARC- versus control-transfected cells, and only the 31-kDa galectin-3 was present (Fig. 4A). In contrast, immunoblot (Fig. 4B) and densitometric (Fig. 4D) analyses of conditioned media showed increased secretion of full-length galectin-3 and the 27-kDa and 22-kDa forms in the SPARC- versus control-transfected cells. The increased secretion of the full-length galectin-3 did not completely account for the increase in galectin-3 processing, as there was a greater percentage of the cleaved forms in the SPARC-transfected cells than in control cells (Fig. 4D), thus indicating an increase in protease activity with increased SPARC expression.

Discussion

We and others have demonstrated that increased SPARC expression correlates with glioma invasion in vitro [12] and in vivo [27,28]. In vivo, we observed that increased SPARC expression promoted tumor invasion of individual cells along blood vessels, through the corpus collosum, and into adjacent brain tissue [28]. cDNA array analysis identified MT1-MMP and MMP-2 as possible candidates that might promote this invasion [13]. In this study, we determined that increased SPARC expression induced an increase in MT1-MMP transcript and protein, an upregulation and activation of MMP-2, and an increase in the secretion and cleavage of galectin-3, which is a target of both MT1-MMP and MMP-2. Although the exact means whereby SPARC increases the expression of these proteins is unknown, these data provide a possible mechanism whereby SPARC promotes glioma invasion by increasing the degradation of the surrounding ECM via MMP activation and/or altering tumor cell adhesion and motility via galectin-3 secretion and/or cleavage.

The role of MMPs in glioma invasion is well documented [2,8,23]. Differential roles have been suggested whereby MMP-9 contributes primarily to invasion along established blood vessels,

but MT1-MMP and MMP-2 may regulate both invasion and angiogenesis [9]. Indeed, activation of pro-MMP-2 requires MT1-MMP [11]. In gliomas, MT1-MMP expression correlates with tumor grade [18,19] and invasiveness [15]. Furthermore, transfection of U251 cells with MT1-MMP displayed prominent activation of MMP-2 and increased invasive growth *in vitro* [17], and promoted invasion of C6 cells in central nervous system white matter [1].

Increased MMP-2, along with increased MMP-9 and MMP-3, has also been observed in a genetically defined model of SPARC-induced glioma invasion [27]. Invasion was attributed to MMP-3. However, the effects of MMP-2 on invasion may have been clouded by the expression of the other MMPs. That study did not evaluate MT1-MMP expression.

A benefit to the U87MG cell line used here is the lack of MMP-9 expression, thereby eliminating possible confounding effects, especially with respect to cleavage of galectin-3 [20]. Our data suggest that SPARC-induced upregulation and activation of MT1-MMP and MMP-2 may contribute to SPARC-induced invasion.

Although reports on the expression of galectin-3 in gliomas have been conflicting, galectin-3 is expressed in a number of glioma cells lines [16]. We previously reported [5] that galectin-3 expression increases during glioma progression, whereas other studies have found a global decrease in expression [7,14]. This discrepancy may be due to the specific regions of tumors analyzed, as higher galectin-3 expression is associated with the invasive regions *in vivo*. Indeed, glioblastoma cells demonstrated greater motility on galectin-3 *in vitro*, suggesting that increased galectin-3 promotes invasion [7]. In addition, galectin-3 expression is not restricted to the tumor cells. Therefore, galectin-3 expression from other normal cell sources must also be considered, as heterogeneous expression within the tumors results from various cell types including microglia and endothelial cells [30].

The increasing expression of MMP-2 and MMP-9 associated with glioma progression could result in more cleavage of galectin-3. Since the cleaved fragment is present and stable *in vivo* and can compete for cell surface or ECM binding, it has been speculated that cleavage would result in changes in cell adhesion and motility [22]. Our results show that there is an increase in galectin-3 secretion by the more invasive SPARC-expressing cells, and much of the secreted protein is cleaved. Galectin-3 cleavage in these cells is likely mediated by MMP-2 and/or MT1-MMP. Indeed, here we confirmed that MT1-MMP can readily accomplish the degradation of galectin-3 to the ~22-kDa product by hydrolyzing the same peptide bond cleaved by gelatinases.

In summary, these studies suggest a novel mechanism whereby SPARC promotes glioma invasion, and this function of SPARC warrants further investigation.

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Figure 1.

Increased SPARC correlates with increased MMP-2 expression and activity. **A.** Immunoblot analysis demonstrating increased SPARC in lysates and conditioned media of SPARC- (A2bi, A2b2) versus control- (B1b2, C2a4) transfected cells. +C/SPARC: SPARC positive control. Actin: loading control. **B.** Zymography demonstrating increased MMP-2 expression and activation in lysates and conditioned media of SPARC- versus control-transfected cells. (+C/MMP2) and (+C/MMP9): MMP-2 and MMP-9 positive controls. The latent, intermediate, and active forms are indicated. **C.** Densitometric analyses demonstrating increased active MMP-2 in the lysates (L) and intermediate MMP-2 in the conditioned medium (CM) of the SPARC-

versus control-transfected cells. (The active MMP-2 signals in CM were too weak to quantitate). Representative results from n = 3 experiments.

Figure 2.

SPARC increases expression of MT1-MMP. **A.** RT-PCR analysis demonstrating an increase in MT1-MMP transcript in SPARC- (A2b2, A2bi) versus control- (C2a2, B1b2) transfected cells. GAPDH serves as the internal control. Immunoblot analysis demonstrating increased MT1-MMP in SPARC- versus control-transfected cells using the AB-815 antibody to the hinge region or the antibody to the cytoplasmic tail. Actin: loading control (only one actin blot is illustrated). Note: The lanes for the protein standard were moved closer from another region of the same blots. **B and C.** Densitometric analyses demonstrating increased MT1-MMP transcript (**B**) and protein (densitometry for the cytoplasmic tail antibody immunoblot is shown

[C]) in the SPARC- versus control-transfected cells. Representative results from $n = 3$ experiments.

Figure 3.

MT1-MMP cleaves galectin-3. Recombinant human galectin-3 was incubated with a recombinant catalytic domain of human MT1-MMP in a 1:10 molar ratio of MT1-MMP to galectin-3. Samples were taken at the indicated time points and resolved by reducing 12% SDS-PAGE followed by immunoblot analysis using anti-galectin-3 antibody recognizing full length and cleaved galectin-3.

Figure 4.

SPARC increases galectin-3 secretion and cleavage. **A and C.** Immunoblot and densitometric analysis demonstrating no difference in galectin-3 levels in lysates of SPARC- (A2bi, A2b2) versus control- (B1b2, C2a2) transfected cells. Actin (A) is the loading control used for normalization in (D). **B and D.** Immunoblot and densitometric analysis showing the increase of full-length and both cleaved forms of galectin-3 in conditioned media from SPARC versus control-transfected cells. The relative levels of the 31-kDa (black), the 27-kDa (grey), and the 22-kDa (white) proteins are illustrated (D). The bars in total indicate relative levels of total secreted galectin-3 by each cell line. Representative results from $n = 3$ experiments.