SPARC-induced migration of glioblastoma cell lines via uPA-uPAR signaling and activation of small GTPase RhoA

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Abstract. Secreted protein acidic and rich in cysteine (SPARC) is highly expressed in human gliomas where it promotes invasion and delays tumor growth, both in vitro and in vivo. SPARC, which interacts at the cell surface, has an impact on intracellular signaling and downstream gene expression changes, which might account for some of its effects on invasion and growth. Additionally in vitro studies demonstrated that SPARC delays growth, increases attachment, and modulates migration of tumor cells in an extracellular matrix-specific and concentration-dependent manner. Because the signaling aspect of this migration is neither well understood nor characterized, we overexpressed SPARC in both the minimally-invasive U87 cell line and in the most aggressive invasive cell line, SNB19. We first performed RT-PCR analysis and observed an upregulation of uPA and its receptor, uPAR. We also observed increased expression levels of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9). Western blot analysis confirmed these results, and the enzymatic activity of the metalloproteinases and uPA was further supported by zymography. Downstream of the uPA-uPAR interaction, upregulation of PI3-K occurred in cells overexpressing SPARC. Using GST-TRBD, we showed the upregulation of active GTP-bound RhoA, but neither Rac1 nor Cdc42 were activated. The inhibition of uPA and uPAR downregulated PI3-K activity and cell migration, as shown by matrigel invasion assay. A dorsal skin-fold chamber model revealed the high angiogenic activity of SPARC, though the proliferation of SPARC overexpressing cells was unaffected. Our results show that the small GTPase RhoA was a critical mediator of invasion or migration in the uPA-uPAR/PI3-K signaling pathway.

Introduction

Expressed during many stages of development in a variety of organisms, the matricellular protein SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM-40) is expressed primarily in tissues that undergo consistent turnover or in sites of injury/disease in adult vertebrates (1). The capacity of SPARC to bind to several resident proteins of the extracellular matrix (ECM), to modulate growth factor efficacy, to affect the expression of matrix metalloproteinases (MMPs), and to alter cell shape as a counter adhesive factor, supports the idea that SPARC acts to regulate cell interaction with the extracellular milieu during development and in response to injury in vivo.

In an attempt to clarify current information and to propose functions for SPARC in living tissues, Golembieski and Rempel (2) confirmed that SPARC was expressed at high levels in neurogliomas and melanomas, respectively. It has been demonstrated that SPARC is present in high levels in Grade 2 and 3 bladder cancers (3), while others have implicated elevated levels of SPARC in renal cell carcinomas with subsequent tumor development and neovascularization and/or invasion (4). Further two groups have highlighted the fact that SPARC is found in high levels in invasive tumor cells (5,6).

The uPA-uPAR complex is a multifunctional system involved in wound healing, immune response, vascular...
remodeling and cancer. Invasion of cancer cells is a complex process in which the tumor cells migrate from the primary site through the ECM barrier into surrounding normal tissue by degrading the ECM. This complex process involves many proteolytic enzymes which are either serine proteases, especially those constituting the plasminogen activator/plasmin system (7), or matrix metalloproteases (8). Urokinase-type plasminogen activator (uPA) is often expressed at high levels in malignant tumors (9), and its activity is high and localized at the invasive edge of these tumors (10). A large body of evidence suggests that uPA plays a key role in tumor progression and invasion by virtue of its ability to activate plasminogen, which degrades many ECM components and activates latent collagenases (11). The effects of uPA-uPAR are not restricted to localizing proteolysis on the cell surface, but are mediated by various signaling pathways activated when uPA binds to uPAR, as well as by uPA-independent functions of uPAR. Signaling cascades induced by uPA-uPAR have been widely studied and found to be exclusively cell specific (12).

PI3-K is a heterodimer composed of a regulatory p85 subunit and a 110-kDa catalytic subunit. The ability of p85 to associate with other proteins links PI3-K to distinct signaling cascades required for proliferation, adhesion, and motility (13). PI3-K has been shown to act downstream of FAK in cell migration and survival. Kusch et al (14) showed that uPA induces PI3-K activation, which has a role in vascular smooth muscle cell migration. PI3-K and small Rho GTPases such as Cdc42 and Rac1 are key effectors that regulate cell migration by means of dynamic changes in the actin cytoskeleton (15,16). The present study was undertaken to assess the importance of SPARC activation of uPA-uPAR signaling in cell migration via activation of the PI3-K pathway involving the GTPase RhoA. The results of the present study demonstrate an additional function to SPARC: activation of the PI3-K/Akt pathway via the upregulation of uPA-uPAR signaling in tumors.

Materials and methods

Cell culture and isolation of stable cell line. SNB19 and U87 glioblastoma cells were cultured in advanced DMEM supplemented with 2% FBS in a humidified CO2 incubator at 37°C. SNB19 and U87 cells were transfected with SPARC cDNA-containing vectors using FuGene 6. Stable transfectants were selected with medium containing 600 μg/ml of G418. Cells transfected with an empty expression vector served as a control. Medium containing 250 μg/ml of G418 was used to maintain transfected cells expressing SPARC (pSPARC). Transwell inserts with 8-μm pores were coated with 0.7 mg/ml Matrigel (Collaborative Research, Inc., Boston, MA). SNB19 and U87 cells were transfected with pEV or the vector overexpressing SPARC (pSPARC). Transwell inserts were subjected to SDS-PAGE and were analyzed by the respective antibodies using standard Western blot protocol.

Matrigel invasion assay. The invasiveness of Matrigel was tested in vitro with the Boyden chamber invasion assay after transfection with either the pEV or the vector overexpressing SPARC (pSPARC). Tranwell inserts with 8-μm pores were coated with 0.7 mg/ml Matrigel (Collaborative Research, Inc., Boston, MA). SNB19 and U87 cells were detached using 5 mM EDTA, and 500 μl of the cell suspension (1x10^6 cells/ml) was added to the wells. After incubation for 24 h at 37°C, the cells were stained with Hema-3. The cells and Matrigel on the top surface of the filter were carefully removed with a cotton swab. The invasive cells adhering to the bottom surface of the filter were quantified under a light microscope (x20). The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Experiments were performed in triplicate filters and were counted at five areas.

Immunofluorescence microscopy. We used a previously described protocol (17-19) with minor changes. Briefly, the cells on the cover slips were washed once with PBS after the stated incubation time, and fixed in 4% paraformaldehyde (w/v) in PBS for 15 min at RT, followed by three PBS washes. Cells were then permeabilized with 0.5% Triton X-100 (w/v) in PBS for 10 min, followed by three PBS washes. Finally, the cells were blocked with 1% BSA (w/v) in PBS overnight at 4°C. For staining, the cells were incubated in uPAR antibody solution. The primary antibodies on the cell were detected by counter-reaction with secondary antibody conjugated with fluorescent materials. For double staining, the cells were also incubated in anti-SPARC primary antibody and the corresponding secondary antibody, as described above, followed by three washes with PBS and mounted onto a glass-slide in Vectashield. The results were documented using a fluorescence microscope.
Rho protein pull-down assay. GST-TRBD and GST-PAK PBD fusion proteins were expressed in Escherichia coli and affinity purified. Glutathione agarose beads containing 20-30 μg of GST-TRBD or GST-PAK PBD were incubated with lysates of transfected and non-transfected SNB19 and U87 cells for 60 min. The proteins precipitated on the agarose beads were washed three times with wash buffer and then eluted by boiling in loading buffer at 95°C for 10 min. The samples were subjected to SDS-PAGE and immunoblotting with corresponding antibodies.

Fibrin zymography. Zymography was used to determine the activity of the plasminogen activators as previously described (20). The samples were subjected to SDS-PAGE with 10% gels that contained fibrinogen and plasminogen. Following electrophoresis, the gels were washed twice with 2.5% (v/v) Triton X-100 for 30 min each time to remove SDS. Finally, the gels were incubated with 0.1 M glycine buffer (pH 7.5) at 37°C overnight, stained with amido black, and then destained. Final gel had a uniform background except in regions to which uPA had migrated and cleaved its substrate.

Gelatin zymography. Gelatin-substrate gel electrophoresis was performed as described previously (21). SNB19 and U87 cells were transfected with pEV or pSPARC. To collect conditioned media, cells were washed once with serum-free medium and incubated with fresh serum-free medium. After 12-14 h, conditioned media were collected, centrifuged to remove cellular debris, and protein concentrations were determined. Equal amounts of protein were subjected to 0.1% gelatin SDS-PAGE, under non-reducing conditions. Gels were washed in 2.5% Triton X-100 and incubated overnight in Tris-CaCl2 buffer. The gels were then stained with 0.2% Coomassie blue for 30 min and destained in 20% methanol and 10% acetic acid. The clear bands represent gelatinase activity.

Dorsal skin-fold chamber model. Athymic nude mice (n=12, nu/nu, male, 30-35 g) were bred and maintained within a specific pathogen-free environment. At the implantation site for the U87 and SNB19 glioma cells, we used the dorsal skin-fold chamber preparation that contains 2 layers of the membrane on both sides of a ring. This model is characterized by both solid and diffuse infiltrative glioma growth patterns and represents a versatile technique for the non-invasive analysis of glioma microcirculation and hemodynamics (6).

Results

Overexpression of SPARC in the U87 and SNB19-transfected cells. To study the effects of SPARC overexpression, we transfected SNB19 and U87 cell lines with a pcDNA mammalian expression vector harboring SPARC sequences driven by a CMV promoter. Increase in mRNA levels was determined by the semi-quantitative reverse transcription PCR. Total RNA was isolated from the cells 48 h after transfection, and first-strand cDNA was prepared using oligo-dT primed Superscript III reverse transcriptase. PCR analysis was carried out using the primers for SPARC. Clear amplification was observed in the transfected cells at approximately 440 bp (SPARC) as compared to non-transfected cells. The levels of GAPDH remained unchanged in all treatment groups, indicating uniform levels of first-strand cDNA taken in all cases (Fig. 1A).

Western blot analysis confirmed that SPARC overexpression increased the levels of SPARC in SNB19 and U87 transfected with pSPARC compared to control and pEV-transfected cells (Fig. 1B).

Levels of MMP-9, uPAR and uPA protein expression. The levels of uPAR protein in the total cell lysate and uPA protein in the conditioned media were assessed by Western blotting and fibrin zymography respectively. Western blot analysis using the anti-uPAR antibody revealed a significant increase in the levels of uPAR after cells were transfected with pSPARC (Fig. 2A).

PCR analysis was performed using the primers for MMP-9, uPAR and uPA. Clear amplification was observed in the transfected cells with pSPARC at approximately 455 bp with the primers for MMP-9, 280 bp with the primers for uPAR and 780 bp with primers for uPA as compared to non-transfected cells. The levels of GAPDH remained unchanged (Fig. 2B).

FACS analysis showed the upregulation of uPAR in the pSPARC-transfected cells as compared to the controls (Fig. 2C). Further immunofluorescence analysis of the localization of uPAR and SPARC revealed that the proteins were co-localized (Fig. 2D). It is well known that uPAR localizes at the leading edge of migrating cells, regardless of their normal or malignant status (Fig. 2D). Gelatin zymography, using conditioned medium from transfected U87 and SNB19 cells with pSPARC increased the levels of MMP-9 when compared to control and pEV-transfected cells (Fig. 2E).
Fibrin zymography, using conditioned medium from treated cells, revealed an increase in the levels of uPA activity in the pSPARC-treated cells as compared to the controls. No significant change was observed in the cells treated with the empty vector (Fig. 2F).

Overexpression of SPARC upregulates the invasive potential of SNB19 and U87 cells. The uPA-uPAR system is known to aid in normal cell migration as well as to promote invasion of tumor cells. To study the effect of SPARC-mediated increases of uPA and uPAR and their invasive potential, SNB19 and U87 were transfected with pSPARC and transferred to matrigel-coated transwell chambers and allowed to invade through the matrigel. Staining of pSPARC transfected cells that invaded through the matrigel was much higher compared to the cells transfected with pEV or control (Fig. 3A). Quantitative analysis of the number of cells showed a two- to three-fold increase in SPARC-transfected cells compared to controls (Fig. 3B). The effect was higher in U87 cells compared to SNB19 cells.

Overexpression of SPARC causes rapid and transient activation of RhoA, but not Rac1 or Cdc42. We addressed the question
of whether or not RhoA, Rac1 and Cdc42 are activated in response to SPARC overexpression using pull-down assays. For Rho proteins, both the TRBD-containing and the PAK-PBD-containing GST fusion proteins were employed. The lysates of control and SPARC-overexpressing cells were incubated with the appropriate beads and the precipitated proteins were analyzed by immunoblotting with anti-RhoA, anti-Rac1 or anti-Cdc42 antibodies. As shown in Fig. 4A overexpression of SPARC led to rapid and significant activation of RhoA. In pull-down experiments performed to evaluate the activation of Rac1 and Cdc42 proteins, we observed no significant changes in the amount of active Rac1 and Cdc42, respectively (Fig. 4B and C). The lower panel in the figure demonstrates that equal cell lysate protein amounts were used for precipitation reactions. In various cell types, RhoA and Rac1 might be activated to different stimuli. PI3-K was shown to be acting upstream of Rho proteins.

**Overexpression of SPARC does not affect proliferation.** The MTT assay is used for the quantitative determination of cellular proliferation and activation. We performed the assay with pSPARC-transfected cells from the U87 and SNB19 lines. We observed no significant effect on cell proliferation from SPARC overexpression (Fig. 5). An earlier study by Rempel (22) also supports these findings.

**Overexpression of SPARC enhances angiogenesis in gliomas.** A strong line of experimental evidence supports the concept that solid glioma growth is critically dependent on angiogenesis, i.e., the formation of new blood vessels that guarantee tissues sufficient oxygen and nutritional supply (23).
Implantation of SPARC-overexpressed U87 and SNB19 glioma cells into the dorsal skin-fold chamber induced a strong angiogenic response as compared to the control glioma cells (Fig. 6A). Between the two cell lines, angiogenic activity was higher in SNB19 cells than in U87 cells. The tumor microvasculature demonstrated chaotic micro-angiarchitecture and enormous amounts of new blood vessel formation, which has a ‘curly’ appearance compared to the pre-existing blood vessel. Our results are in accordance with the findings of a study by Rempel (22), in which the results demonstrated the angiogenic effect of SPARC in C6 rat gliomas.

Further, we demonstrated the involvement of the uPA-uPAR system in the SPARC-mediated invasion/migration or angiogenesis. We addressed this question by downregulating uPA (puPA), uPAR (puPAR) and both (pU2) using the siRNA constructs in the SPARC overexpressed SNB19 stable cell line. We performed Western immunoblot analysis using the whole cell extracts from these transfected cells and probed
with PI3-K and FAK, which are downstream of uPA-uPAR signal transduction pathway, and found that in the cells transfected with bicistronic constructs, there was a considerable amount of reduction in both PI3-K and FAK compared to the pEV-, puPA- and puPAR-transfected cells (Fig. 6B). Fig. 6C shows in vivo angiogenic assay results using the dorsal skin-fold chamber which support the above finding by no neovascularization in the cells transfected with the bicistronic constructs.

Discussion

In this study, we suggest that SPARC functionally contributes to glioma invasion through the modulation of several mechanisms that regulate tumor growth and migration. It has been reported that SPARC expression is high in cancer cells relative to normal cells, and also that the aggressiveness and migratory capacity of the cancer cells depends on the concentration of SPARC in the ECM. As a result of these experiments, we propose that SPARC is a target candidate for the design of therapies to specifically treat glioma invasion.

Our immunocytochemistry studies as well as earlier reports (24) support the contention that the majority of SPARC is localized to the cytoplasm. However, we cannot rule out the possibility that a small, immunohistochemically undetectable amount of SPARC may be localized to the nucleus. A recent report demonstrated that exogenous SPARC was taken up into endothelial cells and translocated into the nucleus (25) where it could potentially exert its effect on cell division. Thus, we need to consider that SPARC’s effects on gliomas may occur both extracellularly and intracellularly. Experiments are currently underway to determine whether SPARC functions via independent mechanisms that affect cell motility and proliferation. In our immunocytochemical analysis, we found that SPARC-transfected cells showed co-localization of uPAR and SPARC. In addition, after probing the cells with anti-uPAR antibody, FACS analysis demonstrated the upregulation of uPAR in SPARC-transfected cells as compared to control and pEV-transfected cells. It has been reported by many researchers that uPAR localizes at the leading edge of migrating cells. This may be one explanation for the high invasive capacity of cells with elevated SPARC expression.

The degradation of existing extracellular matrix (ECM) involves proteolytic enzymes such as plasminogen activators (e.g., uPA) and matrix metalloproteinases (MMPs) and their respective inhibitors (e.g., PAI-1 and TIMP-1) (26-28). The link between uPA-uPAR system regulates the migration of various cell types and has an important role in the progression of cancer. Our RT-PCR result confirms the upregulation of uPA and its receptor at the mRNA level, supporting earlier reports that the cancer cells overexpress uPA and uPAR (30). In addition, the FACS analysis shows the upregulation of uPAR in SPARC clones and pSPARC-transfected cells. However, we suspect that MMP-2 and MMP-9 activation may, in part, contribute to SPARC's promotion of invasion in gliomas. It has been shown by cDNA array analysis that MMP-2 expression can be upregulated by SPARC (2). Further, SPARC has been shown to induce the activation of MMP-2 in breast cancer cell lines (31). Zymography results revealed MMP-2 activation in the SPARC-transfected and stable clones as compared to the controls, therefore supporting previously reported data. In our studies, we also found that SPARC clones and transfected cells showed a high content of PI3-K. Previous reports of PI3-K involvement in uPAR signaling may explain the results observed in the present study; PI3-K may also be involved in the activation of Rho GTPases.

We demonstrate here that the small GTPase RhoA is involved in the SPARC-induced migration of SNB19 and U87 cells. RhoA was highly activated in response to SPARC, as demonstrated by the GST-TRBD pull-down assay. Once the G-protein coupled receptors (GPCR) are activated, the Rho proteins are also activated. Because uPAR lacks a transmembrane domain, it requires some additional protein to transduce extracellular stimuli into the cell. The adaptor molecule that is best known in uPAR signal transduction is the integrin αvβ3. Here, the integrin αvβ3 may also be required to transduce the signal to the interior of the cell. Recently, small GTPases, particularly RhoA and Rac1, were shown to be upregulated and exhibited high activity in isolated growing lamellipodia (32). We did not find any change in the amount of GTP-Rac1 and GTP-Cdc42 (data not shown) in our pull-down assays. A possible explanation is that the activation and deactivation of these molecules or redistribution of the active form of the protein may occur in a negligible amount as compared to RhoA. Additionally, redistribution of Cdc42 to the leading edge of the migrating cells was not observed (32).

Taken together, these results clearly support our hypothesis. Unlike normal cells in culture that express SPARC yet are adherent, U87 cells are weakly adherent and show low to intermediate levels of SPARC. Highly aggressive SNB19 cells show more SPARC expression. SPARC transfection induced intermediate to high adherence in our study. However, exceptionally high levels of SPARC can promote de-adhesion and, therefore, can also induce an intermediate level of adherence (22,33). This represents the contradictory situation that both low and high levels of SPARC can promote more aggressive invasion than intermediate levels.

SPARC may contribute to mechanisms involving uPA-uPAR in cell migration. SPARC binds to the inhibitor of uPA (34), and thus, could indirectly promote tumor infiltration by permitting uPA greater receptor interaction, leading to downstream signaling involving PI3-K and small GTPases. Also, its interaction with the ECM molecule vitronectin (34), which is found in high abundance along vessel basement membranes, may directly contribute to tumor invasiveness by facilitating migration along those structures.

In terms of the function of SPARC, we have characterized two aspects: the proliferation and angiogenesis of glioma cells. Proliferation in the overexpressed SPARC cells was not affected when compared to the controls, as shown by the MTT assay. During angiogenesis, endothelial cells proliferate and differentiate under stimulation from various factors such as basic fibroblast growth factor (bFGF), tumor necrosis factor (TNF-α), angiopoietin-1 (ANG-1), vascular endothelial growth factor (VEGF), heparin-binding EGF-like growth factor (HBEGF), and insulin-like growth factor 1 and 2 (IGF-1 and -2). These angiogenic factors (IGF-2, VEGF, BFGF,
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SPARC overexpression are closely associated and involve SPARC-mediated invasion/migration of gliomas.
In conclusion, our data highlight that tumor invasion and SPARC overexpression are closely associated and involve PI3-K and RhoA GTPase. These results shed light on potential targets for inhibiting tumor cell infiltration in gliomas and, possibly, other cancers.

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