Secreted Protein Acidic and Rich in Cysteine Promotes Glioma Invasion and Delays Tumor Growth in Vivo

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ABSTRACT

Secreted protein acidic and rich in cysteine (SPARC) is highly expressed in human astrocytomas, grades II–IV. We demonstrated previously that SPARC promotes invasion in vitro using the U87MG-derived clone U87T2 and U87T2-derived SPARC-transfected clones, A2b2, A2bi, and C2a4, in the spheroid confrontation assay. Additional in vitro studies demonstrated that SPARC delays growth, increases attachment, and modulates migration of tumor cells in extracellular matrix-specific and concentration-dependent manners. Therefore, we propose that SPARC functionally contributes to brain tumor invasion and delays tumor growth in vivo, and that the effects of SPARC are related to the level of SPARC secreted into the extracellular matrix. To test these hypotheses, we stereotactically injected these clones into nude rat brains (six animals were injected per clone). Animals were sacrificed on day 7 to assess growth and invasion for all clones at the same time in tumor development. To determine whether SPARC delayed but did not inhibit growth, rats were injected with U87T2 or clone A2b2, and the animals were sacrificed on days 9 (U87T2) and 20 (A2b2), when the animals demonstrated neurological deficit. Brains were removed, fixed, photographed, paraffin embedded, and sectioned. Sections were then serially stained with H&E for morphological assessment of invasion and to measure tumor volume, immuno-histochromically stained to visualize SPARC, subjected to in situ hybridization with the human AluII DNA-binding probe to identify human cells, and immuno-histochromically stained with MIB-1 to measure proliferation index. The results demonstrate that SPARC promotes invasion in vivo at day 7. Both the low (A2b2) and the high (A2b2) SPARC-secreting clones produced invasive tumors, invading with fingerlike projections and satellite masses into adjacent brain, as well as along the corpus callosum. The intermediate SPARC secreting clone (C2a4) primarily migrated as a bulk tumor along the corpus callosum. SPARC significantly decreased tumor growth at day 7, as measured both by adjusted MIB-1 proliferation indices (U87T2 = 95.3 ± 1.4 versus A2b2 = 73.4 ± 4.0, A2b2 = 30.8 ± 6.7 and C2a4 = 15.7 ± 13.0) and tumor volumes (U87T2 = 13.4 ± 0.6 mm³ versus A2b2 = 4.5 ± 0.6 mm³, A2b2 = 1.1 ± 0.1 mm³, and C2a4 = 0.4 ± 0.1 mm³). Furthermore, SPARC delayed but did not inhibit tumor growth. The patterns of invasion and the extent of growth delay correlated with the level of SPARC expression. We propose that the ability of SPARC to promote invasion depends on the level of its secretion and the resultant modulation of the level of adherence and motility induced. This demonstration that SPARC functionally contributes to brain tumor invasion in vivo suggests that SPARC is a candidate therapeutic target for the design of therapies directed toward inhibition of the invasive phenotype.

INTRODUCTION

The average survival time for patients with low-grade astrocytomas is approximately 6–8 years, 3 years for patients with anaplastic astrocytomas, and 12–18 months for patients with glioblastomas. Significantly, almost all astrocytoma patients eventually succumb to this deadly form of cancer. The major reason for these short survival times is that all of these tumors are highly infiltrative. They not only spread into adjacent brain tissue, giving rise to recurrences, but also migrate to distant sites in the brain, giving rise to separate tumors. Undoubtedly, the identification and characterization of the genes that regulate invasion would have a significant impact on designing treatment therapies that would specifically target invasion. With this objective in mind, we have performed previously subtractive hybridization to identify genes that were up-regulated early in astrocytoma progression and found that the matricellular protein SPARC is highly expressed in astrocytoma grades II–IV (1).

SPARC is a developmentally regulated glycoprotein that is secreted into the ECM (2). It functions as a counteradhesive protein, modifying cell shape through the dissociation of focal adhesions (3), and modulates cell-matrix interactions, presumably by binding to ECM components (2). It delays cell cycle progression in endothelial cells and fibroblasts by concentration-independent and -dependent mechanisms, respectively (4). This delay is probably accomplished, in part, by the direct interference of SPARC with platelet-derived growth factor (5) and vascular endothelial growth factor (6) receptor interactions and by indirect effects on basic fibroblast growth factor mechanisms (7). Therefore, SPARC influences several biological processes including differentiation, migration, and proliferation.

Early in development, SPARC is expressed in tissues undergoing remodeling (8) and during vascular morphogenesis (9). In the adult, expression becomes more restricted but can be activated in controllable pathological situations such as wound healing (9). However, increasing evidence in the literature suggests that SPARC expression plays a role in certain malignancies, including melanoma (10) and breast (11), colon (12), esophageal (13), and prostate (14) cancers. Our studies examining SPARC expression in human astrocytomas (1) and meningiomas (15) and an in vivo skin-fold model of invasion (16) indicated that SPARC was highly expressed in invading brain tumor cells. Therefore, our results, combined with reported data, suggest that SPARC might functionally contribute to the invasive phenotype.

To assess the effects of SPARC on tumor invasion, we selected the established cell line U87MG because it has low levels of endogenous SPARC (1), and it is not invasive when stereotactically injected into the nude rat brain. U87MG cells were transfected with the transactivator, and a selected parental clone designated as U87T2 was transfected with SPARC. Doxycycline-regulatable, SPARC-transfected clones, designated A2b2, A2bi, and C2a4, were established for further analyses. Using these clones, we demonstrated that SPARC promoted invasion in vitro using the spheroid confrontation assay (17). Additional in vitro studies demonstrated that SPARC modulated growth, attachment, and migration of tumor cells in vitro (18). Increased SPARC expression correlated with delayed cell growth and altered attachment to brain ECM proteins in ECM-specific and concentration-dependent manners (18).

Although all clones acted similarly in the growth and attachment assays, the clones differed from one another with respect to migration on the ECM proteins. Assessing our results in the context of a model
of adherence proposed by Greenwood and Murphy-Ullrich (3), we hypothesized that the ability of the SPARC-transfected clones to migrate on the ECMs correlated with the amount of SPARC secreted and, consequently, the level of adherence induced (18). Furthermore, we proposed that the effects of SPARC were complex, such that both lower and higher levels of secreted SPARC promoted a level of adherence conducive for migration, whereas intermediate levels would promote stronger adherence and less migration (18). In addition, we hypothesized that the increased invasion would be accompanied by delayed cell growth (18). Therefore, to test these hypotheses and determine whether SPARC promotes invasion and delays growth in vivo, we have used these same clones and stereotactically injected them into nude rat brain. Tumors were allowed to grow for either 7, 9, or 20 days before brain removal, fixation, paraffin embedding, and sectioning. Sections were then serially H&E stained for morphological assessment of invasion and to measure tumor volume, immunohistochemically stained to visualize SPARC, subjected to in situ hybridization with the human 

**MATERIALS AND METHODS**

**Cells.** Derivation of the U87MG-derived U87T2 and SPARC-transfected clones (A2b1, A2b2, and C2a4) was reported previously (17). Cells were grown in selective DMEM + 10% FBS at 37°C with 5% CO₂. The SPARC-transfected clones were grown in 400 μg/ml G418 (neomycin) and 1 μg/ml puromycin, whereas T2 was grown only in the presence of G418. The L, H, and I designations now included with the clone names A2b1 (L), A2b2 (H), and C2a4 (I) stand for low, high, and intermediate, respectively. They are included for clarity regarding the level of SPARC expression and secretion.

**Brain Xenograft.** Before implantation, 85–90% confluent cells were trypsinized, rinsed with DMEM + 10% FBS, and centrifuged at 1000 rpm for 10 min, and the pellet was resuspended in PBS (1 × PBS; pH 7.0–7.2; Life Technologies, Inc.). Viable cells were counted using a Hauser Scientific Brightline hemocytometer, and the concentration was adjusted to 5 × 10⁴ cells/5 μl of PBS. The cells were placed on ice. For each cell line, six nude rats were injected with 5 μl each. Injections were performed in sets of two, and the cells were freshly prepared before each set of injections. Using Institutional Animal Care and Use Committee-approved protocols, each nude rat was anesthetized and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA), and the skull was exposed. A hole was made 3 mm to the right of the bregma, and the cells were injected using a 10-μl Hamilton (#2701) syringe with a #4 point 26 s-gauge needle mounted in a stereotactic holder. The syringe was lowered to a depth of 3.5 mm and then raised to a depth of 2.5 mm. The tumor cells were injected at a rate of 0.5 μl every 10 s. On completion, a 2-min waiting period was observed before withdrawing the syringe slowly to prevent any reflux. The skull was then cleaned, the hole was sealed with bone paste, and the incision was sutured. Tumors were allowed to grow, and animals were sacrificed on day 7 (U87T2 and SPARC-transfected clones) or on day 9 (U87T2) and on day 20 (SPARC-transfected clone A2b2), when the animals developed signs of neurological deficit and had to be sacrificed. Before being sacrificed, the animals were anesthetized, and death followed cardiac puncture, perfusion with 250 ml of sterile 0.9% saline solution, and fixation with 250 ml of 10% formalin. The brains were removed and stored in 10% formalin for at least 24 h.

**Sections.** Formalin-fixed rat brains were placed in a coronal rat brain matrix (Activational Systems, Inc., Warren, MI) for 200–400 g rats, and slices into 2-mm blocks. These blocks were then processed routinely, paraffin embedded, and serially sectioned at 7 μm. Sections for H&E staining were placed onto uncoated slides. Sections intended for immunohistochemistry and in situ hybridization were placed onto positively charged Opti Plus Barrier Slides (BioGenex, San Ramon, CA). Sections from all six tumors were used as follows. Serial sections were: (a) routinely H&E stained for histomorphological assessment of invasion; (b) immunohistochemically processed for SPARC expression and localization; (c) subjected to AluII in situ hybridization to identify human tumor cells; and (d) immunohistochemically processed for MIB-1 expression to assess proliferative index. Three tumors from each set of six were completely sectioned, H&E stained to visualize the tumor, and used for tumor volume measurements.

**SPARC and MIB-1 Immunohistochemistry.** The 7-μm sections were dried in a 60°C oven for 1 h and routinely deparaffinized to water. Heat-induced antigen retrieval using citrate buffer (pH 6.0) was performed using a hot plate as described previously (1, 15). The sections were then processed using the BioGenex staining system. Unless indicated, all reagents used were provided by BioGenex as predilutes supplied in kit form. All steps were performed at room temperature using room temperature reagents. The slides were labeled with a BioGenex protocol barcode, placed on the stainer, and processed automatically according to the indicated protocol. Briefly, for SPARC and MIB-1 detection, sections were blocked with hydrogen peroxide for 5 min, rinsed in wash buffer, blocked with Power Blocking reagent for 5 min, and rinsed in wash buffer. Sections were then incubated for 1.5 h with anti-SPARC antibody (Hematologic Technologies, Inc.): 2 μl diluted in 20 ml of 0.25% BSA in PBS or for 30 min with MIB-1 anti-human Ki-67 antibody (Dako Corp.: 1:100 dilution in 0.5% BSA in PBS). Control sections were processed omitting the primary antibody or substituting with the appropriate immunoglobulin isotype. Sections were then mounted in supersensitive multilink reagent for 20 min, and rewedashed. The sections were then mounted in supersensitive streptavidin/horseradish peroxidase reagent for 20 min, and washed. For MIB-1, DAB substrate (16 drops of chromagen, 8 drops of peroxide in 20 ml of substrate buffer) was applied to the sections for 8 min. For SPARC detection, the slides were removed from the system and hand processed because of time constraints. DAB substrate was applied for 15 s.

**AluII in Situ Hybridization.** The 7-μm sections were dried in a 60°C oven. The slides were submersed in BioGenex Nucleic Acid Retrieval solution, and the loosely covered container was placed in a microwave at full power. When the solution started to boil, the container was removed, and the slides were quickly immersed in another staining container of Nucleic Acid Retrieval solution. The sections were then air-dried to subject to the BioGenex in situ hybridization protocol. The following water washes were performed: deionized water or BioGenex buffer wash as indicated. Briefly, the sections were treated in solution I for 5 min, washed, incubated with BioGenex AluII probe for 23 min, water washed, and rewedashed in AluII probe for 23 min. Control sections were performed omitting the AluII probe. The sections were then washed in water and stringency washed in 2 × SSC twice for 5 min each with a water rinse in between and after. Sections were then stringency washed in 0.5 × SSC for 5 min, followed by three rinses in buffer wash. Protein Block was applied for 20 min, and sections were washed in buffer, blocked in Peroxide Block for 10 min, washed in buffer, blocked in avidin/biotin blocking reagents for 12 min each, and washed in buffer. Sections were then exposed to Link 1 (mouse antifluorescein in PBS) for 20 min, washed in buffer, exposed to Link 2 (biotinylated Fab), fragments of anti-mouse immunoglobulins in PBS) for 8 min, washed in buffer, incubated with Label (peroxidase-labeled streptavidin in PBS) for 20 min, washed in buffer, exposed to DAB substrate (prepared as above) for 10 min, washed in buffer, rinsed in water, counterstained for 8 s in Gill II hematoxylin, blued in ammonia water, dehydrated, cleared, and coverslipped.

**Tumor Volume.** Images of H&E-stained sections containing tumor were captured in Photoshop 5.0 with a SPOT model 1.3 camera (Diagnostic Instruments, Inc.) using either the 1 × or 2 × objective. Using the NIH Image 1.62 software, the tumor in each section was manually outlined using the freehand selection tool to measure tumor area in mm². The area was then multiplied by the section thickness to achieve a section volume measurement. The volumes of all of the sections were added to arrive at the total volume for each tumor. Tumor volumes for three animals/tumor cell line were measured, and their volumes averaged. Results are presented as the mean ± SD.
MIB-1 Proliferation Index. For assessment of proliferative index, the tumor core was used. Images of MIB-1 staining were captured as described above using a ×20 objective. Two fields were used to cover the majority of the A2b2 and C2a4 tumors, whereas four fields were required to cover the majority of the U87T2 and A2bi tumors. Using Photoshop 5.0 software, the images were overlaid with a grid, and both total and MIB-1 positively stained cells were counted. The proliferation index was calculated as the number of MIB-1-positive cells divided by the total number of cells multiplied by 100. The proliferation indices for the six tumors/cell line were averaged. Results are presented as the mean ± SD.

MIB-1 Adjusted Proliferation Index. The in situ hybridization results indicated that the tumors contained various amounts of nonstaining cells that were either rat brain or vessel cells. Therefore, the tumor proliferation indices were corrected for these non-tumor cells. The same fields used for MIB-1 measurement were assessed on the adjacent sections subjected to in situ hybridization. The number of AluII positively stained cells were divided by the total number of cells counted to obtain the fraction of tumor cells within the tumor. The adjusted proliferation index was then calculated as the proliferation index multiplied by the fraction of tumor cells. The adjusted proliferation indices for the six tumors/cell line were averaged. Results are presented as the mean ± SD.

Statistical Analyses. Three animals/group were used for tumor volume calculations. Six animals/group were used for the MIB-1 proliferation index and adjusted proliferation index. Student’s t tests were used to calculate the Ps. Within each experimental group, an adjustment for multiple testing was applied using the Hochberg’s method.

RESULTS

SPARC Promotes Glioma Invasion in Vivo. Day 7 H&E-stained cross-sections were assessed for SPARC-induced changes in invasion
Parental

A

+ SPARC

D

U87T2

B

C

A2bi (L)

E

F

A2b2 (H)

G

H

I

C2a4 (I)

J

K

L

Fig. 2. Immunohistochemical analysis of SPARC expression at the brain-tumor interface. Representative regions demonstrating the most aggressive invasion observed in the set of six animals for each clone. The U87T2 clone (A–C) produced noninvasive, well-circumscribed tumors. The clones expressing the least [A2bi (L); D–F] and the most [A2b2 (H); G–I] SPARC invaded with fingerlike projections and distinct tumor satellites into adjacent brain parenchyma, as well as along the corpus collosum. The clone [C2a4 (I)] expressing the intermediate level of SPARC invaded as a bulk mass along the corpus collosum (J and K). Individual SPARC-positive tumor cells were visible within the corpus collosum (A–K at ×10; L at ×40).

(Fig. 1, A–H), and adjacent sections were immunohistochemically evaluated for SPARC expression and localization (Fig. 1, I–L). A comparison of the immunohistochemical staining patterns (Fig. 1, I–L) indicates that, as expected, all of the SPARC-transfected clones expressed SPARC at levels greater than the endogenous level of the parental U87T2 clone. However, differences were observed between the clones, with A2bi expressing the least, A2b2 expressing the most, and C2a4 expressing an intermediate level. These results are consistent with our previously reported Western results demonstrating that all clones secrete more SPARC than the parental U87T2; however, when comparing clones, A2bi secretes the least, A2b2 the most, and C2a4 an intermediate level of SPARC (18). Therefore, for clarity regarding the level of SPARC expression and secretion, the L, H, and I designations are now included with the clone names as follows A2bi (L), A2b2 (H), and C2a4 (I).

Although the parental U87T2 clone gave rise to a well-circumscribed tumor (Fig. 1, A and E), the SPARC-transfected clones all produced invasive tumors (Fig. 1, B and F; C and G; D and H). Whereas both the A2bi (L) and the A2b2 (H) clones (Fig. 2A) produced tumors invading into adjacent brain (Fig. 1, B, F, F; C, G, K), as well as migrating along the corpus collosum, the clone C2a4 (I) primarily invaded as a bulk tumor along the corpus collosum (Fig. 1, D, H, L). Therefore, although all of the SPARC-transfected clones were invasive, distinct patterns of invasion were observed.

SPARC Expression Levels Relative to the Invasion Patterns. To further assess the differences in the invasive patterns of the parental and SPARC-transfected clones, images of the tumor-brain interface and regions representative of the most aggressive invasion into adjacent brain were captured at lower magnification (Fig. 2). The parental tumors neither invaded adjacent parenchyma nor traveled along the corpus collosum, consistently giving rise to well-circumscribed tumors (Fig. 2, A–C). Both A2bi (L) (Fig. 2, D–F) and A2b2
SPARC-promoted changes in overall tumor growth, the tumor volumes were measured (Fig. 3A). Although the parental U87T2 clone gave rise to large tumors by day 7 (13.4 ± 0.6 mm³), the SPARC-transfected clones all produced smaller tumors; however, the tumor volumes differed among the clones. Of the SPARC-transfected clones, tumor volume was greatest for the A2bi tumors (4.5 ± 0.6 mm³), intermediate for A2b2 (1.1 ± 0.1 mm³), and lowest for C2a4 (0.4 ± 0.1 mm³).

**SPARC Decreases Glioma Growth in Vivo as Assessed by Adjusted MIB-1 Proliferation Index.** To determine whether SPARC affected growth at the level of cell proliferation, MIB-1 immunohistochemistry was performed (Fig. 4, A, C, E, and G) and the proliferation index (Fig. 3B) within the tumor core was calculated for U87T2 (75.8 ± 1.0), A2bi (54.0 ± 3.0), A2b2 (20.3 ± 4.4), and C2a4 (9.3 ± 7.7). An adjusted proliferation index (Fig. 3C) was calculated for U87T2 (95.3 ± 1.4), A2bi (73.4 ± 4.0), A2b2 (30.8 ± 6.7), and C2a4 (15.7 ± 13.0) that corrected for rat cells present within the tumor as detected by *in situ* hybridization (Fig. 4, B, D, F, and H). (The high SD for the C2a4 clone is attributable to the fact that two of the tumors had no detectable MIB-1 signal.) The results indicate that both the proliferative index and the adjusted proliferative index were reduced for all of the SPARC-transfected tumors. The level of proliferation correlated with the size of tumor in that the greater the adjusted proliferation index (Fig. 3C), the larger the tumor volume (Fig. 3A).

To determine whether tumor proliferation differed in the region of invasion versus the tumor core, the MIB-1 proliferation was measured (Fig. 1, M–P) and adjusted for rat cells (Fig. 1, Q–T) in each of these regions for individual sections. For example, the adjusted proliferation indices for the core versus the invading edge were: 80.3 versus 68.5 for A2bi (Fig. 4C versus Fig. 1N), 24.2 versus 15.0 for A2b2 (Fig. 4E versus Fig. 1O), and 23.5 versus 14.0 for C2a4 (Fig. 4G versus Fig. 1P), respectively. Therefore, the adjusted proliferative index at the invading edge was reduced 1.2–1.7-fold for A2bi, A2b2, and C2a4, respectively, in comparison with the tumor core and, on average, 1.5-fold for all of the SPARC-transfected clones.

**SPARC Delays but Does Not Inhibit Growth in Vivo.** Because our *in vitro* data indicated that SPARC delays but does not inhibit growth (18), we determined whether SPARC had a similar affect *in vivo*. The parental U87T2 and SPARC-transfected A2b2 clones were injected, and the tumors were allowed to grow until animals exhibited neurological deficits and had to be sacrificed. Rats were sacrificed on day 9 (parental) and day 20 (A2b2). Brains were imaged before paraffin embedding and demonstrated that the tumors from the SPARC-transfected clone continued to grow larger over the 20 days, surpassing the size of the tumors derived from the parental clone (Fig. 5, A and D). H&E-stained cross-sections of the parental and A2b2 tumors demonstrated that the parental clone produced a well-circumscribed tumor (Fig. 5B) with edema (Fig. 5C), whereas the A2b2 tumors gave rise to highly invasive tumors (Fig. 5, E and G). Furthermore, in addition to the fingerlike projections and distinct satellite tumors noted previously, these tumors also contained regions of frank necrosis surrounded by pseudopalisading cells, a hallmark of glioblastomas (Fig. 5, F and H). As was observed with day 7 tumors, SPARC expression was observed in the small invading tumor satellite cells (Fig. 5, I and J) and in more distant invading tumor masses (Fig. 5, K and L).

**Statistical Analyses.** SPARC was found to significantly reduce tumor volume for all SPARC-transfected clones, A2bi (*P* = 0.0004), A2b2 (*P* = <0.0001), and C2a4 (*P* = 0.0017), compared with the U87T2 parental clone, although the sample size was small. However, this trend was visually observed in all six animals. SPARC was found to significantly reduce (*P* = <0.0001) the proliferation index and
adjusted proliferation index for all clones compared with the parental U87T2 clone.

DISCUSSION

In this report, we stereotactically implanted the parental clone U87T2 and three parental-derived, SPARC-transfected clones into nude rat brain to determine whether SPARC functionally contributes to brain tumor invasion in vivo. Our results indicate that SPARC promotes brain tumor invasion, it delays but does not inhibit brain tumor growth, and that the effects of SPARC on invasion and growth correlate with the amount of SPARC expressed by the tumor cells. These data suggest that SPARC functionally contributes to glioma invasion through the modulation of several mechanisms that regulate tumor growth and migration and depend on the concentration of SPARC in the ECM. As a result of these experiments, we propose that SPARC is a candidate therapeutic target for the design of treatment therapies to specifically target glioma invasion.

To assess the effects of SPARC in vivo, we used the well-characterized nude rat brain xenograft model that permits an adequate brain size for tumor development and growth comparisons. To determine whether SPARC promotes invasion, we correlated SPARC expression with morphological evidence of invasion. Both lower and higher levels of SPARC correlated with invasion by fingerlike projections and satellite tumor masses into the brain parenchyma, whereas the intermediate level of secreted SPARC promoted a tumor that appeared to invade more by bulk movement along the corpus colliculsum than into adjacent parenchyma. These differences in the ability of the clones to promote invasion in vivo are in agreement with the differences ob-

![Fig. 4. Immunohistochemical analysis of MIB-1 and in situ hybridization with Alu II probe. Brains were sectioned and MIB-1 stained, and the number of MIB-1 positively staining cells and the total number of cells in a ×20 field were counted. Representative images are presented for 6 animals/clone examined (A, C, E, and G). Serial sections were subjected to in situ hybridization with the Alu II probe to detect human tumor cells in a ×20 field. Representative images are presented for 6 animals/clone examined (B, D, F, and H).](image-url)
served between clones in our reported in vitro confrontation assays, where both A2bi and A2b2 clone spheroids were better able to invade fetal brain aggregates (17). These data are consistent with the contention that SPARC promotes glioma invasion via its ECM interactions that modulate the extent of cell adherence and, thereby, cell migration (18).

Although we chose to investigate several clones so that we could compare effects relative to the amount of SPARC secreted, an alternative method would have been to choose the parental and one clone and examine invasion in the absence and presence of doxycycline. However, the use of doxycycline in vivo would likely confound our results because of its own ability to affect gene expression and protein activation. Of particular relevance, doxycycline not only inhibits MMP-2 activity but also reduces enzyme expression at the transcriptional level (19). However, we suspect that MMP-2 activation may, in part, contribute to SPARC's promotion of invasion in gliomas because we found MMP-2 expression to be up-regulated by SPARC using cDNA array analysis of the U87T2 and A2b2 clones (20), and SPARC has been shown to induce the activation of MMP-2 in breast cancer cell lines (11). Therefore, when dealing with the in vivo model, we thought that it was better to examine SPARC-specific effects on invasion.

The results also indicated that although SPARC promoted tumor invasion, it also decreased overall tumor growth, and that this decrease was accompanied by a decrease in cell proliferation as assessed by MIB-1 proliferation index. Because the AluII in situ hybridization results indicated that some of the cells contained within the tumor were not human, the MIB-1 proliferation index was adjusted to remove these cells. We found that increased SPARC corresponded with a statistically significant decrease in the adjusted proliferation index in all clones. However, a concentration effect was noted in that the lowest level of SPARC was associated with a higher proliferation index than those observed for the intermediate and high levels of SPARC. These data are consistent with our flow cytometry data that demonstrated that increased SPARC increased the percentage of cells in G0-G1 for C2a4 and A2b2 versus a greater percentage of cells in G2-M for A2bi (18). Therefore, as hypothesized, we observed that the lower level of secreted SPARC correlated with more proliferation but sufficient adherence to increase infiltrative invasion, the highest level of secreted SPARC correlated with less proliferation and increased infiltrative invasion, whereas the intermediate level of secreted SPARC correlated with the least proliferation and bulk invasion.

Our in vivo data are also consistent with our in vitro data demonstrating that SPARC delays but does not inhibit tumor cell growth (18). When the cells were implanted and allowed to grow until neurological deficit was observed, the parental U87T2 clone gave rise to well-circumscribed tumors on day 9 (similar to those observed on day 7), whereas the SPARC-transfected clone A2b2 gave rise to tumors on day 20 that were much larger than the parental clone. These tumors were not only highly invasive but also presented with regions of frank necrosis surrounded by pseudopalisading cells, features consistent with human glioblastomas. This concomitant SPARC-induced...
invasion and decreased proliferation is interesting relative to ongoing scientific debate as to whether cells can both divide and move at the same time. The inverse relationship we observed supports the hypothesis that cells can either "go" or "grow" but not do both (21), and the concept that the delay in cell cycle progression provides a mechanism whereby cells might temporarily exit the cell cycle to facilitate migration (22). These concepts were further supported by the additional decrease in proliferation associated with tumor cells at the invading edge versus in the tumor core. Tumor cells for both A2b2 and C2a4 were less proliferative at the invasive periphery than cells within the tumor core. Thus, these combined data support the concept that a decrease in proliferation is more conducive for the invasive phenotype.

The MIB-1 signal for the A2bi clone, however, was almost as high at the invading edge as in the tumor core, suggesting that these tumor cells might be both invading and dividing. Because the MIB-1 antigen Ki-67 is present throughout the cell cycle and its presence cannot be determined by the MIB-1 signal for the A2bi clone, we conclude that the cells secreted the MIB-1 clone to progress past G0-G1 and stall further in the cell cycle. This interpretation is consistent with the tumor volume data demonstrating that SPARC decreased tumor growth for all clones, and the tumor volume for clone A2bi was more similar to that of A2b2 and C2a4. These data suggest that tumor growth for A2bi was impacted more than the MIB-1 proliferation index would suggest.

 Whether the effects of SPARC on invasion and proliferation are induced by mechanisms that are linked or mutually exclusive is not yet known. For example, attenuation of cell cycle progression may be attributable independently to the direct interference of SPARC with growth factors and their receptors and the resultant attenuation of the growth signaling pathways (5, 6). The effects on tumor invasion are likely a result of the effects of SPARC on focal adhesions and cytoskeletal structure, which result in different levels of cell adhesion and migration capacity (3). It is not known whether SPARC induces these changes solely by modulating interactions with integrins and/or ECM proteins at the cell surface or through a signaling mechanism via its putative receptor (3). However, our cDNA array analyses suggest that the effects of SPARC on tumor invasion and growth are accomplished, in part, through signaling mechanisms that alter the expression of proteins that participate in the regulation of tumor migration and cell proliferation (20).

In summary, these data indicate that SPARC functionally promotes glioma invasion and delays cell growth in vivo. Because it is up-regulated in all grades of human gliomas, SPARC is a candidate therapeutic target for the design of treatment strategies to inhibit glioma invasion in glioma tumors for all grades of glioma progression.

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