Neuro-Oncology

SPARC affects glioma cell growth differently when grown on brain ECM proteins in vitro under standard versus reduced-serum stress conditions[1]

Satya V. Vadlamuri, Joe Media, Steadman S. Sankey, Alexander Nakeff, George Divine, and Sandra A. Rempel[2]

The Hermelin Brain Tumor Center (S.V.V., S.A.R.), Josephine Ford Cancer Center (J.M., A.N.), and the Department of Biostatistics and Epidemiology (S.S.S., G.D.), Henry Ford Hospital, Detroit, MI 48202, USA

Secreted protein acidic and rich in cysteine (SPARC) has a suppressive effect on U87 glioma cell proliferation when assessed in vitro and in vivo using parental U87T2 and U87T2-derived SPARC-transfected clones. Since SPARC interacts with extracellular matrix (ECM) proteins, we examined the effect of SPARC secretion on proliferation, morphology, and cell density of glioma cells grown in vitro, in the absence and presence of ECM proteins under standard (10% fetal bovine serum [FBS]) and reduced (0.1% FBS) serum stress conditions. Under standard conditions, MTT (3-(4,5-cimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) growth curves, morphology, and Western blot analyses demonstrated that SPARC had a suppressive and biphasic effect on growth that was not grossly modulated by the ECMs. The SPARC-induced changes in morphology observed at 24 h were not altered by the presence of ECMs. Under reduced-serum stress conditions, Western blot, morphological, and flow cytometric analyses indicated that the SPARC-induced suppressive growth effects were eliminated when the cells were grown on plastic. However, ECM-specific changes in growth were observed, some of which correlated with secreted SPARC levels. These results indicate that the differential effects of SPARC and ECMs on proliferation are dependent on culture conditions. Since the results obtained under standard conditions agree with our in vivo observations, we conclude that the ability of SPARC to suppress proliferation is regulated to a greater degree by the level of SPARC and that this suppressive effect is not influenced by the presence of any of the ECMs examined.
Neuro-Oncology 5, 244–254, 2003 (Posted to Neuro-Oncology [serial online], Doc 03-005, August 27, 2003. URL http://neuro-oncology.mc.duke.edu; DOI: 10.1215/S1152 8517 03 00005 X

Secreted protein acidic and rich in cysteine (SPARC),[3] also known as osteonectin and BM40, is a member of the matricellular proteins (Bornstein and Sage, 2002) that regulate diverse biological functions, including cell morphogenesis; cell differentiation; and attachment, migration, and proliferation from within the extracellular matrix (ECM). Although SPARC's mechanisms of action are not clearly delineated, some of its effects on cell attachment and motility are likely due to its disruption of integrin-ECM interactions (Greenwood and Murphy-

Received February 3, 2003; accepted June 17, 2003.

[1] This work was supported by NIH grant number R01 CA86997.

[2] Address correspondence to Sandra A. Rempel, Barbara Jane Levy Laboratory of Molecular Neuro-Oncology, Hermelin Brain Tumor Center, Department of Neurosurgery, Room 3096, Education and Research Building, Henry Ford Hospital, 2799 West Grand Blvd., Detroit, MI 48202 (nssan@neuro.hfh.edu).

[3] Abbreviations used are as follows: ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; dox, -doxycycline; ECM, extracellular matrix; FBS, fetal bovine serum; MMP, matrix metalloprotease; MTT, 3-(4,5-cimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SPARC, secreted protein acidic and rich in cysteine; TA, transactivator; TBS, Tris-buffered saline.

Ullrich, 1998; Murphy-Ullrich, 2001; Sage and Bornstein, 1991). We have shown that SPARC is highly expressed in astrocytomas (Rempel et al., 1998) and have demonstrated that SPARC functionally promotes glioma invasion in vitro (Golembieski et al., 1999) and in vivo (Schultz et al., 2002). Our analyses to characterize SPARC-ECM interactions regarding adhesion and migration indicated that an increase in SPARC promoted ECM-specific, concentration-dependent attachment, and that the amount of SPARC secreted by tumor cells may be important in determining its effects on migration (Rempel et al., 2001) and invasion (Schultz et al., 2002).

SPARC has a complex, but mainly suppressive influence on proliferation when added to cells in culture (Funk and Sage, 1991, 1993; Sage et al., 1995). The finding of this suppressive function of SPARC is supported by the observation that cells isolated from the tissues of Sparc knockout mice grow faster in culture than cells from Sparc-expressing wild-type mice (Bradshaw et al., 1999). Our initial studies using parental U87T2 and U87T2-derived SPARC-transfected clones also demonstrated that SPARC has a suppressive effect on glioma cell proliferation, delaying cell growth on plastic in vitro (Rempel et al., 2001), and delaying tumor growth in rat brains in vivo (Schultz et al., 2002). Furthermore, we demonstrated by flow analysis that the higher the level of secreted SPARC, the greater the percentage of cells in G_0/G_1 or G_2/M phase (Rempel et al., 2001). Therefore, we hypothesize that, for our transfectants, SPARC delays cell cycle progression, but the amount of SPARC is important in determining where in the cell cycle the delay is manifested.

This growth suppression may be accomplished by SPARC's interference with growth factor-growth factor receptor interactions (Kupprion et al., 1998; Pichler et al., 1996; Raines et al., 1992) or by an unknown indirect mechanism (Hasselaar and Sage, 1992). Further, its effects may be cell-type specific and/or concentration dependent. For example, it has been reported that SPARC at any concentration inhibits the incorporation of thymidine and delays the onset of the S-phase of endothelial cells; however, it exhibits biphasic effects on fibroblasts such that low concentrations increase tritiated thymidine incorporation, but higher concentrations decrease tritiated thymidine incorporation (Funk and Sage, 1993). These in vitro studies were performed with endothelial cells or fibroblasts cultured on plastic under serum-free or reduced-serum (1%–2% fetal bovine serum [FBS]) conditions for 2–5 days, trypsinized, replated, and then restimulated to grow in standard medium in the presence of SPARC or SPARC peptides. This experimental design is useful to examine where SPARC affects cell cycle progression in vitro; however, it is not known whether this reflects the in vivo situation, where SPARC may be continuously expressed throughout the stress conditions and where it may be in contact with and influenced by ECM molecules.

SPARC is known to bind to collagens I–IV (Mayer et al., 1991; Sasaki et al., 1997), and it interacts with vitronectin (Rosenblatt et al., 1997). Furthermore, we have demonstrated that SPARC increased attachment to specific ECMs found in the brain, including collagen, laminin, hyaluronic acid, and tenascin, but not vitronectin and fibronectin (Rempel et al., 2001). Since cell-ECM interactions and the level of cell attachment to substrate are important in the regulation of cell proliferation (Bornstein and Sage, 2002; Gladson, 1999; Murphy-Ullrich, 2001), we sought to determine whether the suppressive effects of SPARC on cell proliferation are due, in part, to specific SPARC-ECM interactions.

Therefore, the present study was performed to determine whether specific ECMs modulate SPARC's suppression on proliferation under (1) standard (Dulbecco's modified Eagle medium [DMEM] + 10% FBS) growth conditions, and (2) a modified reduced-serum stress regimen (standard medium for 24 h, DMEM + 0.1% FBS for 48 h, and standard medium for 24 h) in which SPARC is continuously expressed by the transfected clones throughout the stress. This regimen is similar to those described above to examine SPARC effects on proliferation, the difference being that the cells are not trypsinized and replated after serum withdrawal, so that we could examine SPARC effects under conditions that more closely mimic those in vivo.

For the first analysis, 3-(4,5-cimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) growth curves were generated to compare the growth of the parental U87T2 with growth of the U87T2-derived SPARC-transfected clones (A2b2, A2bi, and C2a4) on collagen IV, laminin, hyaluronic acid, tenascin, vitronectin, fibronectin, and plastic over 16 days. For the second analysis, flow cytometry was used to compare the cell cycle distribution of the clones grown on the ECMs under the standard conditions and reduced-serum stress conditions. To determine whether stress, alone or in combination with ECMs, alters SPARC-induced effects on morphology, digital images of the clones were captured on ECM surfaces after 24 h of growth under standard and reduced-serum conditions. Western blot analysis was performed on the conditioned standard and stress media to evaluate the morphology relative to the level of secreted SPARC.

Materials and Methods

Cell Culture

The derivation and development of parental U87T2 and U87T2-derived SPARC-transfected clones A2b2, A2bi, and C2a4 have been previously described (Golembieski et al., 1999). Briefly, U87MG cells were transfected with the transactivator expression vector pUHD15-1neo. This vector encodes the tetracycline-controllable transactivator (TA) needed to induce transcription from the pUHD10-3 expression vector. A neomycin-resistant clone designated U87T2 was cotransfected with the SPARC-encoding pUHD10-3 and puromycin-resistant pJ6Ω vectors. Of the 37 clones selected and expanded, 3 clones designated A2b2, A2bi, and C2a4 were selected for further study (Golembieski et al., 1999; Rempel et al., 2001). These transfected clones were PCR-verified before and after this study to have retained the TA regulator. The A2b2, A2bi,

and C2a4 clones were verified to have retained the SPARC-transfected sequence (Golembieski et al., 1999; Rempel et al., 2001). For standard conditions, the parental clone was maintained in DMEM plus 10% FBS with G418 (0.4 mg/ml) medium, while the SPARC-transfected clones were maintained in the same medium plus puromycin (0.001mg/ml; Sigma Chemical Company, St. Louis, Mo.). For the reduced-serum stress conditions, the cells were grown in the same medium, but with 0.1% FBS. Cells were grown to 65% to 70% confluency before passaging. This controllable system was originally chosen for the ability to examine SPARC's effects by comparing SPARC-expressing cells (-doxycycline [dox]) versus SPARC-inhibited cells (+ dox). However, our recent cDNA array results (Golembieski and Rempel, 2002) suggest that dox is incapable of inhibiting all of SPARC's downstream effects. Furthermore, recent publications indicate that dox itself has effects on the expression of genes, in particular upregulating matrix metalloproteases (MMPs), which degrade ECM proteins (Axisa et al., 2002; Lamparter et al., 2002; Uitto et al., 1994). Since these changes overlap directly with reported SPARC-modulated changes on matrix-associated molecules such as MMP2, and since these experiments are examining ECM effects on SPARC's role in proliferation, we did not want to confound our results with nonrelated dox effects. We therefore chose not to include dox in this study.

ECM Coating

Plates (96-well) or flasks (T75) were coated with one human ECM protein including either collagen IV, vitronectin, laminin (Invitrogen Life Technologies, Carlsbad, Calif., or Chemicon International, Temecula, Calif.), tenascin (Chemicon), fibronectin (BD Biosciences, Franklin Lakes, N.J.), or hyaluronic acid (Sigma). All ECMs were used at a final concentration of 0.005 mg/ml, except for tenascin, which was used at 0.001 mg/ml. Collagen IV was dissolved in 0.25% filter-sterilized acetic acid, whereas vitronectin and fibronectin were dissolved in sterile distilled water. Laminin, hyaluronic acid, and tenascin were dissolved in sterile, $Ca^{2+}Mg^{2+}$-free 1X phosphate-buffered saline (PBS), pH 7.1. The 96-well plates were coated with 0.040 ml of one of the ECM protein solutions. The T75 flasks were coated with 3.0 ml of one of the ECM protein solutions. The Parafilm (American National Can, Greenwich, Conn.) wrapped plates and sealed flasks were stored overnight at 4°C with gentle shaking. Next, the wells and flasks were rinsed with $Ca^{2+}Mg^{2+}$-free 1X PBS and then blocked with sterile 1% bovine serum albumin (BSA) in $Ca^{2+}Mg^{2+}$-containing 1X PBS at room temperature for 1 h. The 96-well plates were used for the MTT growth curves, and the T75 flasks were used for the morphological documentation and flow cytometry.

MTT Growth Curves

After the BSA blocking solution was removed from the wells by gentle suction, the wells were rinsed with $Ca^{2+}Mg^{2+}$-containing 1X PBS and then seeded with 500 cells per well of the respective clones. For each clone, cells were plated onto uncoated plastic (control) or ECM-coated 96-well plates. Clones were plated in triplicate for time points 1, 2, 4, 6, 8, 10, 12, 14, and 16 days. The medium was changed every second day. At each time point, 0.050 ml of freshly prepared MTT (5 mg/ml; Sigma) in $Ca^{2+}Mg^{2+}$-containing PBS was added to each well, and the plates were incubated at 37°C for 4 h. The MTT-containing medium was then removed, and the plates were allowed to dry overnight. White mineral oil (0.1 ml; Sigma) was then added to each well, and the plates were rotated on a shaker for 24 h. The intensity of the released formazan product was measured at 540 nm (Fusion spectrophotometer; Packard Biosciences).

Cell Cycle Analysis

U87T2 and SPARC-transfected clones were plated at equal density (1.5×10^6 cells) on uncoated plastic (control) or ECM-coated T75 flasks. Cultures were grown for 24 h in standard media. The medium was then aspirated, and the cells were washed with $Ca^{2+}Mg^{2+}$-containing 1X PBS. The cells were stressed by serum reduction (0.1% FBS) for 48 h. The medium was then removed, and the cells were then restimulated to grow with standard medium for 24 h. The cells were harvested as described previously (Rempel et al., 2001). Briefly, the cells were trypsinized, diluted in medium, and collected following centrifugation at 800 rpm for 5 min at room temperature. The medium was decanted, and the cell pellets were suspended in 1X PBS and counted with a hemocytometer. The PBS was removed after centrifugation at 800 rpm for 5 min, and the cells were resuspended in ice cold 70% ethanol that was added dropwise with shaking. The final concentration was 2×10^6 cells per milliliter. The cells were stored at 4°C until analysis by flow cytometry. For flow cytometric analysis, the cells were centrifuged at 200 $\times$ *g* for 5 min at room temperature. After the supernatant was decanted, the cells were washed once more with ice-cold PBS. The cell pellets were then resuspended in a propidium iodide/RNase mixture (0.050 mg/ml and 5 U/ml, respectively). The cells were mixed thoroughly by vortexing and analyzed for DNA content with a Becton Dickinson LSR flow cytometer (BDIS, San Jose, Calif.). Cell cycle distribution was determined from DNA histogram analyses using ModFitLT software (Verity, Topsham, Me.).

Morphological Assessment Under Standard Versus Reduced-Serum Conditions

After the BSA blocking solution was removed from the T75 flasks, clones were plated equally onto uncoated (control) and coated flasks (1.5×10^6 cells per flask). To assess morphology under standard conditions, cells were plated for 24 h in standard medium. To assess morphology after stress, the cells were plated for 24 h in standard medium, washed, cultured for 48 h in reduced-serum medium, and restimulated with standard medium for 24 h. Representative fields were photographed by using a Nikon Coolpix 990 digital camera (Nikon, Inc., Melville,

N.Y.) and exported to Photoshop (Adobe Systems, Inc., San Jose, Calif.) for documentation.

Western Blot Analysis

To determine the amount of SPARC secreted under the standard conditions used for morphological assessment, cells were plated equally (1×10^6 cells per T75 flask) onto uncoated flasks, and the media samples were collected after 24 h of growth. To assess the levels of SPARC secreted after stress conditions, an equal number of cells were grown in standard medium for 24 h, then for 48 h in reduced-serum medium containing 0.1% FBS, and subsequently in reduced-serum medium for 24 h as described previously. For each experiment, duplicate Western blots were prepared, along with duplicate control gels that were Coomassie Blue-stained to document sample loading. Media aliquots (0.010 ml) were mixed with an equal volume of 2X sodium dodecyl sulfate (SDS) reducing buffer (100 mM Tris; pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% glycerol, 0.2% bromophenol blue), boiled 3 min, and electrophoresed with a Benchmark prestained protein ladder (0.010 ml; Gibco-BRL, Bethesda, Md.) through 10% polyacrylamide SDS Tris-glycine gels. The resolved proteins were transferred to an Immobilon P membrane (Millipore, Bedford, Mass.). All the following procedures were performed at room temperature unless otherwise indicated. Membranes were dried, pre-wet in methanol, rinsed in Tris-buffered saline (TBS), pH 7.5, and blocked for 1 h in 5% fat-free dry milk (Biorad, Hercules, Calif.) in TBS. Membranes were incubated with primary SPARC antibody (0.0011 mg/ml; Haematologic Technologies, Inc., Essex Junction, Vt.) in blocking solution containing 5% fat free milk in TBS and 0.1% Tween-20 overnight at 4°C. Membranes were washed 3 times in TBS for 10 min, with shaking. The membranes were incubated with secondary HRP-linked anti-mouse Ig whole antibody (1:2500 dilution: Amersham Biosciences, Piscataway, N.J.) with blocking buffer containing 5% fat-free milk in TBS and 0.1% Tween-20 for 1 h and subsequently washed 3 times in TBS. SPARC protein expression was detected by chemiluminescence with an ECL Western blotting kit according to the manufacturer's directions (Amersham Biosciences). Membranes were immersed in the detection agent for 1 min and then exposed to film for 15 s, 30 s, or 1 min and developed. The level of SPARC was determined by measuring the intensity of the signals on X-ray film with NIH Image analysis software version 6.2 (NIH, 2003). The multiple exposures were measured to ensure that the signals used were within the linear range of the X-ray film, as previously reported (Rempel et al., 2001). The increase in SPARC was determined by comparison with the SPARC level of U87T2. Experiments were performed at least twice.

Statistical Methods

To determine whether SPARC's ability to suppress tumor cell growth was affected by ECM proteins, the growth of the SPARC-transfected clones was compared to that of the parental U87T2 on individual ECMs for a period of 1 to 16 days. Growth curves were plotted with StatView 5.0 software (SAS Institute, Cary, N.C.). Analysis was performed 2 ways. Each ECM was evaluated for the differences in the growth of the 4 clones. Each clone was examined for its ECM-specific differences in growth. To determine the effects of reduced serum and ECM on SPARC's ability to alter the number of cells in G_0/G_1, flow cytometry was performed for all SPARC-transfected clones and the parental U87T2 clone. For both the growth curves and the flow cytometric analyses, 2-way analysis of variance (ANOVA) was the primary method of analysis. The factors of interest were clones (4 types) and ECMs (6 types plus plastic controls [1 for the growth curves and 2 for the flow cytometry]). All effects were considered fixed. For the analysis of growth curves, interactions involving time were present. Therefore, 2-way ANOVAs were conducted separately at each time point. The methodology used at each time point was the same as that used for the flow cytometry experiment. Fisher's least-significant difference was used in the two-way ANOVA to adjust type I error at each time point for growth curve analysis, as well as for the flow analysis. This method maintains the experimentwise type I error rate at the 0.05 level. This is a 2-step procedure in which the overall F-test was first examined, and pairwise *t*-tests were then performed at the same alpha level, conditional on a significant result. If the F-test was not significant at the 0.05 level, the *t*-tests were not performed, and all pairwise comparisons were declared nonsignificant. For all group comparisons, a *P*-value less than 0.05 was considered statistically significant.

Results

SPARC and Glioma Growth on Different ECMs

MTT growth curves for the parental U87T2 and SPARC-transfected clones A2b2, A2bi, and C2a4 on each ECM are shown in Fig. 1A. The results are plotted as the mean (A540 nm) plus or minus standard error over a period of 16 days. In agreement with previous results comparing growth on plastic using standard growth curves (Golembieski et al., 1999), all the SPARC-transfected clones grew more slowly than the parental U87T2. The onset and continuation of statistically significant changes were observed throughout the growth curves from day 6 to day 14 for clones A2b2, A2bi, and Ca4. A similar trend was observed on all ECMs. For fibronectin, vitronectin, and laminin, the 3 SPARC-transfected clones also showed significantly reduced growth compared to the parental U87T2, the onset of suppression occurring consistently by day 4 for all 3 clones. For collagen, hyaluronic acid, and tenascin, the onset of suppression was the same as on plastic, occurring consistently by day 6 for all 3 clones. However, individual differences were observed between the clones for the earlier time points, whether they were grown on an ECM or on plastic. In the majority of cases, the C2a4 clone grew the slowest. The A2bi clone generally grew faster than clone A2b2, although

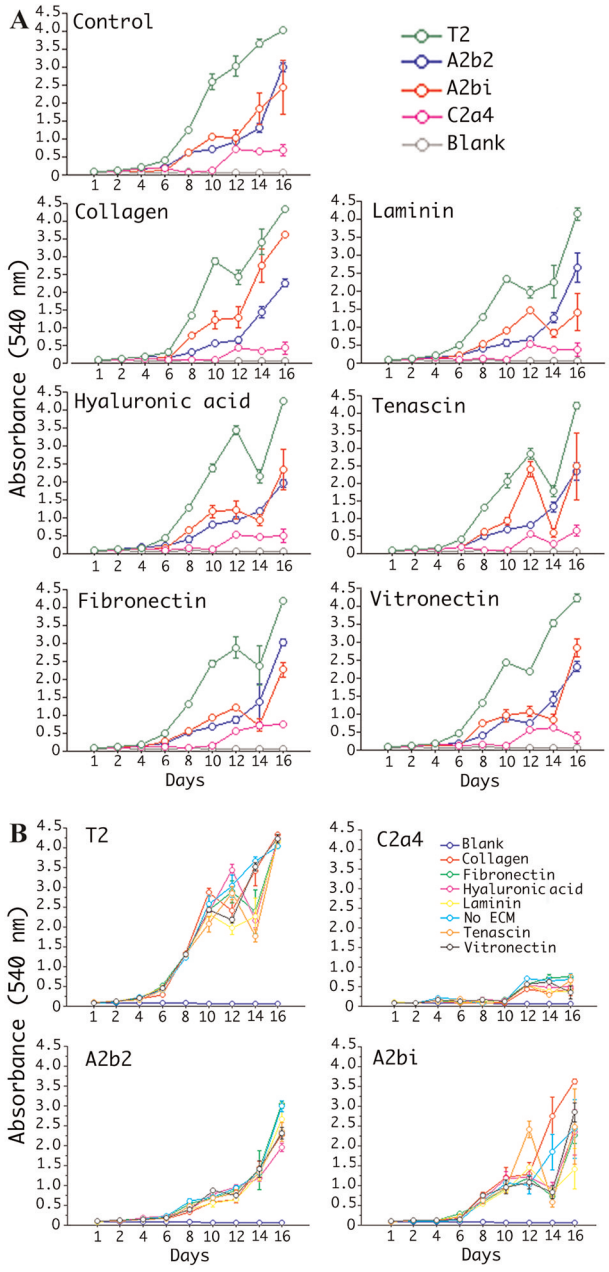

Fig. 1. Growth curve analysis for U87T2 (T2) parental versus SPARC-transfected clones A2b2, A2bi, and C2a4 in the absence and presence of extracellular matrix (ECM) proteins. For the blank control, no cells were added to the wells. A. An equal number of cells (500 per well) were seeded onto 96-well uncoated (control) plates or plates coated with human ECM, including collagen, laminin, hyaluronic acid, tenascin, fibronectin, and vitronectin, in triplicate for each time point. The number of viable cells was measured with the MTT assay on the days indicated. The absorbance was measured at 540 nm, and the results were plotted as the mean growth (A_{540} nm) plus or minus the standard error. ANOVA analysis indicated that the SPARC-transfected clones grew significantly slower than parental U87T2 clone ($P < 0.05$). B. The results were replotted to assess individual clonal growth on the ECMs. Results are presented as the mean growth (A_{540} nm) plus or minus the standard error. ANOVA analysis indicated that no consistent pattern was observed between the clones in comparison to the parental clone.

starting on day 8, A2b2 grew better on tenascin, on day 14 better on vitronectin, and on day 16 better on fibronectin and laminin. ANOVA analysis comparing all clones for a given ECM indicates that clonal effects were significant ($P < 0.05$), and all SPARC-transfected clones were significantly different from the parental U87T2 ($P < 0.05$) on each ECM. Overall, growth was ranked U87T2 > A2bi > A2b2 > C2a4 across all ECMs. The amount of SPARC secreted by these clones in log phase (data not shown) was the same as that previously reported. A biphasic relationship was found in which the clones with the lowest and highest levels of secreted SPARC affected growth more similarly than the clone with the intermediate levels of secreted SPARC (Rempel et al., 2001).

To determine whether ECM-specific changes in the growth patterns for individual clones occurred, separate growth curves were generated for each clone grown on all ECMs (Fig. 1B). Although ANOVA analysis indicated that ECM-specific effects on proliferation were observed for individual clones starting on day 4, no consistent pattern of ECM effect was observed for all clones or in a comparison of effects on the SPARC-transfected clones versus the parental U87T2 clone.

ECM Effects on SPARC-Mediated Changes in Morphology

To determine whether the SPARC-transfected clones exhibited different morphology on the ECMs in comparison with the parental clone, images were captured at 24-h growth in standard medium (Fig. 2). In agreement with our previously published results, the SPARC-transfected clones exhibited a flatter morphology in comparison with the parental U87T2 clone on plastic (Rempel et al., 2001). All the clones retained the same morphological features when grown on an ECM, with the exception of U87T2, which was less adherent on hyaluronic acid. Therefore, the morphology of the SPARC-transfected clones, while different from the parental clone, was not dependent on the ECM.

Secreted SPARC Levels and Morphology Under Standard Conditions

To evaluate the 24-h morphology of the clones in the context of the level of secreted SPARC, Western blot analysis was performed on media collected at 24 h for all clones grown on plastic. Figure 3A illustrates a representative Western blot and the fold increases of the secreted SPARC levels in the clones relative to the levels secreted by U87T2. U87T2 cells secreted low levels of endogenously synthesized SPARC, as expected. A2b2 secreted the highest level (9.0-fold), followed by A2bi (6.0-fold) and C2a4 (1.5-fold). Therefore, increased SPARC secretion accompanied changes in morphology, as previously reported (Golembieski et al., 1999; Rempel et al., 2001). The relative fold increases between the clones differ from the previous report, as the clones were not in log phase. Therefore, the results reflect differences in the secretion rate of SPARC by the clones at this selected time point.

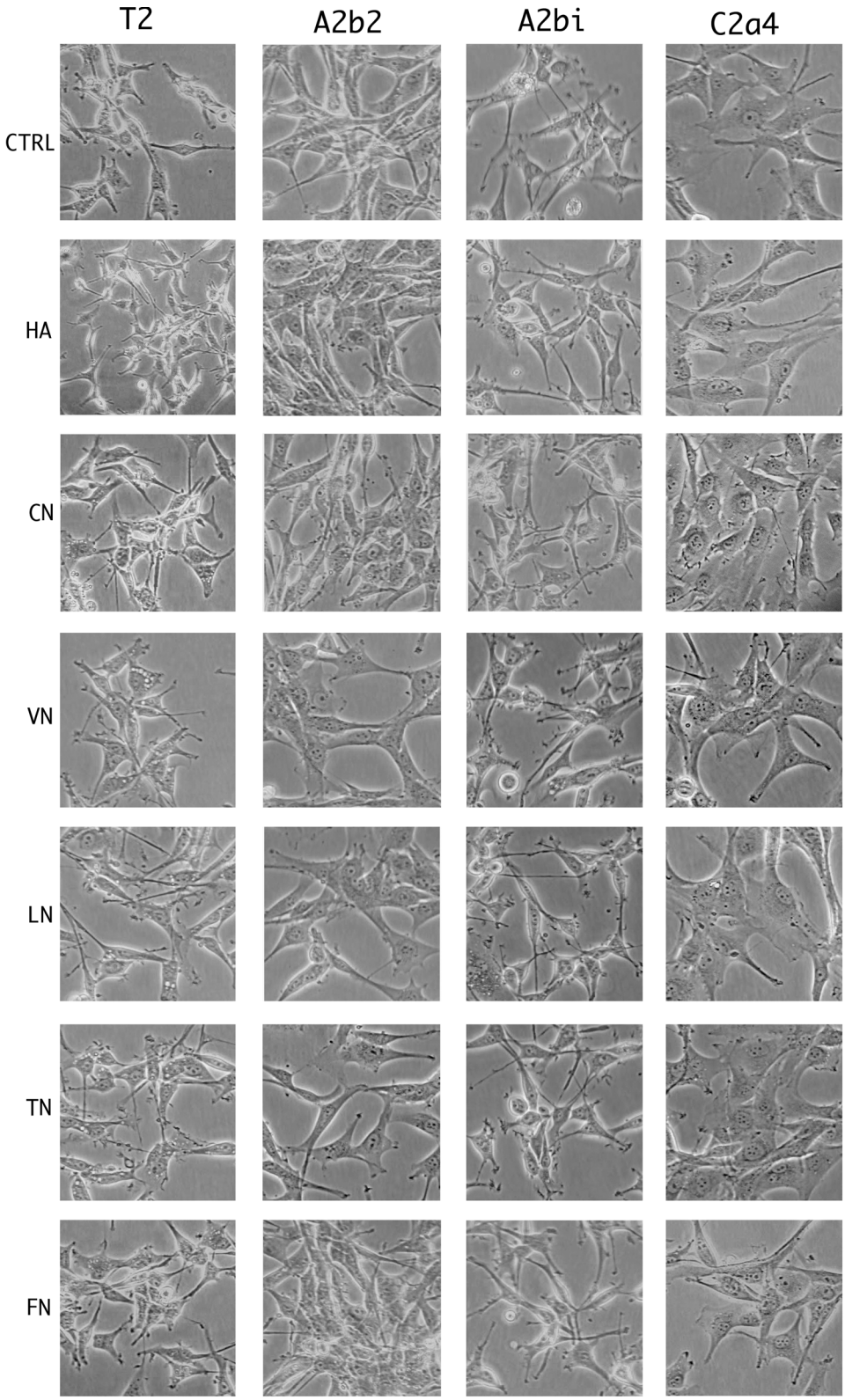

Fig. 2. Morphology of U87T2 parental (T2) and SPARC-transfected clones A2b2, A2bi, and C2a4 after 24 h of growth on various ECMs under standard conditions. An equal number of cells were plated in T75 flasks uncoated (CTRL) or coated with hyaluronic acid (HA), collagen (CN), vitronectin (VN), laminin (LN), tenascin (TN), or fibronectin (FN). The cells were grown for 24 h under standard conditions, and images were taken from representative fields using a Nikon Coolpix 990 digital camera. Increased SPARC induced increased adhesion as previously reported (Rempel et al., 2001). There are minimal or no ECM-dependent changes on morphology of SPARC clones due to the presence of an ECM. T2 exhibited a mild degree of altered morphology on HA after 24 h.

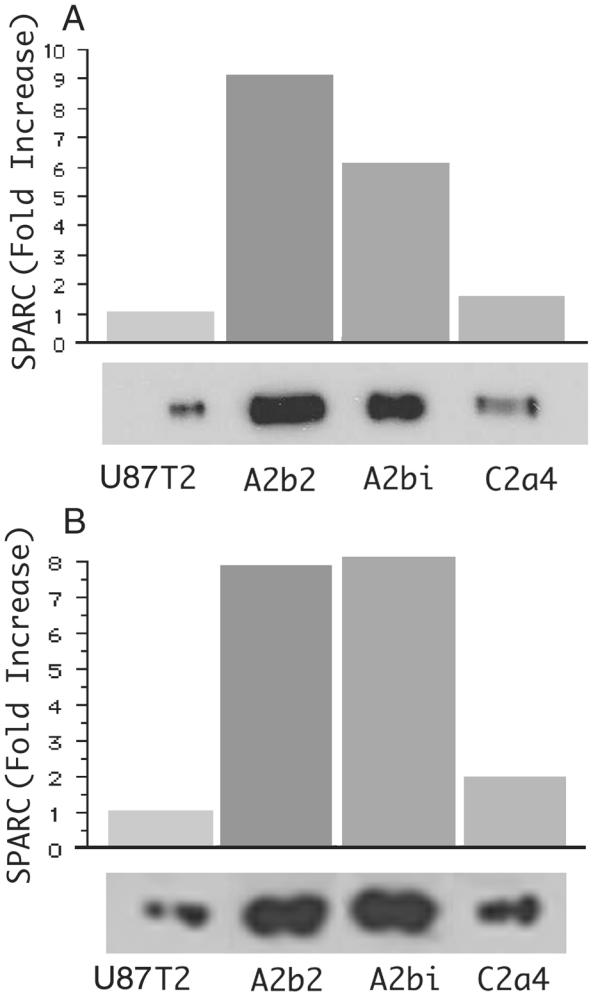

Fig. 3. Quantitation of SPARC secreted by U87T2 parental (T2) and SPARC-transfected clones A2b2, A2bi, and C2a4 under standard and reduced-serum stress conditions. A. Standard conditions. An equal number of cells were plated in standard growth medium. The media samples were collected at 24 h. The levels of secreted SPARC were assessed by Western blot analysis. Results were quantitated by using NIH Image (NIH 2003) analysis software. SPARC levels are presented as the fold increase above the level of SPARC secreted by parental U87T2: A2b2, 9.0-fold; A2bi, 6.0-fold; and C2a4, 1.5-fold. B. Stress conditions. An equal number of cells were plated in standard growth medium for 24 h. The medium was then replaced with serum-reduced medium (0.1% FBS) for 48 h. The serum-reduced medium was replaced with standard medium for 24 h prior to media sample collection. Quantitation of the level of SPARC was assessed as in panel A as the fold increase above the level of SPARC secreted by parental U87T2: A2b2, 7.8-fold; A2bi, 7.6-fold; and C2a4, 1.9-fold.

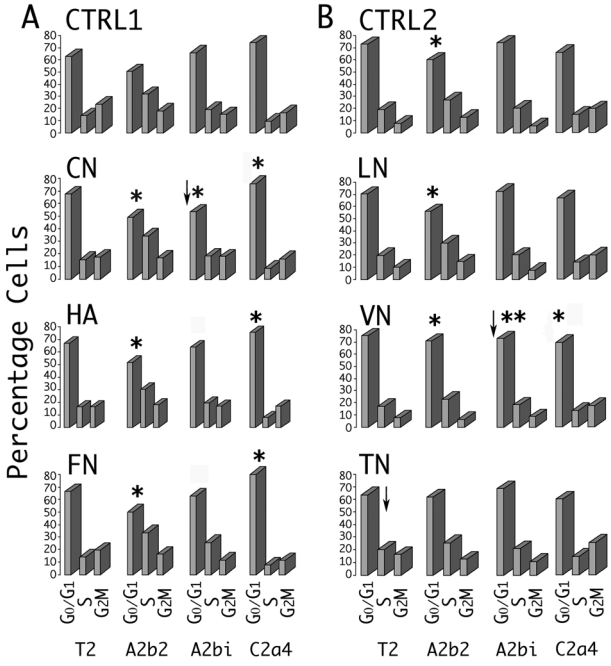

Fig. 4. Flow cytometric analysis of U87T2 parental (T2) and SPARC-transfected clones A2b2, A2bi, and C2a4 under reduced-serum stress conditions on plastic or ECM proteins. A. Plastic (CTRL1), collagen (CN), hyaluronic acid (HA), and fibronectin (FN). B. Plastic (CTRL2), laminin (LN), vitronectin (VN), and tenascin (TN). Equal numbers of cells were seeded in T75 flasks coated with ECMs. The cells were stabilized for 24 h under standard conditions, stressed for 48 h with media containing 0.1% FBS, and restimulated for 24 h under standard conditions. The cells were harvested and subjected to flow analysis. Flow histograms are illustrated for all clones grown on all ECMs. *Significant difference from parental U87T2 (T2) with respect to the percentage of cell in G0/G1. **Marginal significant difference ($P \leq 0.10$). The arrow indicates a significant difference ($P \leq 0.05$) compared to that clone grown on plastic. On CN, HA, and FN, clones having greater levels of secreted SPARC had a tendency to have a lower percentage of cells in G_0/G_1. On TN and LN, growth was independent of SPARC levels. On VN, any increase in SPARC correlated with increased growth (smaller percentage of cells in G_0/G_1).

Reduced-Serum Stress and ECM Effects on SPARC-Mediated Changes in Cell Cycle Progression

To determine whether stress of the transfected cells in the absence or presence of ECMs altered the percentage of cells in G_0/G_1, flow analyses were performed for the parental U87T2 and SPARC-transfected clones grown either on plastic or on individual matrix proteins (Fig. 4).

There was little or no difference in the percentage of cells in G_0/G_1 when the clones were grown and stressed by reduced serum on plastic. Only A2b2 was significantly different from U87T2 in 1 of 2 control experiments. This is unlike earlier results demonstrating that increased SPARC promotes an increase in the percentage of cells in G_0/G_1 or G_2/M when grown to log phase on plastic in the absence of stress (Rempel et al., 2001).

With respect to stress effects when the clones were grown on the ECMs, the U87T2 clone had a higher percentage of cells in G_0/G_1 than A2b2 (except on tenascin), a lower percentage of cells than C2a4 (except on tenascin and laminin), and a higher percentage of cells than A2bi, but only on collagen and vitronectin. Although differences were observed between the SPARC-transfected clones, ECM-specific patterns of proliferation were deduced between the stressed parental and SPARC-transfected

clones, as follows: On hyaluronic acid, collagen, and fibronectin, the clones A2b2 and A2bi had fewer cells in G_0/G_1 than the parental clone, whereas the C2a4 clone had a greater percentage of cells in this phase of the cell cycle. All SPARC-transfected clones had fewer cells in G_0/G_1 than the parental clone when grown on vitronectin. No consistent difference was observed between the SPARC-transfectants and U87T2 on tenascin and laminin.

Reduced-Serum Stress and ECM Effects on SPARC-Mediated Changes in Morphology and Cell Density

To determine whether the stressed SPARC-transfected clones exhibited grossly different morphology on an ECM in comparison with the stressed parental clone, the morphology was captured just prior to collection of the cells for cell cycle analysis (Fig. 5). On plastic, the parental U87T2 clone exhibited a flatter morphology after stress (compare Figs. 2 and 5) and was more similar to the SPARC-transfected clones. In contrast, the stress did not appear to grossly alter the morphology of the SPARC-transfected clones on plastic (compare Figs. 2 and 5). Generally, clones grew better on the ECMs than on plastic. However, the presence of the ECMs clearly facilitated the growth of the clones in ECM-specific and clone-specific manners that agreed with the proliferation patterns observed by the flow cytometric analysis.

Secreted SPARC Levels and Morphology Under Reduced-Serum Stress Conditions

To evaluate the 24-h poststress morphology of the clones in the context of the level of secreted SPARC, Western blot analysis was performed on media samples collected at 24 h poststress for all clones grown on plastic. Figure 3B illustrates a representative Western blot and the fold increases of the secreted SPARC levels in the clones relative to U87T2 (A2b2, 7.8-fold; A2bi, 7.6-fold; and C2a4, 1.9-fold). These results differ from previously reported results, likely because of the time point in media sampling and differences in secretion rates by the SPARC-transfected clones.

Summary of ECM Effects on SPARC Modulation of Proliferation and Cell Density Under Reduced-Serum Stress Conditions

In the presence of vitronectin, any increase in SPARC secretion by the SPARC-transfected clones promoted increased proliferation and cell density. In the presence of collagen, hyaluronic acid, and fibronectin, proliferation and cell density varied with the level of secreted SPARC; the higher the level of secreted SPARC, the lower the G_0/G_1, and the higher the cell density. In the presence of tenascin and laminin, cell proliferation and cell density increased for the parental as well as the SPARC-transfected clones and were, therefore, independent of SPARC secretion.

Discussion

Our in vitro (Rempel et al., 2001) and in vivo (Schultz et al., 2002) data have demonstrated that SPARC has a suppressive effect on U87 glioma growth. In addition, our cDNA array analyses indicate that increased SPARC expression is accompanied by a decrease in the expression of the cell cycle regulating genes, cyclins D1and D3, and the cyclin-dependent kinase regulatory subunits 1 and 2 (Golembieski and Rempel, 2002). These data support the hypothesis that SPARC may indirectly regulate cell proliferation via a signal transduction mechanism, for example, through the reported interference with growth factor-growth factor receptor interactions (Pichler et al., 1996; Raines et al., 1992). However, since SPARC is known to bind to ECM proteins (Gladson, 1999; Mayer et al., 1991; Rosenblatt et al., 1997), it is possible that a signaling mechanism that regulates proliferation might also be influenced by SPARC's disruption of cell-ECM interactions.

Therefore, in this report, we examined the effect of transfected SPARC secretion on proliferation, morphology, and cell density of glioma cells grown in vitro, in the absence and presence of ECM proteins under standard and reduced-serum stress conditions. We have demonstrated that, under standard conditions, (1) increased SPARC secretion was accompanied by decreased growth, (2) SPARC-induced changes in growth were not grossly modulated by the ECMs, (3) SPARC induced morphological changes on plastic by 24 h, and (4) these SPARC-modulated changes in morphology were not altered by the presence of ECMs. Thus, ECM molecules had little effect in modulating SPARC's suppression of proliferation under standard conditions. We further demonstrated that, under reduced-serum conditions, (1) stress alone was sufficient to eliminate the SPARC-induced increase in G_0/G_1 seen under standard conditions, (2) any increase in transfected SPARC secretion correlated with increased growth on vitronectin, (3) increasing levels of SPARC secretion correlated with increasing growth on collagen, hyaluronic acid, and fibronectin, (4) the level of secreted SPARC did not correlate with increased growth on tenascin and laminin, and (5) the changes observed in morphology and cell density were consistent with the flow cytometric data. Therefore, the stress conditions alone were sufficient to induce morphological changes of the parental clone, reducing the SPARC-induced differences. Furthermore, while the ECM effects on the SPARC transfectants were absent under standard conditions, ECM-specific effects were apparent under the stress conditions in our model. Importantly, we observed that a change in the culture conditions could alter the outcome with respect to the analysis of SPARC and its regulation of tumor cell proliferation.

For these studies, we assessed growth under standard conditions using the MTT growth assay rather than the previously reported standard growth curves (Rempel et al., 2001). This allowed a better discrimination of the clonal differences in growth over a longer period of time (i.e., 16 days rather than 10 days). A similar growth profile was reproduced on plastic; all the SPARC-transfected

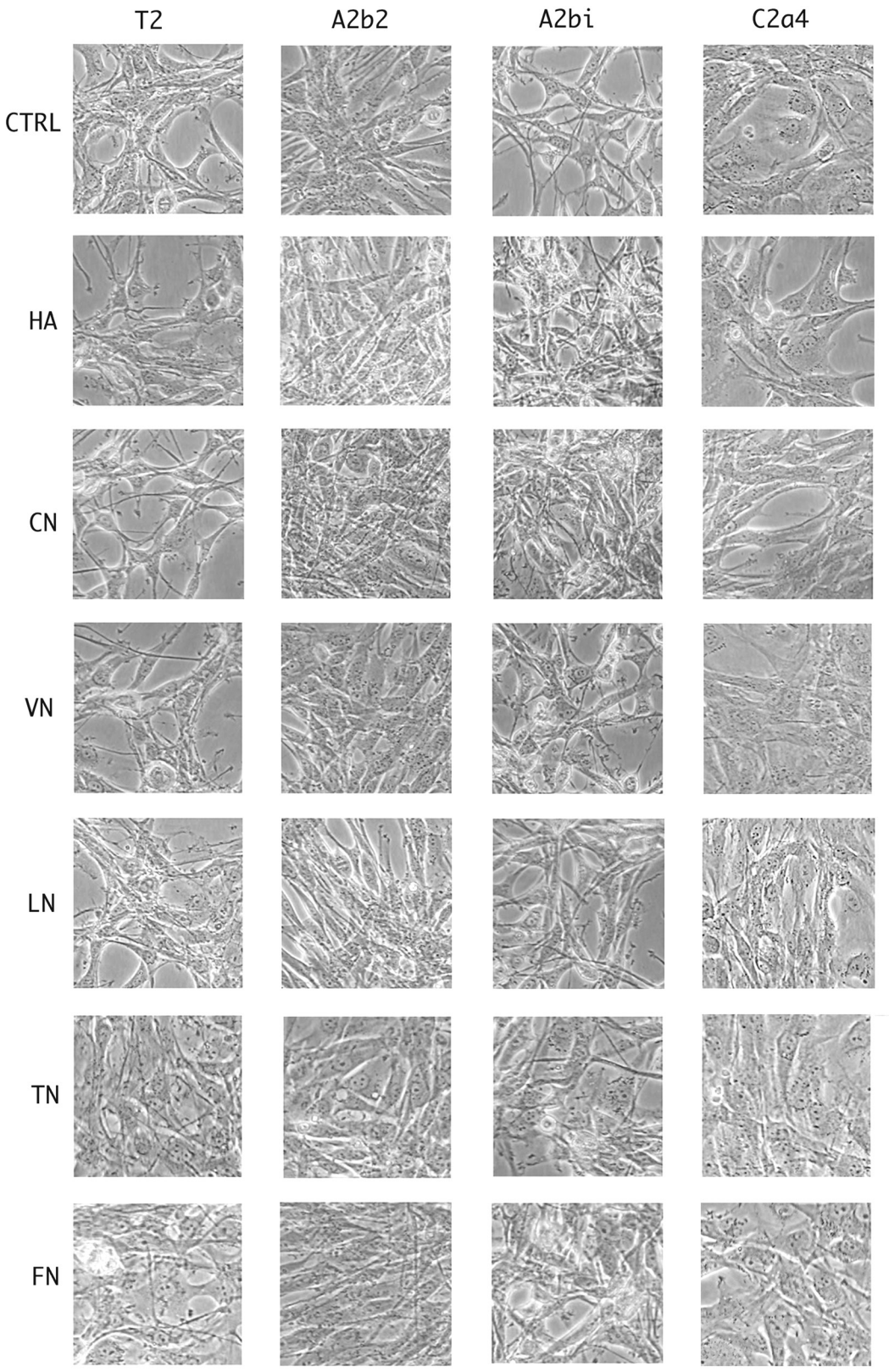

Fig. 5. Morphology of U87T2 parental (T2) and SPARC-transfected clones A2b2, A2bi, and C2a4 after 24 h of growth on individual ECMs under reduced-serum stress conditions. An equal number of cells were plated on uncoated (CTRL) or T75 flasks coated with collagen (CN), laminin (LN), hyaluronic acid (HA), tenascin (TN), fibronectin (FN), or vitronectin (VN). The cells were grown for 24 h at standard conditions, grown in serum-reduced conditions (0.1% FBS) for 48 h, and restimulated to grow for 24 h under standard conditions. Images were taken as described in Fig. 2. ECM-specific effects on growth were as observed for the flow data (see Fig. 4).

clones grew slower than the parental U87T2, and the same growth patterns were observed in the clones; A2bi grew faster than A2b2, which grew faster than C2a4. Having established similar results, we evaluated the effects of 6 ECM proteins known to be components of either the brain parenchyma or blood vessel basement membranes. We observed no changes in growth patterns from their growth profiles on plastic under standard conditions (with the exception of U87T2, which was less adherent on hyaluronic acid).

Although the matrix molecules appear to have no effect on proliferation, we cannot rule out the possibility that ECM-mediated effects do exist. It is possible that the SPARC transfectants lay down their own matrix, the level of which is dictated by the amount of SPARC secreted by the clones, of sufficient concentration to override the effects of any single ECM protein. Reports indicate that SPARC has an effect on ECM secretion, either increasing or decreasing matrix production in a cell-type-specific manner (Kamihagi et al., 1994; Lane et al., 1992). Furthermore, it has been demonstrated that astrocytomas can synthesize their own matrix (Gladson, 1999). However, this interpretation is not supported by our observation that the clones retained the same morphological features when grown on either plastic or the ECMs for 24 h. Also, our cDNA array analyses did not demonstrate SPARC-induced changes in matrix production at the level of transcript abundance (Golembieski et al., 2002). Therefore, we interpret these findings to suggest that the amount of SPARC secreted by cells is likely more important than an individual ECM protein in determining its overall effects on proliferation under standard conditions.

Many of the studies evaluating SPARC's effects on proliferation assess cell cycle distribution under stress conditions induced by the withdrawal or reduction of serum, followed by restimulation of growth in the presence of exogenous SPARC or SPARC peptides (Funk and Sage, 1991, 1993; Sage et al., 1995). SPARC is considered to be a stress response gene. Therefore, we investigated whether SPARC's suppression of proliferation was different under standard versus reduced-serum stress conditions, and whether the ECM proteins have an effect. We have previously characterized SPARC's ability to suppress growth by delaying cells in the G_0/G_1 and G_2/M phases of the cell cycle using flow cytometric analysis (Rempel et al., 2001). We therefore examined the effects of stress and ECM proteins on the ability of SPARC to modulate the cell cycle as described previously. The results indicate that SPARC's effects on cell cycle are indeed absent under the reduced-serum stress conditions, as the parental clone had a percentage of cells in G_0/G_1 similar to that of the SPARC-transfected clones. In addition, the stress itself was sufficient to induce a change in morphology for the parental clone, which appeared flatter and more adherent. Western blot analysis indicates that stress did not grossly alter the relative amount of SPARC secreted by the clones before and after stress. Therefore, the stress effects do not appear SPARC-related.

Interestingly, it was under these stress conditions that ECM-specific effects were observed on cell cycle distribution, cell morphology, and cell density, some of which appeared to be, in turn, influenced by the amount of SPARC secreted. Those ECMs that appeared to be influenced by SPARC levels included vitronectin, collagen, hyaluronic acid, and fibronectin. In contrast to the results observed under standard conditions, increased SPARC levels were associated with greater levels of proliferation. Thus, these ECMs appeared to reverse SPARC's suppressive effects. Of these ECMs, SPARC is known to interact with vitronectin (Rosenblatt et al., 1997) and collagen IV (Mayer et al., 1991; Sasaki et al., 1997). We have previously shown that tumors secreting high levels of SPARC migrate less well on vitronectin than tumors secreting lower levels (Menon et al., 2000). This inverse relationship between the amount of SPARC secreted and migration is consistent with the opposite observation that stressed cells proliferate better on vitronectin. With respect to hyaluronic acid, interruption of the binding to its receptor, RHAMM (hyaluronic acid-receptor for hyaluronic acid-mediated motility), has been shown to have a negative effect on the proliferation of astrocytomas (Akiyama et al., 2001), which indicates that the ECM-receptor interaction promotes proliferation. This supports our observation that hyaluronic acid promotes proliferation under stress conditions, even in the presence of high levels of SPARC.

The present experiments indicate that an assessment of ECM effects on SPARC's ability to suppress growth in vitro depends heavily upon the culture conditions used to assess growth. A comparison of the in vitro results with those obtained when the clones were injected into rat brains (Schultz et al., 2002) indicates that growth of the clones under the standard in vitro conditions most closely resembles the results obtained in vivo. Thus, we suggest that the individual ECM proteins studied do not play a role in SPARC's ability to suppress glioma growth. However, the results obtained under our modified stress regimen are also of interest because of *SPARC*'s role as a stress response gene. It may be that under certain pathological conditions, the stress elicits a similar ECM-modulated response, overriding *SPARC*'s suppression of proliferation. Such a response may be necessary in conditions of wound healing, for example. This may also be relevant in the study of human glioma invasion, as we have previously shown that SPARC is upregulated in reactive astrocytes in the adjacent brain responding to tumor invasion (Rempel et al., 1998). These are highly synthetic cells, producing ECM proteins. The co-secretion of SPARC and ECM proteins by these cells may influence the local environment, resulting in enhanced tumor growth. Thus, cell-type-specific responses and differences in the local environment may influence the role of SPARC in the tumor, such that it promotes tumor invasion in some regions, whereas in others it promotes tumor growth.

Acknowledgements

Dr. Rempel thanks the Levy family for their continued support of the Barbara Jane Levy Laboratory of Molecular Neuro-Oncology.

References

Akiyama, Y., Jung, S., Salhia, B., Lee, S., Hubbard, S., Taylor, M., Mainprize, T., Akaishi, K., van Furth, W., and Rutka, J.T. (2001) Hyaluronate receptors mediating glioma cell migration and proliferation. *J. Neurooncol.* **53**, 115–127.

Axisa, B., Loftus, I.M., Naylor, A.R., Goodall, S., Jones, L., Bell, P.R., and Thompson, M.M. (2002) Prospective, randomized, double-blind trial investigating the effect of doxycycline on matrix metalloproteinase expression within atherosclerotic carotid plaques. *Stroke* **33**, 2858–2864.

Bornstein, P., and Sage, E.H. (2002) Matricellular proteins: Extracellular modulators of cell function. *Curr. Opin. Cell Biol.* **14**, 608–616.

Bradshaw, A.D., Francki, A., Motamed, K., Howe, C., and Sage, E.H. (1999) Primary mesenchymal cells isolated from SPARC-null mice exhibit altered morphology and rates of proliferation. *Mol. Biol. Cell* **10**, 1569–1579.

Funk, S.E., and Sage, E.H. (1991) The Ca^{2+}-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* **88**, 2648–2652.

Funk, S.E., and Sage, E.H. (1993) Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts. *J. Cell. Physiol.* **154**, 53–63.

Gladson, C.L. (1999) The extracellular matrix of gliomas: Modulation of cell function. *J. Neuropathol. Exp. Neurol.* **58**, 1029–1040.

Golembieski, W.A., and Rempel, S.A. (2002) cDNA array analysis of SPARC-modulated changes in glioma gene expression. *J. Neurooncol.* **60**, 213–226.

Golembieski, W.A., Ge, S., Nelson, K., Mikkelsen, T., and Rempel, S.A. (1999) Increased SPARC expression promotes U87 glioblastoma invasion *in vitro*. *Int. J. Dev. Neurosci.* **17**, 463–472.

Greenwood, J.A., and Murphy-Ullrich, J.E. (1998) Signaling of de-adhesion in cellular regulation and motility. *Microsc. Res. Tech.* **43**, 420–432.

Hasselaar, P., and Sage, E.H. (1992) SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. *J. Cell. Biochem.* **49**, 272–283.

Kamihagi, K., Katayama, M., Ouchi, R., and Kato, I. (1994) Osteonectin/SPARC regulates cellular secretion rates of fibronectin and laminin extracellular matrix proteins. *Biochem. Biophys. Res. Commun.* **200**, 423–428.

Kupprion, C., Motamed, K., and Sage E.H. (1998) SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. *J. Biol. Chem.* **273**, 29635–29640.

Lamparter, S., Slight, S.H., and Weber, K.T. (2002) Doxycycline and tissue repair in rats. *J. Lab. Clin. Med.* **139**, 295–302.

Lane, T.F., Iruela-Arispe, M.L., and Sage, E.H. (1992) Regulation of gene expression by SPARC during angiogenesis *in vitro*. Changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. *J. Biol. Chem.* **267**, 16736–16745.

Mayer, U., Aumailley, M., Mann, K., Timpl, R., and Engel, J. (1991) Calcium-dependent binding of basement membrane protein BM-40 (osteonectin, SPARC) to basement membrane collagen type IV. *Eur. J. Biochem*. **198**, 141–150.

Menon, P.M., Gutierrez, J.A., and Rempel, S.A. (2000) A study of SPARC and vitronectin localization and expression in pediatric and adult gliomas: High SPARC secretion correlates with decreased migration on vitronectin. *Int. J. Oncol.* **17**, 683–693.

Murphy-Ullrich, J.E. (2001) The de-adhesive activity of matricellular proteins: Is intermediate cell adhesion an adaptive state? *J. Clin. Invest.* **107**, 785–790.

NIH. National Institutes of Health. (2003) NIH Image. Available at http://rsb.info.nih.gov/nih-image/about.html, accessed June 27, 2003.

Pichler, R.H., Bassuk, J.A., Hugo, C., Reed, M.J., Eng, E., Gordon, K.L., Pippin, J., Alpers, C.E., Couser, W.G., Sage, E.H., and Johnson, R.J. (1996) SPARC is expressed by mesangial cells in experimental mesangial proliferative nephritis and inhibits platelet-derived-growth-factor-mediated mesangial cell proliferation *in vitro*. *Am. J. Pathol.* **148**, 1153–1167.

Raines, E.W., Lane, T.F., Iruele-Arispe, M.L., Ross, R., and Sage, E.H. (1992) The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc. Natl. Acad. Sci. USA* **89**, 1281–1285.

Rempel, S.A., Golembieski, W.A., Ge, S., Lemke, N., Elisevich, K., Mikkelsen, T., and Gutiérrez, J.A. (1998) SPARC: A signal of astrocytic neoplastic transformation and reactive response in human primary and xenograft gliomas. *J. Neuropathol. Exp. Neurol.* **57**, 1112–1121.

Rempel, S.A., Golembieski, W.A., Fisher, J.L., Maile, M., and Nakeff, A. (2001) SPARC modulates cell growth, attachment and migration of U87 glioma cells on brain extracellular matrix proteins. *J. Neurooncol.* **53**, 149–160.

Rosenblatt, S., Bassuk, J.A., Alpers, C.E., Sage, E.H., Timpl, R., and Preissner, K.T. (1997) Differential modulation of cell adhesion by interaction between adhesive and counter-adhesive proteins: Characterization of the binding of vitronectin to osteonectin (BM40, SPARC). *Biochem. J.* **324**, 311–319.

Sage, E.H., and Bornstein, P. (1991) Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin. *J. Biol. Chem.* **266**, 14831–14834.

Sage, E.H., Bassuk, J.A., Yost, J.C., Folkman M.J., and Lane, T.F. (1995) Inhibition of endothelial cell proliferation by SPARC is mediated through a Ca^{2+}-binding EF-hand sequence. *J. Cell Biochem.* **57**, 127–140.

Sasaki, T., Gohring, W., Mann, K., Maurer, P., Hohenester, E., Knauper, V., Murphy, G., and Timpl, R. (1997) Limited cleavage of extracellular matrix protein BM-40 by matrix metalloproteinases increases its affinity for collagens. *J. Biol. Chem.* **272**, 9237–9243.

Schultz, C., Lemke, N., Ge, S., Golembieski, W.A., and Rempel S.A. (2002) Secreted protein acidic and rich in cysteine promotes glioma invasion and delays tumor growth *in vivo*. *Cancer Res.* **62**, 6270–6277.

Uitto, V.J., Firth, J.D., Nip, L., and Golub, L.M. (1994). Doxycycline and chemically modified tetracyclines inhibit gelatinase A (MMP-2) gene expression in human skin keratinocytes. *Ann. N.Y. Acad. Sci.* **732**, 140–151.