ABSTRACT

Neuroblastoma (NB), a common pediatric neoplasm, consists of two main cell populations: neuroblastic/ganglionic cells and Schwann cells. NB tumors with abundant Schwannian stroma display a more benign clinical behavior than stroma-poor tumors. Recent studies suggest that Schwann cells influence NB tumor growth via secreted factors that induce differentiation, suppress proliferation, and inhibit angiogenesis. Two angiogenesis inhibitors, suppress epithelium-derived factor and tissue inhibitor of metalloproteinase-2, have been detected in Schwann cell secretions. Here, we isolated another Schwann cell-derived secreted inhibitor of angiogenesis, a 43-kDa protein identified as SPARC (secreted protein acidic rich in cysteine), an extracellular matrix protein. We found SPARC to be critical for the antiangiogenic phenotype of cultured Schwann cells. We also show that purified SPARC potently inhibits angiogenesis and significantly impairs NB tumor growth in vivo. SPARC may be an effective candidate for the treatment of children with clinically aggressive, Schwannian stroma-poor NB tumors.

INTRODUCTION

NB, a common pediatric neoplasm that arises from neural crest tissue, has a broad spectrum of clinical behavior (1–4). Although numerous factors including stage (5), patient age (6), tumor histology (7), molecular markers (8, 9), and genetic abnormalities (10–13) have been shown to be predictive of outcome in children with NB, the mechanisms responsible for the highly variable clinical behavior of NB remain largely unknown. Several recent studies implicate angiogenesis in the regulation of NB growth. In primary NB tumors, high vascular index correlates with MYCN amplification, metastases, and poor outcome, whereas low tumor vascularity is associated with a better prognosis, localized stage, and favorable histology (14). Advanced-stage NB is associated with high levels of angiogenic stimuli and αvβ3 and αvβ6 integrins, both markers of active angiogenesis (15, 16). Overexpression of exogenous MYCN results in enhanced malignant growth of NB cells and reduced levels of activin A, an inhibitor of angiogenesis (17). Expression of the neurotrophin receptor TrkA also causes down-regulation of angiogenesis stimulatory and impaired tumorigenicity in a mouse xenograft model (18). Furthermore, preclinical studies have shown effective reduction of NB tumor growth in vivo by a variety of antiangiogenic agents (16, 19–23).

SPARC Is a Key Schwannian-derived Inhibitor Controlling Neuroblastoma Tumor Angiogenesis

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SPARC expression is inversely correlated with the vascular index in NB tumors and suggests that a shift in the angiogenic balance results in in vivo tumor maturation. Immature NB tumors are composed of undifferentiated neuronal cells and a paucity of Schwannian stroma, whereas larger, ganglion-like cells and abundant Schwannian stroma are seen in maturing NB tumors. The Schwann cells are thought to be normal cells that infiltrate the NB tumor (25), although recent reports suggest that the Schwann cells may also be malignant (26). The favorable prognostic impact of the presence of Schwannian stroma has been emphasized in the pathological classification system of Shimada et al. (24) and the International NB Pathology Classification System (7). It has been speculated that Schwann cells influence NB tumor growth by secreting molecules that serve as antiproliferative and differentiating factors for neuronal cells (25, 27, 28). Schwann cells also produce a spectrum of angiogenesis inhibitors (29, 30), suggesting that Schwann cells may also influence NB growth by restricting angiogenesis.

The angiogenesis inhibitors PEDF and TIMP-2 have been previously found in media conditioned by Schwann cells, and both factors appear to contribute to the antiangiogenic activity of the SCM (29, 30). In this study, we isolated an additional angiogenic inhibitor in SCM, identified as SPARC. SPARC, also known as osteonectin, BM-40, and 43K protein, is a highly conserved calcium-binding matricellular glycoprotein (31–33). SPARC is spatially and temporally regulated during development, and it is transiently expressed in derivatives of the three primitive germ layers in mouse embryos (34). This glycoprotein is highly expressed in bone and in basement membranes as well as in a variety of cell types associated with remodeling tissues and high cellular turnover (35). Although its precise function is unclear, SPARC plays a modulatory role in cell-matrix interactions (36). SPARC induces cell rounding, blocks cell spreading and adhesion, and inhibits endothelial cell migration (37–39). SPARC also appears to contribute to vascular morphogenesis and cellular differentiation (36). However, the contradictory reports regarding the role of SPARC in cell growth and tumor formation (40–42) suggest that its effects are cell type specific and may be dependent on concentration, extracellular matrix components, and the ability of the cell to proteolyze SPARC (42–44).

We report that SPARC expression is inversely correlated with the degree of malignant progression in NB tumors. We also demonstrate that SPARC is one of the key contributors to the antiangiogenic activity of the SCM. Purified SPARC blocks angiogenesis in vitro and in vivo and significantly impairs NB tumor growth in vivo. Our observations stress the cumulative nature of the angiogenic equilibrium in NB tumors and suggest that a shift in the angiogenic balance may be prompted by even slight disturbances in the complex array of inhibitors and stimuli in the endothelial cell microenvironment.

MATERIALS AND METHODS

Cell Culture and CM Collection. Primary human Schwann cells were purified from adult nerves or from Schwannian stroma-dominant NB tumors and expanded as described previously (29, 30, 45). Tumor-derived Schwann...
cells were maintained at 5% CO₂ in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (US Biotechnologies, Inc., Parkerford, PA). 50 ng/ml recombinant human heregulin β1 (R&D Systems, Minneapolis, MN), 1% penicillin/streptomycin, 2.5 µg/ml amphotericin, 0.5 µm isobutylmethylxanthine (Sigma, St. Louis, MO), and 0.5 µm forskolin (Sigma). NB cell lines used in this study have been described previously (46–49), with the exception of NBL-L and NBL-R, MYCN-amplified lines established in our laboratory from clinically aggressive NB tumors. NB cells were grown at 5% CO₂ in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. For some experiments, 10 µM all-trans-RA (Sigma) or 6 µM BrdUrd (Sigma) was added where indicated, and the cells were harvested at the indicated time intervals. CM from NB cell lines and SCM were collected as described previously (29, 30).

**Isolation of SPARC from SCM.** Concentrated SCM were dialyzed against PBS and fractionated on a HiTrap Q-Sepharose column (Amersham, Piscataway, NJ) with the inhibitor's flow-through fractions cut from a Coomassie Blue-stained gel and submitted for sequence analysis at the Harvard University Microchemistry Facility.

**SPARC Expression Analysis.** To examine SPARC mRNA expression levels, total RNA was isolated from cultured cells using Trizol reagent (Life Technologies, Inc., Flanders, NJ) was used to highlight endothelial cells on frozen tumor sections. MVD was quantified by counting 10 consecutive fields at ×100 magnification, and the average MVD counted in the 10 fields was reported as MVD/mm².

**In Vitro Angiogenic Assay.** Migration assays were performed with human umbilical vein endothelial cells [National Cancer Institute Preclinical Repository (Bethesda, MD) and VEC Technologies (Rensselaer, NY)] as described previously (29). Test substances were assayed in Opti-MEM media (Life Technologies, Inc.) with or without 3 ng/ml bFGF (National Cancer Institute Preclinical Repository). Purified human platelet osteonectin (referred to hereafter as SPARC) was obtained from Calbiochem (San Diego, CA). To generate dose-response curves, the data were normalized as percentage of maximum migration using the difference between bFGF/Opti-MEM-induced migration and background migration in Opti-MEM alone as 100% control. For some assays, neutralizing anti-SPARC antibody (Zymed) or isotype-matched control antibody was added to the media at 10 µg/ml after dialysis against PBS. Control mouse IgG was obtained from Lab Vision (Fremont, CA).

**Endothelial Cell Apoptosis Assay.** Bovine adrenal capillary endothelial cells were treated overnight with SCM or control Opti-MEM. Dialyzed anti-SPARC antibody (Zymed) and control antibody were added at 10 µg/ml in some assays. Apoptotic cells were visualized using the ApopTag in situ Apoptosis Detection kit (InterGen, Gaithersburg, MD). Each assay was performed in triplicate, and the percentage of apoptotic cells was calculated as the number of green terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells with DNA fragmentation divided by the total number of Hoechst-counterstained nuclei.

**In Vivo Angiogenesis Assay.** Female Fischer 344 rats (Harlan, Madison, WI) were used to perform rat corneal assays using previously described methods (29, 52). Briefly, 5 µ Hydron pellets (IFN Sciences, New Brunswick, NJ) prepared with 25 µg/ml SPARC (Calbiochem) with or without 50 ng/ml bFGF were implanted into the corneas of anesthetized rats. Control studies were performed with pellets containing PBS with or without bFGF. Additional experiments were performed with pellets also containing 50 µg/ml anti-SPARC antibody (Zymed). After 7 days, the animals were sacrificed and perfused with water drawing water (Sanford, Bellwood, IL) by intracardiac injection. The eyes were fixed in 10% neutralized formalin overnight. The corneas were examined, and dense capillaries reaching the pellet were scored as positives. Animals were treated according to NIH guidelines for animal care and use, and protocols were approved by the Animal Care and Use Committee at Northwestern University.

**In Vivo Inhibition of NB Growth.** NB xenografts were grown in female 4–6-week-old homozygous athymic nude mice (Harlan) after s.c. inoculation of 5 × 10⁶ SMS-KCNR NB cells into the right flank. Once tumors were palpable, animals were anesthetized, and ALZET osmotic pumps (Durect, Cupertino, CA) containing purified SPARC (Calbiochem; n = 3) or PBS (n = 3) were implanted s.c. SPARC was released s.c. by the pump at a rate of 62.5 ng/h. Tumor volume was measured weekly using the formula: tumor volume = (length × width²)/2 (53). Mice were sacrificed after 3 weeks of treatment, and tumors were resected for histological analysis. Tumor volume was analyzed using Student’s t test to compare control and treatment groups.

**RESULTS**

**Schwann Cells Secrete Antiangiogenic SPARC.** We have previously reported antiangiogenic activity in SCM collected from normal and NB tumor-derived Schwann cells (29) and demonstrated the presence of several angiogenic inhibitors including PEDF and TIMP-2 (29, 30). Seeking additional angiogenesis inhibitors in SCM, we subjected it to multiple-step chromatography and tested fractions for the ability to block bFGF-induced endothelial cell migration and cause endothelial cell apoptosis. Fractions capable of both activities contained a 43-kDa protein (Fig. 1) that was identified as SPARC using sequence analysis of the tryptic peptide fragments. No contaminating sequence was detected in the SPARC band.

**SPARC Was Expressed by Schwann Cells and Differentiated NB Cells in Vitro and In Vivo.** SPARC expression was evaluated by semiquantitative RT-PCR in Schwann cells, a panel of NB cell lines, and in phenotypically distinct subclones of NB cell lines [neuroblastic (N-type) and substrate adherent (S-type)] that exhibit different malig-
nant potentials (Refs. 54 and 55; Fig. 2 A). Although SPARC mRNA was detected in all NB cell lines with the exception of NBL-W-N, mRNA levels were significantly higher in the Schwann cells and nontumorigenic S-type subclones than in tumorigenic N-type subclones and NB cell lines. SPARC protein levels in CM collected from the cells paralleled the mRNA levels (Fig. 2 B). NB cells can be induced to differentiate in vitro with a number of agents including all-trans-RA or BrdUrd (56–59), and real-time quantitative RT-PCR demonstrated up to a 10-fold increase in SPARC mRNA in differentiated NB cells (Fig. 2 C).

To investigate whether SPARC was expressed within NB tumors, histological sections from NB tumors displaying varying degrees of differentiation and abundance of Schwannian stroma and from ganglioneuromas were stained with antibody against human SPARC. Schwannian stroma-poor tumors were composed predominantly of neuroblasts and showed minimal or no staining for SPARC (Fig. 2 D). Conversely, in maturing and mature tumors, SPARC could be detected not only in Schwann cells (Fig. 2 E) but also in differentiating neuroblasts/ganglion cells (Fig. 2 F).

**SPARC in SCM Inhibited Migration and Induced Apoptosis of Endothelial Cells.** The previously reported inhibition of bFGF-induced endothelial cell migration by SCM (29) was SPARC dependent because migration was largely restored in the presence of neutralizing antibody against SPARC (Fig. 3 A). We also showed that SPARC-dependent induction of endothelial cell apoptosis by SCM was effectively neutralized by the same antibody (Fig. 3 B). Consistent with earlier studies (38), purified SPARC blocked bFGF-induced endothelial cell migration in a dose-dependent manner at concentrations ranging from 0.05 to 5 μg/ml (Fig. 3 C). However, endothelial cell migration inhibition was not observed at higher concentrations of SPARC. Biphasic responses have similarly been reported with angiogenesis inhibitor thrombospondin-1 (60, 61). SPARC also triggered endothelial cell apoptosis, with maximal induction at 20 μg/ml (Fig. 3 D).

**SPARC Inhibited Angiogenesis and Impaired Tumor Growth In Vivo.** Purified SPARC blocked bFGF-induced angiogenesis in vivo in the rat corneal neovascularization assay (Fig. 4; Table 1). Furthermore, the addition of the anti-SPARC antibody fully restored angiogenesis by bFGF, indicating that this inhibitory effect was indeed due to SPARC. Angiogenesis was not observed when SPARC was tested alone (Fig. 4). To our knowledge, the ability of SPARC to inhibit angiogenesis has not previously been tested in a rat corneal model. The effect of SPARC on NB growth in vivo was tested in a mouse xenograft model where SPARC was delivered continuously for 3 weeks using osmotic pumps. During the first 2 weeks, tumor growth was completely arrested in the SPARC-treated group, whereas in the control animals carrying PBS-charged pumps, the volume of the
tumors doubled every 5–6 days (Fig. 5, A and B). During the third week of treatment, a slight increase in tumor size was observed in the treatment group. After 3 weeks of treatment with SPARC, the average tumor volume was significantly smaller than that observed in control animals (152 ± 44 mm$^3$ versus 919 ± 317 mm$^3$; $P = 0.03$). Histological comparison revealed decreased vascularity in SPARC-treated tumors (MVD = 23/mm$^2$) compared with control tumors (MVD = 47/mm$^2$) as assessed by the number of structures that stained positively with an anti-CD31 antibody (Fig. 5, C and D).

### DISCUSSION

Schwann cells secrete substances that promote NB cell survival and differentiation and inhibit angiogenesis (25, 27, 29, 30). It is thought that cross-talk between neuroblasts and Schwann cells is responsible for the more benign nature of Schwannian stroma-rich/stroma-dominant NB tumors (25, 29). Seeking factors produced by Schwann cells that could contribute to this clinically less aggressive tumor phenotype, we identified a factor present in SCM that was capable of inhibiting angiogenesis.

#### Table 1 In vivo antiangiogenic activity of SPARC

<table>
<thead>
<tr>
<th>Sample</th>
<th>bFGF</th>
<th>Positive corneas/total implanted</th>
</tr>
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<tr>
<td>PBS</td>
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<td>PBS</td>
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<td>9/9</td>
</tr>
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Fig. 4. SPARC inhibits angiogenesis in vivo. 

**Top panels**. SPARC inhibits bFGF-induced angiogenesis in the rat corneal assay. Addition of anti-SPARC antibody restored angiogenesis by bFGF. Bottom panels, in the absence of bFGF, control media, purified SPARC, or purified SPARC plus anti-SPARC neutralizing antibody did not induce angiogenesis in rat corneal assays.
SPARC, a known member of the matricellular protein family, appears to be a key contributor to the cumulative antiangiogenic activity produced by Schwann cells. We confirmed a known ability of SPARC to block chemotaxis of endothelial cells induced by angiogenic stimuli (38) and report its previously unknown function to induce endothelial cell apoptosis.

Previous studies have indicated that SPARC contributes to the regulation of tumor formation, although its role appears to be cell type specific. SPARC expression is down-regulated in rat and chick embryo fibroblasts transformed with c-Jun and v-Src (40, 62), and its reintroduction counteracts tumorigenesis by these cells (41). In addition, SPARC slows in vitro growth, induces apoptosis, and reduces tumorigenicity of ovarian cancer cells (44). In contrast, suppression of SPARC expression abrogates the tumorigenicity of melanoma cells (42).

SPARC is also involved in angiogenesis. SPARC blocks the G1 to S transition of endothelial cells manifested by decreased proliferation rates (63, 64) and antagonizes the activity of the potent angiogenic activators bFGF and VEGF (38, 39). SPARC-null mice display substantially higher tumor invasion and angiogenesis compared with their wild-type littermates (65), indicating that SPARC may interfere with tumor angiogenesis. However, other studies point to proangiogenic activity of SPARC (66): it was found at high levels in breast cancer and colon cancer (67, 68), in metastatic melanoma (69), and in invasive meningiomas (70). Our studies suggest that disparate data regarding the role of SPARC in tumor growth and angiogenesis may be explained by its biphasic effect on the endothelial cells, where higher concentrations appear inactive or even stimulatory. Such biphasic effects are not uncommon for antiangiogenic factors and are shared by at least two more inhibitors, thrombospondin-1 (60, 61) and PEDF.

Our study revealed an inverse correlation between SPARC expression levels and the degree of malignant progression in NB tumors. Whereas tumorigenic cell lines and N-type subclones showed low or no detectable levels of SPARC mRNA and secreted protein, non-tumorigenic S-type NB subclones and NB cells induced to differentiate in vitro expressed SPARC at high levels. In maturing and mature NB tumors rich in Schwannian stroma, Schwann cells and differentiated neuroblasts/ganglion cells showed strong positive staining for SPARC, whereas little to no staining was detected in undifferentiated, Schwannian stroma-poor tumors. Furthermore, we demonstrated antiangiogenic activity of SPARC in a mouse NB xenograft model.

The mechanisms underlying these highly variable cell type-specific activities of SPARC remain unknown. However, similar to thrombospondin-1 (60, 61), concentration-dependent activity of SPARC may be explained if two distinct receptors are present on vascular endothelium, where a low-affinity receptor activated by higher ligand concentrations conveys a proangiogenic function of SPARC, and a high-affinity receptor is antiangiogenic. SPARC function may also be altered via posttranslational modifications. SPARC subspecies secreted by normal fibroblasts and by melanoma tumors differ in size and glycosylation pattern (69). It is tempting to speculate that turnover rates and receptor affinity of SPARC produced by malignant melanoma cells may be disparate from those of SPARC from normal stroma. Specific cleavage of SPARC by tumor cells may be glycosylation dependent and lead to an altered function. This hypothesis appears more feasible because the peptides from distinct structural domains of SPARC affect diverse cell phenotypes including growth rate, cell shape, matrix attachment, and angiogenic potential (66, 69, 71). Changes in any of these functions alone may lead to decreased

D. W. Dawson, unpublished data.

R. Schodlu et al., Augmentation of choroidal neovascularization in a laser induced mouse model by PEDF, in preparation.
angiogenesis and slow down tumor progression. However, it is likely that it is the ability of SPARC, shared with the majority of the angiogenesis inhibitors (reviewed in Refs. 72 and 73), to induce apoptosis in the endothelial cells of remodeling vasculature that allows it to interrupt angiogenesis and to delay tumor progression.

Our data point to SPARC as a key contributor to the antiangiogenic activity of factors secreted by the Schwann cells. However, other inhibitors of angiogenesis including PEDF and TIMP-2 also appear to be involved in the cross-talk between Schwann cells and the neuronal component of NB tumors (29, 30, 74). Our data clearly demonstrate that the angiogenic balance is complex: it is not a result of a single inducer-inhibitor combination but a composite value determined by all of the inducers and inhibitors present (reviewed in Ref. 75).

Changes in angiogenic phenotype may occur gradually (76) or in a single step (77, 78). In Schwannian stroma-rich/stroma-dominant NB tumors, a spectrum of angiogenic inhibitors appears to be important for maintaining the net inhibitory phenotype, possibly because the nature of the angiogenic balance in NB tumors is quite complex. In addition to its effect on angiogenesis, PEDF also promotes survival and differentiation of the neuronal component of the tumors and thus addition to its effect on angiogenesis, PEDF also promotes survival.

Changes in angiogenic phenotype may occur gradually (76) or in a single step (77, 78). In Schwannian stroma-rich/stroma-dominant NB tumors, a spectrum of angiogenic inhibitors appears to be important for maintaining the net inhibitory phenotype, possibly because the nature of the angiogenic balance in NB tumors is quite complex. In addition to its effect on angiogenesis, PEDF also promotes survival and differentiation of the neuronal component of the tumors and thus addition to its effect on angiogenesis, PEDF also promotes survival.

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