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Secreted protein acidic and rich in cysteine as a regulator of murine ovarian cancer growth and chemosensitivity

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Abstract

OBJECTIVE—Secreted protein acidic and rich in cysteine (SPARC) influences the growth of several solid tumors. Our objectives were to determine the effect of SPARC on the growth and response to cisplatin therapy of platinum-resistant ovarian cancer.

STUDY DESIGN—SPARC expression was determined in 4 platinum-resistant ovarian cancer cell lines. The effect of increasing SPARC on cell proliferation was determined in vitro. The effect of host-derived SPARC on tumor growth and response to therapy was determined in vivo using the murine ovarian cancer cell line, OSEID8, which was injected into the peritoneum of wild-type (WT) and SPARC-null (SP^{-/-}) mice.

RESULTS—Forced expression of SPARC decreased growth of platinum-resistant ovarian cancer cell lines in vitro. In vivo, tumor growth was more aggressive in the absence of host-derived SPARC resulting in decreased survival compared with *WT* mice ($P = .005$). Cisplatin did not improve survival of *WT* mice. In contrast, cisplatin therapy resulted in a significant survival advantage ($P = .0048$) and decreased tumor volume ($P = .02$) in *SP^{-/-}* animals.

CONCLUSION—We conclude that SPARC is an important extracellular matrix protein that regulates the growth and chemosensitivity of ovarian cancer. In general, SPARC appears to control tumor cell growth but also impede the efficacy of cisplatin therapy. Therefore, selective inhibition of SPARC may provide an attractive strategy for increasing the efficacy of therapy in platinum-resistant ovarian tumors.

Keywords

Chemosensitivity; extracellular matrix; murine; ovarian cancer; secreted protein acidic and rich in cysteine

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Secreted protein acidic and rich in cysteine (SPARC) is a 32-kDa calcium binding glycoprotein. It is antiadhesive and antiproliferative, and it binds to many extracellular matrix (ECM) proteins.^{1,2} In this capacity, SPARC has been shown to have tumor-suppressor effects and significant function in the host response to tumor development.^{2,3} SPARC expression is altered in many cancers. For instance, there is increased expression of SPARC in melanoma, glioma, colorectal, and breast carcinomas compared with their respective normal tissues.⁴ In these tumors, high levels of SPARC are often associated with enhanced invasion and metastasis.⁴ In contrast, expression of SPARC by pancreatic carcinoma cells is limited because of promoter hypermethylation, whereas stromal cells in the tumor express increased levels of SPARC.⁵ Whether produced by tumor cells or stromal (host) cells, SPARC is often found at tumor-stromal interfaces; in tumor capsules; and in areas of desmoplasia, angiogenesis, and vascular remodeling.⁶ Thus, the context of SPARC expression in the microenvironment is critical for understanding its influence on cancer growth.

Previously epithelial ovarian cancer cells have been shown to express low levels of SPARC, whereas adjacent stroma expresses higher levels.^{2,7,8} Mok et al⁹ published 1 of the earliest studies on the function of SPARC in ovarian cancer. They found that SPARC-expressing transfectants had reduced growth in vivo compared with control cells. Furthermore, Yiu et al² demonstrated that forced expression of SPARC or addition of recombinant SPARC induced apoptosis of ovarian cancer cell lines in vitro. A more recent study by Said and Motamed³ evaluated the effect of host-derived SPARC on ovarian cancer growth, in vivo, demonstrating more rapid and aggressive tumor growth in SPARC-deficient animals.

SPARC has been shown to modulate response to chemotherapy. A recent study by Tai et al¹⁰ demonstrated that, in colorectal cancer, addition of exogenous SPARC and/or forced expression of endogenous SPARC improved tumor cell response to chemotherapy in vitro. The authors also found in vivo evidence to suggest that SPARC can sensitize colon cancer cells to chemotherapy.¹⁰

These findings were validated by demonstration of enhanced apoptosis in colon cancer cells and decreased blood vessel formation in tumors after exposure to or forced expression of SPARC.¹⁰ If and how SPARC affects chemosensitivity of ovarian cancer is unknown. However, given these findings, SPARC is well placed to participate in the host response to tumor progression and therapy of ovarian cancer.

Epithelial ovarian cancer is considered a chemoresponsive disease.¹¹ Initial response rates to cytoreductive surgery followed by platinum therapy are as high as 80%; whereas, with advanced stage disease, survival is poor because of recurrence and drug resistance.¹¹ Patients who progress or recur 6 months after primary therapy are considered platinum resistant.^{12,13} Standard second-line treatments are limited and include various single-agent chemotherapies.¹³ More recent clinical trials have looked at biologics and antiangiogenic therapies. Few clinical trials have looked at ways to overcome drug resistance to primary platinum therapy or considered host pharmacogenomic pathways.¹¹

Our primary objective was to determine the effect of modulating the level of SPARC on the growth of known platinum-resistant ovarian cancer. Our secondary objective was to determine whether SPARC modifies chemosensitivity of platinum-resistant ovarian cancer.

Materials and Methods

Cell culture and reagents

Platinum-resistant human ovarian cancer cell lines SKOV3, ES2 (American Type Culture Collection, Manassas, VA), and HCC60 from the Hamon Center for Therapeutic Oncology Research (gift of Dr John D. Minna) and a platinum-resistant murine ovarian cancer cell line (OSEID8)¹⁴ obtained from Dr Paul F. Terranova (University of Kansas Medical Center, Kansas City, KS) were used. All cell lines were grown in RPMI 1640 (Invitrogen Corp, Carlsbad, CA) or Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5–15% fetal bovine serum (Gemini Bio-Products, Woodland, CA), and 1% antibiotic/antimycotic (Invitrogen) at 37°C (5% CO₂). The mouse monoclonal anti-body (MAb) 303 was produced and purified as described previously.¹⁵ MAb303 has been shown to inhibit SPARC-induced activation of AKT in glioma cells.¹⁶

SPARC expression

Cells at 80–90% confluence were harvested along with the corresponding cell-conditioned media. Cell lysates were extracted from cell pellets using lysis buffer containing 2.5 mM of 1 M Tris at pH 7.4, 1% Nonidet P-40, 50 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN), 0.5 M NaF, 20 mM Na₃VO₄, and 1 M β -glycerophosphate. Equal volumes of cell-conditioned media, consisting of supernatant obtained from plates with 80–90% confluence, was used to evaluate secreted SPARC levels. Western blotting was performed as described previously¹⁷ using MAb303 as primary antibody. Signal was developed by incubating with a horseradish peroxidase-conjugated anti-mouse secondary antibody and chemiluminescent substrate (Pierce, Rockford, IL) prior to exposure to X-ray film (Phenix Research Products, Hayward, CA). The level of β -actin was used as the loading control, and recombinant SPARC protein was used as a positive control.

Tumor-associated SPARC modulation

Transient expression of SPARC was induced by transfection with pCMV-SPORT6 plasmid (Invitrogen Corp) containing complementary deoxyribonucleic acid (cDNA) for human SPARC. Cells were also transfected with the empty vector as a control. Western blot was performed at 72 hours to determine SPARC expression in the transfected cells. After harvesting transfected cells, direct cell counts (at 24, 48, and 72 hours) and liquid culture colony formation assays (at 14 days) were performed to determine the functional impact of endogenous SPARC on ovarian cancer cell growth. Two independent experiments were performed in duplicate 6-well plate format.

Chemosensitivity testing with SPARC modulation

In vitro—To determine the effect of SPARC on cisplatin sensitivity, cells at 80–90% confluence were trypsinized, pelleted, resuspended in media, and counted. Cells were seeded at a density of $1\text{--}2 \times 10^3$ cells/well in 96-well plates and allowed to attach for 24 hours, after which they were treated with cisplatin, MAb303, or a combination of the 2, in the presence of 10% serum. Six days after treatment, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H)- tetrazolium reagent] (Promega, Madison, WI) was added to each well.

After an incubation of 4 hours, absorbance was read using the Emax Precision microplate reader (Molecular Devices Corp, Sunnyvale, CA) at 490 nm. Cell growth was normalized to untreated cells. SoftMax Pro 4.7.1 (Molecular Devices) converted the absorbance values into cell concentration compared with control. The concentration of cisplatin resulting in

50% inhibition (IC₅₀), in the presence or absence of MAb303, was calculated. All cell proliferation assays were performed in replicates of 8, using 96-well plates in 2 independent experiments.

In vivo—All experiments were carried out in accordance with University of Texas Southwestern Institutional Animal Care and Use Committee Policies and Guidelines. Then 5×10^6 OSEID8 cells¹⁴ were injected intraperitoneally into female SPARC null (*SP*^{-/-}) C57BL/6 mice ($n = 12$), generated as described by Norose et al,¹⁸ and age-matched, 8-week old wild-type (*WT*) C57BL/6 female mice ($n = 12$; Charles River Laboratories, Wilmington, MA).

Both the *SP*^{-/-} and the *WT* groups were divided equally into observation and treatment arms, resulting in a total of 4 arms with 6 animals in each arm. After observation for 41 days, the treatment group ($n = 6$ /genotype) was given 5 mg/kg cisplatin, intraperitoneally, weekly for 4 weeks. Animals were observed for evidence of disease (ascites, body weight, performance status) and killed for decreased performance status or recurrent ascites. Tumor location and volumes (cubic millimeters) were determined after the animals were killed by direct visualization and measurements of length $\times$ width $\times$ thickness were compared. Mice were observed for 117 days and survival was documented as time to the animals were killed or death.

Statistical analysis

Statistical analysis included Student *t* test for growth and volume comparisons with alpha set at 0.05. Kaplan-Meier survival curves were compared using Mantel-Cox log-rank test with alpha set at 0.05. Calculations, figures, and tables were produced with Graph Pad Prism version 5.00 (GraphPad Software, San Diego, CA).

Results

SPARC expression

SPARC was variably expressed in the cell lines tested. Two of the platinum-resistant human ovarian cancer cell lines (ES2 and HCC60) and the platinum-resistant murine ovarian cancer cell line (OSEID8) showed expression of cellular SPARC in cell lysates (Figure 1, A). When testing conditioned media, we found 2 of the cell lines (ES2 and OSEID8) unequivocally secreted SPARC (Figure 1, B). There was little or no detectable SPARC in the conditioned media from HCC60 cells and questionable SPARC secretion in SKOV3, as compared with the control media lane. Although MAb303 reacts preferentially with mouse and human SPARC, there is some cross-reactivity with bovine SPARC as shown in the media/fetal bovine serum lane (Figure 1, B). Despite performing 2 separate experiments in duplicate, we cannot conclusively state that SKOV3 is or is not a SPARC secretor.

Tumor-associated SPARC modulation

To determine the effect of increasing SPARC expression in ovarian cancer, we transfected all the cell lines with a SPARC cDNA and empty vector plasmid. Direct cell counts showed that forced expression of SPARC had a varied effect on cell growth at 24 and 48 hours. At 72 hours, however, all cell lines showed decreased growth compared with controls transfected with empty vector, with a significant difference seen in OSEID8 and ES2 cells (Figure 2, A; $P = .02$ and $P = .004$, respectively). HCC60 and SKOV3 showed decreased growth after transfection with the SPARC plasmid; however the difference was not significant ($P = .6$ and $P = .5$, respectively), with error bars overlapping for HCC60. Western blot analysis confirmed transfection in all cell lines at 72 hours after transfection (Figure 2, B and data not shown). We con-firmed that forced expression of SPARC significantly

reduced growth in the OS-EID8 and ES2 cells by the use of a colony-formation assay (Figure 2, C). In summary, transfection with SPARC cDNA decreased ovarian tumor cell growth.

Chemosensitivity testing with SPARC modulation

In vitro—To determine what effect altering SPARC had on the efficacy of cisplatin, we treated cells with cisplatin alone or in combination with MAb303. MAb303 has been shown to block SPARC-induced survival of glioma cells.¹⁶ Direct treatment with MAb303 produced no significant growth inhibition. Treatment with cisplatin confirmed the relative resistance of the cell lines, with IC50s ranging from 1.79 μ M to 10.68 μ M. Combining MAb303 with cisplatin resulted in no significant decrease in the IC50 for cell lines ES2, HCC60, and SKOV3. However, treatment of OSEID8 cells with MAb303 and cisplatin resulted in a significant ($P = .002$) decrease in the IC50 of cisplatin (Table).

In vivo—To determine the effect of host-derived SPARC on the chemosensitivity of OS-EID8 cells in vivo, we performed tumor studies in *WT* and *SP*^{−/−} animals. Overall tumor progression was enhanced in untreated control *SP*^{−/−} compared with *WT* animals as evidenced by increased tumor volume and reduced survival at time the animals were killed ($P = .19$ and $P = .005$, respectively; Figures 3 and 4).

Therapy with cisplatin was initiated on day 41 after tumor cell injection at which time 4 of the *SP*^{−/−} but none of the *WT* mice presented with ascites (Figure 3, A). Ascitic fluid was removed aseptically from these animals before therapy was administered. The untreated control *WT* and *SP*^{−/−} mice were observed and at last follow-up, 5 of 6 *SP*^{−/−} and 3 of 6 *WT* mice had developed ascites (Figure 3, B). Cisplatin therapy had little effect on tumor burden or ascites development in *WT* mice (Figure 3, B and C), consistent with OSEID8 being a platinum-resistant cell line. In contrast, *SP*^{−/−} animals responded to therapy. In *SP*^{−/−} animals, cisplatin significantly reduced tumor volume (Figure 3, C; $P = .02$, *SP*^{−/−} control vs treatment) and reduced the incidence of ascites from 83% (5/6) in the control animals to 50% (3/6) in the cisplatin-treated animals (Figure 3, B; $P = .55$, *SP*^{−/−} control vs treatment).

The effect of the absence of host-derived SPARC on tumor progression and therapy was also evident by monitoring survival curves (Figure 4). Untreated *SP*^{−/−} mice displayed a significant decrease in overall survival ($P = .0048$) compared with treated *SP*^{−/−} mice (Figure 4, A). Treatment with cisplatin improved the mean survival time by approximately 30 days in *SP*^{−/−} mice (Figure 4, A). In contrast, cisplatin therapy had no significant effect on the survival of *WT* mice (Figure 4, B). Additionally, untreated *SP*^{−/−} mice had a significantly shorter survival than *WT* control mice ($P = .0005$; Figure 4, C). Therefore, SPARC deficient mice with platinum-resistant ovarian tumors had worse survival and greater tumor burden compared to *WT* mice; however, they had significantly improved survival and decreased tumor burden, when treated with cisplatin therapy, compared with untreated SPARC-deficient mice.

Comment

The major findings to emerge from this study are: (1) increased expression of SPARC by ovarian tumor cells inhibits cell growth, in vitro; (2) host-derived SPARC may reduce tumor volume and improve survival in the absence of therapy; (3) cisplatin therapy is more efficacious in tumor-bearing SPARC-deficient animals than wild-type mice.

SPARC is a matricellular glycoprotein that influences tumor cell interaction with the extracellular matrix.^{1,2} Differentiating the function of tumor-associated and host-derived SPARC is a challenge given that SPARC can be secreted by and can act on many different

cells in the tumor microenvironment. Some studies have found tumor-associated SPARC to be an important modulator of tumor progression and metastasis,¹⁹ whereas others have found host-derived SPARC to have a greater influence.^{1,20}

We found that increasing tumor-associated SPARC diminished ovarian cancer cell growth in vitro. Even in cell lines already expressing SPARC, forcing additional expression, the results were a decrease in cell growth. This is consistent with previous findings by Yiu et al.² Cell lines overexpressing SPARC have significantly decreased growth with forced induction of SPARC, suggesting a potential for further SPARC induction specifically in SPARC producing ovarian cancers.

Attempts to block SPARC activity in the murine cell line OSEID8 with MAb303¹⁵ resulted in improved response to cisplatin treatment, but it had little effect on improving the cisplatin response in the 3 human cell lines. The mechanism(s) underlying the enhanced effect of MAb303 and cisplatin is unclear. Shi et al¹⁵ found that MAb303 reduced SPARC-induced activation of AKT in glioma cells. This, however, is counterintuitive, given that increased expression of SPARC by OSEID8 cells reduces cell number rather than promotes cell survival as would be expected if AKT activation was the primary driver of SPARC effects in ovarian cells.

Another intriguing possibility is SPARC-induced modulation of cell shape or adhesion via inhibition of integrin-collagen interaction. It is possible that MAb303, by binding to SPARC, perturbs OSEID8 cell adhesion, resulting in enhanced cisplatin-mediated cell killing. This is supported by in vitro studies demonstrating that MAb303 can induce cell rounding and deadhesion.¹⁴ The use of additional platinum-resistant human ovarian cancer cell lines and other anti-SPARC antibodies such as AON-1, MAb255, or MAb293¹⁴ may provide different effects.

To evaluate the effect of host-derived SPARC on chemoresistant ovarian tumor growth and response to chemotherapy, we compared the growth of OSEID8 cells in *WT* and *SP*^{-/-} animals. Our results are similar to the studies of Said and Motamed,³ the results of which showed increased tumor burden in the absence of SPARC. In particular, we found that *SP*^{-/-} animals had a decreased time to formation of ascites and a larger tumor burden.

Importantly, we show for the first time, in vivo, that platinum-resistant ovarian tumors have increased sensitivity to chemotherapy in the absence of host-derived SPARC. The mechanism by which SPARC affected the sensitivity of these tumor cells remains to be elucidated. Interestingly, because SPARC is known to participate in ECM deposition, it is postulated that the delivery of chemotherapeutic agents to tumor cells themselves may be improved in the absence of SPARC.

The use of cell lines is controversial because of molecular changes that may occur between generations. Because our goal did not depend on the parent cell lines' molecular characteristics, we did not search for alterations from the original generation. Additionally, although SPARC is a protein often found in the stroma, we chose to study cell lines to examine cell associated and secreted levels of SPARC and to determine the comparative platinum resistance between the murine cell line, OSEID8, and known platinum-resistant human OSE cell lines.

Additional limitations of this study include the use of MAb303, which can bind bovine serum in the media to a small extent, and the small cohort of animals used for the in vivo experiment. The use of a transient transfection protocol for inducing SPARC expression prevented evaluation of the effect of altered endogenous SPARC on in vitro chemosensitivity, which would require a transfection that could persist several days.

Questions needing further study include examining whether forced expression or knockdown of SPARC is functionally equivalent to the natural expression pattern of SPARC in ovarian cancer and whether SPARC expression correlates with chemosensitivity. Future studies may include transfection with a stable intracellular modifier of SPARC, development of alternative SPARC modulators, and inoculation with human ovarian cancer cell lines for the in vivo model.

Cisplatin resistance in ovarian cancer may be related to alterations in excision repair proteins, decreased drug accumulation because of alteration in cellular transport, and increased capacity to tolerate DNA damage.^{21,22} Although our study does not elucidate mechanisms by which chemosensitivity is induced in the absence of host SPARC, it does underscore the importance of incorporating the interplay between tumor and host when studying ovarian cancer growth and response to chemotherapy. Our results and those of others reinforce the fact that SPARC is an important factor in regulating ovarian tumor growth.

Our study suggests that SPARC may also modulate the chemosensitivity of ovarian cancer, either as the primary modulator or an effector of an undiscovered modulator. It would be interesting to determine the effect SPARC has on those factors already known to alter chemoresistance, such as tumor suppressor gene products or the cellular matrix action on drug uptake and distribution. Future therapeutic approaches may include immunotherapy directed at inactivating host-SPARC. Further studies are warranted to determine the mechanisms by which SPARC acts. Selective inhibition of SPARC may provide an attractive strategy for increasing the efficacy of chemotherapy in platinum-resistant ovarian tumors.

CLINICAL IMPLICATIONS

SPARC is an important factor in regulating ovarian tumor growth. SPARC may also modulate the chemosensitivity of ovarian cancer, either as the primary modulator or an effector of an undiscovered modulator. Selective inhibition of SPARC may provide an attractive strategy for increasing the efficacy of chemotherapy in platinum-resistant ovarian tumors.

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FIGURE 1. SPARC is variably expressed by platinum resistant ovarian cancer cells in vitro
A, Cell lysates and **B**, corresponding conditioned media from the indicated cell lines were evaluated for SPARC expression by Western blot analysis. β -Actin was used as a loading control.

FIGURE 2. Forced expression of tumor-associated SPARC reduces cell proliferation

Ovarian cancer cells were transiently transfected with SPARC (SP, *open bars*) or a control empty vector (EV, *filled bars*). **A**, Cell number was determined by counting at the indicated time points after transfection. Mean cell counts (Y axis) at 24, 48, and 72 hours after transfection is shown with SEM bars. **B**, Western blot analysis was performed on cell lysates to confirm transfection after 72 hours. HCC60 and SKOV3 are shown. **C**, Liquid culture colony formation assays were also performed on OSEID8 and ES2 to confirm a significant reduction in cell growth. Fourteen days after plating, colony counts were recorded and mean colony-forming units calculated ($\pm$ SEM).

FIGURE 3. Tumor growth and response to cisplatin therapy

Tumor growth and response to cisplatin therapy is enhanced in the absence of host-derived SPARC. OSEID8 cells (5×10^6) were injected intraperitoneally into *WT* and *SP*^{-/-} mice ($n = 12/\text{genotype}$). Starting on day 41 after tumor cell injection, the mice were treated weekly for 4 weeks with either vehicle ($n = 6/\text{genotype}$) or cisplatin (5 mg/kg, $n = 6/\text{genotype}$). **A**, The number of *WT* (filled bars) and *SP*^{-/-} (open bars) mice showing signs of ascites at day 41 or **B**, the last day of assessment is shown. **C**, The extent of tumor burden (volume in cubic millimeters) was determined at the time the animals were killed. Mean volume $\pm$ SEM is shown. A significant decrease in tumor volume is seen in the *SP*^{-/-} mice treated with cisplatin ($P = .02$ comparing *SP*^{-/-} control and *SP*^{-/-} treated mice).

FIGURE 4. Cisplatin improves survival in tumor-bearing *SP*^{-/-} mice

OSEID8 cells (5×10^6) were injected intraperitoneally into *WT* and *SP*^{-/-} mice ($n = 12$ /genotype). Starting on day 41 after tumor cell injection, the mice were treated weekly for 4 weeks with either vehicle ($n = 6$ /genotype) or cisplatin (5 mg/kg, $n = 6$ /genotype). Mice were monitored daily for signs of morbidity and disease. **A–C**, Kaplan-Meier survival curves for each genotype are shown. **A**, Cisplatin improved survival significantly ($P = .0048$) in *SP*^{-/-} mice but not **B**, *WT* mice. **C**, In the absence of therapy, survival of *SP*^{-/-} mice was diminished significantly ($P = .0005$) compared with *WT* controls.

TABLE 1

Combination of anti-SPARC MAb303 with cisplatin reduced the in vitro IC50 of cisplatin in OSEID8 cells

	MAb303 ($\mu\text{g/mL}$)		Cisplatin (μM)		Cisplatin (μM) plus MAb303 (0.4 $\mu\text{g/mL}$)			
	Mean IC50	SD	Mean IC50	SD	Mean IC50 (cisplatin)	SD	Fold change	P value
OSEID8	> 1000	0	10.68	0.43	2.83	0.18	0.26	.002
ES2	869.2	0	2.25	0.11	2.38	0	1.01	NS
HCC60	> 1000	0	1.79	0.14	1.6	0.9	0.89	NS
SKOV3	> 1000	0	6.48	0.87	10.09	0.28	1.56	.03

Addition of MAb303 and cisplatin to the 3 platinum-resistant human ovarian cancer cell lines did not result in significant reduction in the IC50 of cisplatin.