Normalization of the Ovarian Cancer Microenvironment by SPARC


Abstract
Malignant ascites is a major source of morbidity and mortality in ovarian cancer patients. It functions as a permissive reactive tumor-host microenvironment and provides sustenance for the floating tumor cells through a plethora of survival/metastasis-associated molecules. Using a syngeneic, immunocompetent model of peritoneal ovarian carcinomatosis in SP−/− mice, we investigated the molecular mechanisms implicated in the interplay between host secreted protein acidic and rich in cysteine (SPARC) and ascitic fluid prosurvival/prometastasis factors that result in the significantly augmented levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP). Ascitic fluid–enhanced ID8 invasiveness was mediated through VEGF via a positive feedback loop with MMP-2 and MMP-9 and through activation of αv and β1 integrins. Host SPARC down-regulated the VEGF-MMP axis at the transcriptional and posttranscriptional levels. In vitro, SPARC attenuated the basal as well as VEGF-induced integrin activation in tumor cells. SPARC inhibited the VEGF- and integrin-mediated ID8 proliferation in vitro and significantly suppressed their tumorigenicity in vivo. Relative to SP+/+, SP−/− ascitic fluid contained significantly higher levels of bioactive lipids and exerted stronger chemotactic, proinvasive, and mitogenic effects on ID8 cells in vitro. SP−/− ascites also contained high levels of interleukin-6, macrophage chemoattractant protein-1, and 8-isoprostanate (prostaglandin F2α) that were positively correlated with extensive infiltration of SP−/− ovarian tumors and ascites with macrophages. In summary, our findings strongly suggest that host SPARC normalizes the microenvironment of ovarian cancer malignant ascites through down-regulation of the VEGF-integrin-MMP axis, decreases the levels and activity of bioactive lipids, and ameliorates downstream inflammation.

Introduction
Ovarian cancer is the leading cause of death among gynecologic cancers in the United States. Metastatic dissemination to peritoneal and pleural effusions is considered the major cause of patient morbidity and mortality in ovarian cancer. The ability of floating ovarian cancer cells in these malignant effusions to survive, proliferate, and disseminate in the absence of immediate proximity to a solid scaffold and vascular structures has been attributed to the unique, yet poorly defined, permissive microenvironment of these dense exudative fluids (1). The high autonomous proliferation rate of the metastatic ovarian cancer cells, their ability to develop alternative means of survival, as well as the contributions of inflammatory cells present in malignant effusions are believed to be responsible for the pluripotent nature of these malignant effusions (2-5). Ovarian cancer cells and peritoneal mesothelial cells constitutively produce bioactive lipids and cytokines that further promote motogenesis and mitogenesis of ovarian cancer cells in both autocrine and paracrine fashion (6-9). The autocrine secretion of cytokines, peptide growth factors, as well as proteolytic enzymes by malignant cells not only contributes to ascites accumulation but also enhances their survival and dissemination (1, 10-12). Malignant ascites contains inflammatory cells such as macrophages that further contribute to the production of bioactive phospholipids, inflammatory cytokines, and peptide growth factors in the ascitic fluid (13-15). The interplay between different components of ascitic fluid is complex and reflects the tumor-host interactions that support the malignant process. Identifying and dissecting the factors involved in the crosstalk between the components of such microenvironments will not only improve our understanding of the disease but will ultimately enable us to provide better patient care.

Secreted protein, acidic, and rich in cysteine (SPARC) is a matricellular protein involved in the modulation of cell adhesion, motility, and interactions with components of the extracellular matrix (16, 17). Recent reports highlight the role of this molecule as a positive or negative modulator in the pathogenesis of different malignancies (18-24). We and others have shown that SPARC functions as a tumor suppressor in ovarian cancer (25-28). As a tumor suppressor, effect of SPARC in different cancers was attributed not only to its counteradhesive, antiproliferative functions but also to its role in modulating angiogenesis as well as regulation of the production, assembly, and organization of the structural extracellular matrix proteins including type I collagen and fibronectin (23, 25, 29-32). Interestingly, the quality and
quantity of mature collagen bundles reported in tumor stroma were positively correlated with levels of both tumor cell SPARC (32, 33) and host SPARC (19, 25, 34). Thus, it was hypothesized that SPARC-induced changes in the tumor microenvironment were responsible, at least in part, for its anticancer effects (32).

Herein, to assess the role of host SPARC in modulating the ovarian cancer microenvironment, specifically ascitic fluid, we used a syngeneic model of ovarian carcinomatosis in immunocompetent \( SP^{+/+} \) mice by peritoneal implantation of ID8 mouse ovarian cancer cells. The results of our in vitro and in vivo studies have identified SPARC as a novel tumor suppressor that normalizes the ovarian cancer microenvironment, in part, through significant attenuation of the vascular endothelial growth factor (VEGF)-integrin-MMP signaling axis as well as the inflammatory processes that result from, augment, and/or contribute to this axis.

**Results**

**SPARC Inhibits VEGF-Induced Proliferation and Survival of Ovarian Cancer Cells In vitro and In vivo**

VEGF is known to exert an autocrine as well as paracrine mitogenic effect on a variety of tumor cells including melanoma, prostate cancer, and ovarian cancer in vivo (22, 35-38). Our pilot studies indicated that VEGF stimulates the proliferation of ID8 cells in a concentration-dependent manner (data not shown). To investigate the autocrine effect of VEGF and to mimic the human disease, where VEGF is constitutively up-regulated in cancer cells, we used an ID8 cell line stably expressing VEGF164 (ID8-VEGF). This cell line has previously been shown to dramatically accelerate tumor cell growth and survival, tumor angiogenesis, and ascites formation in a syngeneic mouse model of ovarian carcinomatosis (39). ID8-VEGF exhibited higher proliferation rates than ID8 cells (Fig. 1A). In accord with our earlier report (25), the

**FIGURE 1.** SPARC inhibits VEGF-induced survival of ovarian cancer cells in vitro and in vivo. A, Proliferation of ID8 and ID8-VEGF cells was determined by MTS assay. ID8-VEGF cells exhibited significantly higher rates of proliferation than ID8 cells, and both were inhibited by SPARC in a concentration-dependent manner. B, Animals injected intraperitoneally with ID8-VEGF cells exhibited decreased survival rates than those injected with ID8. \( SP^{+/+} \) mice exhibited decreased survival compared to \( SP^{+/+} \) counterparts. C, \( SP^{+/+} \) mice injected with ID8-VEGF developed significantly larger ascites volumes than \( SP^{+/+} \) counterparts. D, \( SP^{+/+} \) mice developed significantly larger ID8-VEGF subcutaneous tumors than \( SP^{+/+} \) counterparts. E, The mean vascular density (MVD) in ID8 and ID8-VEGF tumors in \( SP^{+/+} \) and \( SP^{+/+} \) mice was determined by CD31 immunostaining. Columns, number of CD31-positive vessels counted in five microscopic fields of at least four different tumor samples; bars, SE. \( #, P < 0.05 \), between ID8-VEGF tumors in \( SP^{+/+} \) and \( SP^{+/+} \) mice. \( *, P < 0.05 \), between ID8 and ID8-VEGF tumors in \( SP^{+/+} \) mice.
antiproliferative effect of SPARC on both ID8 and ID8-VEGF was concentration dependent (Fig. 1A). In vivo, all animals injected intraperitoneally with ID8-VEGF cells developed tumors (10 of 10 animals, both SP/C0/C0 and SP+/+) and were associated with decreased survival rate (Fig. 1B) as well as rapid development of ascites and increased ascitic fluid volumes relative to ID8 tumors (Fig. 1C). In the latter, 10 of 10 SP/C0/C0 and 6/10 SP+/+ mice developed intraperitoneal tumors. When injected subcutaneously, ID8-VEGF cells exhibited significantly augmented tumor growth as compared with ID8 cells (Fig. 1D). Tumor growth (Fig. 1D) and vascularization (Fig. 1E) were significantly enhanced in SP/C0/C0 mice relative to SP+/+ controls, as assessed by tumor volume determinations and mean vascular density measurements, respectively. These results indicate that host SPARC antagonizes the mitogenic and proangiogenic effects of VEGF in ovarian cancer cells in vivo as well as in vitro.

Differential Regulation of MMPs and Tissue Inhibitor of Metalloproteinases by Host SPARC

Proteolytic enzymes such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play an active role in ovarian cancer metastasis and have been associated with the release of growth factors, dissemination of tumor antigens, increased cellular invasiveness, metastasis, and suppression of immune surveillance (40-42). A functional interplay between gelatinase activation, VEGF release, and ascites formation in human ovarian carcinoma models has been reported (41). Activated gelatinases, mainly MMP-9, have been shown to increase the release of biologically active VEGF in a time- and concentration-dependent manner in vitro and increase ascites formation in ovarian tumor–bearing mice in vivo (41). Moreover, stromal MMP-9 expression, mainly by tumor-infiltrating macrophages, has been shown to play a critical role in the malignant behavior of ovarian cancers by promoting angiogenesis and tumor

FIGURE 2. SPARC regulates mRNA expression and protein levels of MMPs and TIMPs. A. Reverse transcription-PCR of five representative peritoneal tumors grown in SP−/− and SP+/+ mice showing the differential mRNA expression of MMP-2 and MMP-9 and TIMP-1 and TIMP-2. B. Immunohistochemical staining of SP−/− and SP+/+ peritoneal tumors revealed attenuated levels of MMP-2 and MMP-9 in SP−/− mice concomitant with augmented levels of TIMP-1 and TIMP-2 (magnification, ×400). C. Left, in vitro stimulation of ID8 cells with SPARC (20 μg/mL) for 6 h resulted in significant attenuations in mRNA expression of MMP-2 and MMP-9 as determined by reverse transcription-PCR. Right, relative densitometric analysis of mRNA expression. Representative of three independent experiments. *, P < 0.05, compared with vehicle-treated controls. D. Immunohistochemical staining with murine macrophage marker F4/80 showing the extensive infiltration of peritoneal tumor nodules (i) as well as floating tumor cells (ii) in SP−/− tumors versus SP+/+. Representative of three independent experiments.
growth in mice (43). A reciprocal stimulation of the expression and activity of MMP-2 by VEGF has also been reported in ovarian cancer cell lines (44). Consistent with our previous study (25), ascitic fluid of SP−/− ovarian tumor–bearing mice showed enhanced MMP-2 and MMP-9 proteolytic activity (data not shown). Up-regulated levels of MMP-2 and MMP-9 mRNA in SP−/− tumors were concomitant with the down-regulation of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 levels. Conversely, up-regulated TIMP-1 and TIMP-2 levels in tumors of SP+/+ mice were associated with down-regulation of MMPs (Fig. 2A). Immunostaining of tumor sections also confirmed the increased expression of MMP-2 and MMP-9 and the decreased expression of TIMP-1 and TIMP-2 in SP−/− tumors (Fig. 2B). In vitro, treatment of ID8 cell line with SPARC (20 µg/mL) for 6 h resulted in down-regulation of MMP-2 and MMP-9 mRNA expression (Fig. 2C). However, SPARC treatment did not have a significant effect on regulation of TIMP-1 and TIMP-2 mRNA levels (Fig. 1C). These results indicate that the tumor suppressor effect of SPARC is mediated through both down-regulation of the expression and activity of MMPs and up-regulation of their tissue inhibitors in ID8 tumor cells in vivo and in vitro. The elevated levels and activity of MMPs in SP−/− tumor-bearing mice can also be attributed to the significantly increased tumor-associated and

![Graph A](image)

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**FIGURE 3.** Augmentation of ID8 cell adhesion and invasion by ascitic fluid is VEGF and MMP mediated. **A.** ID8 cells (1 × 10⁶/mL) were preincubated with sFlt2-11 (50 µg/mL), KDR2 inhibitor IV (KDR2 I; 50 μmol/L), VEGF neutralizing antibody (VEGF-Ab; 20 µg/mL), or vehicle control (DMSO) in DMEM-0.4% bovine serum albumin for 30 min before incubation with SP−/− and SP+/+ ascitic fluid (final dilution of ascitic fluid was 1:2 in DMEM). Cells were allowed to adhere to fibronectin-coated wells of 96-well plates for 4 h. Adherent cells were stained and quantified by measuring absorbance (OD) at 590 nm. **B.** ID8 cells were treated exactly the same as in (A), and in the presence of MMP-2/MMP-9 inhibitors (MMP-2/9 I; 50 μmol/L) or with the general MMP inhibitor GM6001 (50 µmol/L) for 30 min before being allowed to invade fibronectin-coated inserts for 5 h. Columns, mean of three independent experiments done in triplicates; bars, SE. *, P < 0.05, compared with vehicle control. #, P < 0.05, between SP−/− and SP+/+. **C.** Basal adhesion of ID8 cells to fibronectin was measured after pretreatment with SPARC (20 µg/mL, 2 h), VEGF neutralizing antibody, Flt2-11, and KDR2 inhibitor IV as described above. Basal fibronectin invasion by ID8 cells after pretreatment with SPARC, VEGF neutralizing antibody, Flt2-11, and KDR2 inhibitor IV was done exactly as described in (A). Basal VEGF-Ab cell adhesion to (E) and invasion of (F) fibronectin were tested as described above. Adhesion or invasion of ID8-VEGF cells treated with vehicle alone (control) was assigned a value of 100%. Adhesion and invasion results are expressed as percent of control. Columns, mean of three independent experiments done in quadruplicates; bars, SE. *, P < 0.05.
tumor-infiltrating macrophages. Interestingly, SP−/− tumor tissues and ascites exhibited a significant increase in macrophage infiltration, as indicated by F4/80 staining relative to their SP+/+ counterparts (Fig. 2D). In accord with our previous study (25), these results were coincident with the up-regulation of the expression of VEGF and its receptors in tumor tissues and VEGF levels and activity in the ascitic fluid of SP−/− mice.

Augmentation of ID8 Cell Adhesion and Invasion by Ascitic Fluid Is VEGF and MMP Mediated

The proinvasive properties of malignant ascites have been shown to be mediated through the interplay of VEGF and MMPs (2, 41, 45-47). Our previous findings that ascitic fluid from SP−/− mice enhanced the invasiveness and, to a lesser extent, the adhesion of ID8 cells (25) led us to investigate the possible mechanisms by which SPARC modulates the interplay of VEGF and MMPs in the milieu of the ascitic fluid. Consistent with our earlier report, the difference between SP+/+ and SP−/− ascitic fluid–induced adhesion was found to be insignificant but markedly inhibited by a VEGF neutralizing antibody, sFlt2-11, and a VEGFR2 (KDR) tyrosine kinase inhibitor, suggesting an autocrine effect of VEGF produced by ID8 cells themselves (Fig. 3A). On the other hand, SP−/− ascitic fluid significantly (>75%) enhanced the invasiveness of ID8 cells compared with SP+/+ ascitic fluid (Fig. 3B).
invasion of fibronectin-coated inserts was not only inhibited significantly by VEGF neutralizing antibody and VEGFR inhibitors but also by MMP inhibitors. These results suggest that the proinvasive and, to a lesser extent, the proadhesive effects of ascitic fluid are mediated through the paracrine effects of VEGF and MMPs. In support of these findings, basal levels of ID8 cell adhesion to and invasion of fibronectin were shown to be significantly attenuated (~35% and 50%, respectively) in the presence of exogenous SPARC (Fig. 3C and D). Inhibitors of VEGF and VEGFR2, but not VEGFR1, showed significant suppression of ID8 cell adhesion (Fig. 3C), whereas inhibitors of VEGF, VEGFR, and MMP all resulted in significant diminutions in ID8 cell invasion (Fig. 3D). These findings suggest an autocrine effect exerted by VEGF that is produced by ID8 cells themselves.

To further investigate the autocrine effect of VEGF, using ID8-VEGF cell line, we found that they exhibited a significant increase in adhesion to fibronectin (~20%) as compared with ID8 controls. This increase in adhesion was significantly inhibited (up to 40%) by exogenous SPARC and inhibitors of VEGFRs but not MMPs (Fig. 3E). Furthermore, ID8-VEGF cells exhibited markedly increased (~37%) fibronectin invasion, relative to ID8 controls, which was significantly (30-70%) inhibited by exogenous SPARC as well as by inhibitors of VEGFRs and MMPs (Fig. 3F).

Modulation of Integrin-Mediated Adhesion and Invasion of ID8 Cells by Host SPARC

It has been shown that adhesion of ovarian cancer cells to the peritoneal mesothelial cells is integrin mediated (48-50). We have previously shown that SPARC significantly inhibits human ovarian cancer cell adhesion to and invasion of extracellular matrix proteins and human mesothelial cells (25), and that these functions were mediated through attenuations of tumor cell surface expression and/or clustering of αv and β3 integrins (28). In the latter study, we found that SPARC inhibited integrin-mediated proliferation of human ovarian cancer cell lines (28). Herein, we show that activation of αv integrin with 2.5 mmol/L MnCl2 significantly stimulated proliferation of ID8 cells plated on fibronectin- and vitronectin-coated wells (~2.2-fold as compared with unstimulated cells, assigned a value of 1; Fig. 4A) as compared with uncoated wells. Exogenous SPARC (10 μg/mL) attenuated MnCl2-induced proliferation by ~1.5-fold. On the other hand, activation of β1 integrin by P4G11 exerted a significant, albeit less pronounced, effect on ID8 proliferation whether they were seeded on uncoated, collagen I–coated, or fibronectin-coated plates. This effect was significantly inhibited by SPARC. These findings led us to investigate whether ascitic fluid–induced ID8 cell adhesion and invasion are also integrin mediated. We found that ascitic fluid–induced adhesion of ID8 cells to fibronectin was significantly suppressed by inhibitors of αv/β3 (LM609), αv/β3 (P1F6), and β1 (P4C10), as well as by RGD peptide (Fig. 4B). However, no significant difference was detected between the proadhesive effects of SP+/− and SP+/+ ascitic fluids. Conversely, the proinvasive effect of SP+/− ascitic fluid was significantly (>70%) higher than that of SP+/+ controls (Fig. 4C). This ascitic fluid–induced invasion of fibronectin was also found to be inhibited by the aforementioned integrin inhibitors. Next, we tested whether SP+/− peritoneal mesothelium supports adhesion of ID8 cells better than SP+/+ controls and whether this adhesion is integrin mediated. Using an ex vivo organ culture system, coinoculation of freshly isolated peritoneal explants, from either diaphragmatic peritoneum or peritoneal lining of the anterior abdominal wall of SP+/− animals, resulted in a significant (~3-fold) increase in the adhesion of ID8-GFP cells relative to their SP+/+ counterparts (Fig. 4D). Therefore, we tested the effect of integrin activation and inhibition on the adhesion of ID8-GFP cells to isolated SP+/− and SP+/+ primary murine peritoneal mesothelial cells in vitro. SP+/− murine peritoneal mesothelial cells consistently showed a significantly increased (25%) basal rate of adhesion to ID8-GFP cells compared with SP+/+ controls (Fig. 4E). Activation of αv and β3 integrins by MnCl2 and P4G11, respectively, resulted in a significant increase in adhesion of ID8-GFP cells over unstimulated controls and was markedly suppressed by function-blocking antibodies against αv/β3 and β1 (Fig. 4E). ID8-GFP adhesion rates to SP+/− murine peritoneal mesothelial cells were consistently (15-25%) higher than SP+/+ control whether integrins were activated by MnCl2 and P4G11 or inhibited by function-blocking antibodies and RGD peptide. These results indicated that although the adhesion of ID8 cells to murine peritoneal mesothelial cells was integrin mediated, the difference in the adhesion rates was correlated with the basal levels, which were mainly attributed to the absence of SPARC in SP+/− murine peritoneal mesothelial cells. At the transcription level, absence of host SPARC had no significant effect on integrin mRNA levels (Fig. 4F) or protein levels (data not shown) in SP+/− and SP+/+ tumors. However, we cannot rule out differences in activation of tumor cell surface integrins due to unavailability of antibodies against mouse-specific, activated integrin subunits. Taken together, these results indicated that the absence of host SPARC augments the adhesion of ID8 cells to extracellular matrix of the peritoneal mesothelium and that the adhesion is also integrin mediated. This integrin-mediated adhesion is independent of mesothelial SPARC but is hypothesized to be due to the effect of soluble SPARC itself on integrin activation and clustering and/or its effects on the soluble factors in ascitic fluid that activate integrins on the cell surface of ID8 cells.

Interplay between VEGF and Integrins Influences Ligand Recognition and the Proinvasive Properties of Ovarian Cancer Cells

VEGF has been reported to activate the key integrins in tumor cells as well as endothelial cells, thus influencing not only angiogenesis but also tumor growth and metastasis (22, 35-37, 51, 52). Ovarian cancer cells synthesize high levels of VEGF, which not only induces tumor angiogenesis but also functions as a permeability factor implicated in the production and maintenance of malignant ascitic fluid, which in turn supports tumor growth and metastasis (2, 47). Having shown that the proadhesive and proinvasive effects of SP+/− and SP+/+ ascitic fluid on ID8 cells are both VEGF and integrin mediated, we investigated whether SPARC itself inhibits integrin activation and/or inhibits integrin activation by VEGF in ID8 cells. We found that ID8 cells exhibited high levels of basal adhesion to fibronectin that was significantly augmented by
integrin activators P4G11 and Mn$^{2+}$ (Fig. 5A). ID8-VEGF exhibited significantly (~40%) higher adhesion to fibronectin than ID8 cells, an increase that was comparable to that of integrin activation. SPARC inhibited both VEGF-mediated and integrin-mediated adhesion to fibronectin by 40% to 50%, respectively (Fig. 5A). To determine the integrins activated by VEGF, we used integrin inhibiting antibodies and RGD peptide. We found that the adhesion of ID8-VEGF cells to fibronectin was inhibited (up to 40%) by function-blocking antibodies to $\alpha_v \beta_3$ (LM609), $\alpha_v \beta_5$ (P1F6), $\beta_1$ (P4C10), as well as RGD peptide.

**A.** ID8 cells were preincubated with SPARC (10 μg/mL) for 2 h before stimulation with P4G11 (10 μg/mL) or MnCl$_2$ (2.5 mmol/L) for 30 min. ID8-VEGF cells were also pretreated with SPARC as mentioned above. Cell suspensions were allowed to bind to fibronectin-coated wells for 4 h. Adhesion of ID8 cells treated with vehicle alone was assigned a value of 100%. Adhesion is expressed as percent change from control untreated ID8 cells, which is assigned a value of 100%. *, $P < 0.05$, versus vehicle- or isotype-stimulated control. **, $P < 0.05$, versus vehicle- or isotype-stimulated ID8 or ID8-VEGF controls.

**B.** ID8-VEGF cells were pretreated with SPARC or integrin inhibitors as described earlier and were plated on fibronectin-coated wells. Adhesion is expressed as percent change from control untreated ID8-VEGF cells, which is assigned a value of 100%. *, $P < 0.05$, versus ID8-VEGF control (C) cells. C. ID8 cells were preincubated with SPARC as in (A) before stimulation with MnCl$_2$ (2.5 mmol/L) for 30 min. ID8-VEGF cells were similarly pretreated with SPARC. Cell suspensions were allowed to bind to vitronectin-coated wells for 4 h. Adhesion is expressed as percent change from vehicle control–treated ID8 cells, which is assigned a value of 100%. *, $P < 0.05$, versus vehicle-treated ID8 control. **, $P < 0.05$, versus vehicle-treated ID8 or ID8-VEGF. D. ID8-VEGF cells were pretreated with SPARC or $\alpha_v$ integrin inhibitors, as described earlier, and were plated on vitronectin-coated wells. Adhesion is expressed as percent change from vehicle control–treated ID8-VEGF cells, which is assigned a value of 100%. *, $P < 0.05$, versus ID8-VEGF cells.

**E.** ID8 cells were preincubated with SPARC as in (A) before stimulation with P4G11 for 30 min. ID8-VEGF cells were similarly pretreated with SPARC. Cell suspensions were allowed to bind to collagen I–coated wells for 4 h. Adhesion is expressed as percent change from control untreated ID8 cells, which is assigned a value of 100%. *, $P < 0.05$, versus isotype control–stimulated ID8 control. **, $P < 0.05$, versus isotype-stimulated ID8 or ID8-VEGF.

**F.** ID8-VEGF cells were pretreated with SPARC or $\beta_1$ integrin inhibitor P4C10, as described earlier, and were then added to collagen I–coated wells. Adhesion is expressed as percent change from vehicle control–treated ID8-VEGF cells, which is assigned a value of 100%. *, $P < 0.05$, versus ID8-VEGF cells.

**FIGURE 5.** SPARC inhibits integrin-mediated and VEGF-enhanced adhesion of ID8 cells. A. ID8 cells were preincubated with SPARC (10 μg/mL) for 2 h before stimulation with P4G11 (10 μg/mL) or MnCl$_2$ (2.5 mmol/L) for 30 min. ID8-VEGF cells were also pretreated with SPARC as mentioned above. Cell suspensions were allowed to bind to fibronectin-coated wells for 4 h. Adhesion of ID8 cells treated with vehicle alone was assigned a value of 100%. Adhesion is expressed as percent change from control untreated ID8 cells, which is assigned a value of 100%. *, $P < 0.05$, versus vehicle- or isotype-stimulated control. **, $P < 0.05$, versus vehicle- or isotype-stimulated ID8 or ID8-VEGF controls. B. ID8-VEGF cells were pretreated with SPARC or integrin inhibitors as described earlier and were plated on fibronectin-coated wells. Adhesion is expressed as percent change from control untreated ID8-VEGF cells, which is assigned a value of 100%. *, $P < 0.05$, versus ID8-VEGF control (C) cells. C. ID8 cells were preincubated with SPARC as in (A) before stimulation with MnCl$_2$ (2.5 mmol/L) for 30 min. ID8-VEGF cells were similarly pretreated with SPARC. Cell suspensions were allowed to bind to vitronectin-coated wells for 4 h. Adhesion is expressed as percent change from vehicle control–treated ID8 cells, which is assigned a value of 100%. *, $P < 0.05$, versus vehicle-tre
peptide (Fig. 5B). To further identify the VEGF-activated integrins, we first used vitronectin, the prototypic ligand of \(\alpha_v\beta_3\), and found that ID8-VEGF exhibited a significantly increased (~50%) adhesion to vitronectin relative to ID8-GFP cells (Fig. 5C) and that the observed increased adhesion is significantly inhibited by LM609 and RGD but not by P1F6 antibodies (Fig. 5D). Next, we used collagen I as a putative ligand for \(\beta_1\) integrins and found that the adhesion of ID8-VEGF was comparable to that of P4G11-activated ID8-GFP cells (Fig. 5E) and was significantly inhibited by P4C10 (Fig. 5F). These data indicate that VEGF regulates adhesion and recognition of extracellular matrix molecules by ovarian cancer cells through activation of \(\alpha_v\) (\(\alpha_v\beta_3\), \(\alpha_v\beta_5\), and possibly \(\alpha_v\beta_1\)) and \(\beta_1\) integrin subunits.

**FIGURE 6.** SPARC inhibits integrin-mediated and VEGF-enhanced ID8 invasion by ID8 cells. ID8 and ID8-VEGF cell lines were treated exactly as described in Fig. 4. ID8 cells treated with SPARC or PBS, in the presence or absence of integrin activators, were added to the upper chamber of fibronectin-coated inserts (A). After 5 h, fibronectin invasion was determined by counting the cells on the undersurface of the inserts. Invasion is expressed as percent change from vehicle- or isotype control antibody–treated ID8 cells, which is assigned a value of 100%. *, \(P < 0.05\), versus ID8 controls. **, \(P < 0.05\), versus matched stimulated ID8 or ID8-VEGF. B, ID8-VEGF cells were treated with SPARC, vehicle, or isotype control antibody (O), or integrin inhibitors, and allowed to invade fibronectin-coated inserts. Invasion is expressed as percent change from control ID8-VEGF cells, which is assigned a value of 100%. *, \(P < 0.05\), versus ID8-VEGF cells.

C. ID8 cells were preincubated with SPARC or PBS in the presence or absence of MnCl₂. ID8-VEGF cells were pretreated with SPARC or PBS and allowed to invade vitronectin-coated inserts. Invasion is expressed as percent change from control ID8 cells, which is assigned a value of 100%. *, \(P < 0.05\), versus untreated ID8 control. **, \(P < 0.05\), versus matched stimulated ID8 or ID8-VEGF.

D. ID8-VEGF cells were pretreated with SPARC, isotype control antibody, or \(\alpha_v\) integrin inhibitors and allowed to invade vitronectin-coated inserts. Vitronectin invasion is expressed as percent change from control ID8-VEGF cells, which is assigned a value of 100%. *, \(P < 0.05\), versus ID8-VEGF cells. E, ID8 cells were preincubated with SPARC in the presence of \(\beta_1\) integrin activator P4G11 or isotype control antibody. ID8-VEGF cells were pretreated with SPARC or PBS and allowed to invade collagen I–coated inserts. Collagen I invasion is expressed as percent change from control untreated ID8 cells, which is assigned a value of 100%. *, \(P < 0.05\), versus isotype-treated ID8 control. **, \(P < 0.05\), versus matched stimulated ID8 or ID8-VEGF. F, ID8-VEGF cells were pretreated with isotype control antibody, SPARC, or \(\beta_1\) integrin inhibitor P4C10, as described earlier, and were added to the upper chambers of collagen I–coated inserts. Invasion is expressed as percent change from control ID8-VEGF cells, which is assigned a value of 100%. *, \(P < 0.05\), versus ID8-VEGF cells.
Next, we tested whether VEGF influences the integrin-mediated invasiveness of ovarian cancer cells. Accordingly, the effect of overexpression of VEGF164 on ID8 cell invasion was assessed. In a transwell system, ID8-VEGF exerted a profound increase in invasion of fibronectin, vitronectin, and collagen I matrices. As shown in Fig. 6A, fibronectin invasion by ID8-VEGF was significantly (>60%) higher than that of ID8-GFP and was comparable to that induced by activating \( \alpha_h \) integrins (using P4G11) and \( \alpha_v \) integrins (using Mn\(^2+\)). This invasion was inhibited substantially (up to 40%) by LM609, RGD peptide, P1F6, P4C10, as well as SPARC (Fig. 6B). To delineate the identity of the activated integrins, we used vitronectin and collagen I as substrates. We found that basal invasion of vitronectin by ID8 cells was significantly increased (>55%) by Mn\(^2+\) and inhibited (>60%) by SPARC (Fig. 6C). ID8-VEGF exhibited a significant (>40%) increase in vitronectin invasion as compared with ID8-GFP. Vitronectin invasion by ID8-VEGF was significantly inhibited (up to 40%) by LM609 and RGD peptide but not by P1F6 (Fig. 6D), suggesting that \( \alpha_v \beta_3 \) is involved in VEGF-mediated invasion of ID8 cells. Similarly, invasion of collagen I by ID8 cells was markedly (~40%) augmented by \( \beta_1 \) integrin activating antibody P4G11, comparable to that of levels found in ID8-VEGF (Fig. 6E). Collagen I invasion by ID8-VEGF was significantly (up to 35%) inhibited by P4C10 and SPARC (Fig. 6F). These results suggest that the constitutive activity of integrins on ID8 cells was induced by a VEGF-dependent autocrine loop through activation of \( \alpha_v \) and \( \alpha_h \) integrins. SPARC attenuated not only the constitutive activity of integrins in tumor cell lines but also their activated levels induced by specific integrin activators and VEGF.

Modulation of the Activity of Bioactive Lipids in the Ascitic Fluid by Host SPARC

We and others have recently shown that biologically active lipids, mainly lysophosphatidic acid (LPA), in the heat-insensitive fraction of mesothelial cell conditioned medium exert a chemotactic and proinvasive effect on human ovarian cancer cells in vitro (9, 27). In the latter study, we also reported that exogenous SPARC significantly inhibited LPA-mediated invasiveness and proliferation of human ovarian cancer cells. These findings prompted us to test whether the proinvasive and

![FIGURE 7. Modulation of the activity of bioactive lipids in the ascitic fluid by host SPARC. Pooled SP\(^{+/+}\) and SP\(^{-/-}\) ascitic fluids with equivalent protein concentration were heated at 95°C for 10 min to inactivate the biologically active peptides and proteins. The effect of heated and unheated samples on fibronectin invasion (A) or migration (B) of ID8 cells was measured as described earlier. The chemotactic effect of heated and unheated SP\(^{+/+}\) and SP\(^{-/-}\) ascitic fluids was tested by adding 600 \( \mu \)L of ascitic fluid to the bottom chamber of fibronectin-coated transwell inserts. ID8 cells (1 \( \times \) 10\(^5\)) were added to the upper chamber of each insert. After 5 h, cells that migrated to the underside of the inserts were stained and counted. *, \( P < 0.05 \), compared with unheated SP\(^{+/+}\) ascitic fluid. **, \( P < 0.05 \), between heated and unheated ascitic fluid.](mcr.aacrjournals.org)}
chemotactic effects of SP+/+ and SP−/− ascitic fluids are mediated by bioactive lipids, especially LPA. We found that the proinvasive activity of ascitic fluids from intraperitoneal tumor–bearing SP+/+ and SP−/− mice was significantly decreased (~23% and 27%, respectively) after heating at 95°C for 10 min (Fig. 7A). A significant (~2-fold) difference in the proinvasive activity was also observed between heated and unheated SP+/+ and SP−/− ascitic fluids. Similarly, we found that the ascitic fluid from SP+/+ mice exerted a significantly more potent chemotactic activity effect on ID8 cells than that of SP+/+ mice (Fig. 7B). As anticipated, heating significantly decreased this chemotactic effect (>33%). Furthermore, the mitogenic effect of ascitic fluids on ID8 cells was also significantly (~35%) inhibited by heating and revealed a statistically significant difference between the proliferative capacities of SP+/+ and SP−/− ascitic fluids (Fig. 7C). These results indicate that bioactive lipids represent ~60% to 65% of the activity of the ascitic fluid. The major identified component of these bioactive lipids was LPA, which influences almost every aspect of ovarian cancer progression (7, 9). In agreement with our recent report (27), LPA was also shown to induce proliferation of ID8 cells directly in a concentration-dependent manner, and addition of exogenous SPARC (10 μmol/L) significantly diminished the proliferation induced by low concentrations (5-10 μmol/L) of LPA but had no significant effect when higher concentrations (>20 μmol/L) of LPA were used (Fig. 7D). These data suggest that the significantly augmented proinvasive, chemotactic, and mitogenic effects of SP−/− ascitic fluid on ID8 cells are mediated, at least in part, by the biologically active lipids in the ascitic fluid, of which LPA is well recognized. Moreover, these pronounced effects contribute to the increased tumor burden in SP−/− mice, which in turn increases LPA production not only from tumor cells but also from mesothelial cells and other stromal components of the ascitic fluid.

SPARC Ameliorates the Peritoneal Tumor–Induced Inflammation

Ovarian cancer cells have been reported to produce cytokines that further promote tumor survival and invasiveness.

**FIGURE 8.** SPARC ameliorates peritoneal tumor–induced inflammation. Determination of the level of MCP-1 (A), 8-isoprostane (B), and IL-6 (C) in SP−/− and SP+/+ ascitic fluids by ELISA. Columns, mean of eight SP−/− and five SP+/+ ascitic fluid samples; bars, SE. *, P < 0.01, compared with SP+/+. D. Chemotactic effect of ascitic fluid on ID8 cells was determined as described in legend of Fig. 7B. IL-6 (50 ng/mL) was added with SP−/− ascitic fluid and IL-6 neutralizing antibody (IL-6 Ab, 50 μg/mL) was preincubated with SP−/− ascitic fluid for 30 min before being added to the bottom chambers. *, P < 0.05, compared with vehicle control–treated cells. **, P < 0.01, compared with SP+/+. E. The proinvasive activity of IL-6 in the ascitic fluid was tested in a fibronectin invasion assay. ID8 cells (1 × 10⁷/100 μL ascitic fluid) were added to the upper chamber of fibronectin-coated inserts in the presence and absence of IL-6 and IL-6 neutralizing antibody as described earlier. The bottom chambers contained DMEM-5% fetal bovine serum. Columns, mean of three independent experiments done in triplicates; bars, SE. *, P < 0.05, compared with control untreated ascitic fluid. **, P < 0.01, compared with the corresponding IL-6 stimulation. F. Cell proliferation of ID8 cells in response to indicated concentrations of IL-6, in the presence and absence of SPARC (10 μg/mL), was assessed by MTS assay. Columns, mean of three independent experiments done in triplicates; bars, SE. *, P < 0.05, compared with control cells that are not stimulated (NS). **, P < 0.05, compared with the corresponding IL-6 stimulation.
High interleukin-6 (IL-6) levels in the ascitic fluid of ovarian cancer patients were correlated with poor prognosis (6). Other studies have highlighted the crucial role of IL-6 in the pathogenesis of ovarian cancer (54, 55). In the present study, we found significantly higher levels of macrophage chemotactrant protein-1 (MCP-1; >2.5-fold) in SP<sup>−/−</sup> ascitic fluid (Fig. 8A), and these levels coincided with the extensive macrophage infiltration in SP<sup>−/−</sup> tumors and ascites shown in Fig. 2D, highlighting a potent anti-inflammatory effect for SPARC. We also found that the levels of arachidonic acid inflammatory mediator, 8-isoprostane, were significantly higher (4.3-fold) in SP<sup>−/−</sup> ascitic fluid compared with SP<sup>+/+</sup> (Fig. 8B), indicating the hypoxic stress in the ascitic fluid microenvironment, which correlates with LPA, tumor burden, as well as inflammation. Consistently, ascitic fluid from SP<sup>−/−</sup> mice contained significantly (~2-fold) higher levels of IL-6 as compared with SP<sup>+/+</sup> fluid (Fig. 8C). These data suggest that SPARC, through down-regulation of MCP-1, decreases tumor infiltration by macrophages, and hence decreases IL-6 and 8-isoprostane proinflammatory mediators in the ascitic fluid. Furthermore, our in vitro data indicated that the augmented chemotactic (Fig. 8D) and proinvasive (Fig. 8E) effects of SP<sup>−/−</sup> over SP<sup>+/+</sup> ascitic fluid were, in part, due to the high IL-6 content, as confirmed by restoration of these activities in SP<sup>+/+</sup> ascitic fluid by exogenous IL-6 and neutralizing them by IL-6 neutralizing antibody in SP<sup>−/−</sup> ascites. Moreover, IL-6 exerted a concentration-dependent increase in SDS polymerase, which was shown to be inhibited by exogenous SPARC (Fig. 8F).

**Discussion**

Of the numerous growth factors involved in the pathogenesis of ovarian cancer, the angiogenic growth factor VEGF plays a central role in the development of exudative ascitic fluid—the hallmark of peritoneal ovarian carcinomatosis (41, 56). Cross talk between VEGF and integrin cell adhesion receptors is believed to play a critical role in modulation of tumor growth, survival, vascularization, invasion, and metastasis (51). The metastatic potential of tumor cells has been correlated with up-regulation of VEGF and VEGF-induced cell-surface expression and clustering of integrins including α<sub>v</sub>β<sub>3</sub>, α<sub>v</sub>β<sub>5</sub>, and β<sub>1</sub> (35-37, 51, 57). Consistent with these reports, our findings in this study provide evidence that (a) the proadhesive and proinvasive effects of ascitic fluid are mediated, in part, through VEGF binding to its cognate receptors and activation of α<sub>v</sub> and β<sub>1</sub> integrins in ovarian cancer cells; (b) host SPARC negatively regulates MMP-2 and MMP-9 levels and activity in peritoneal tumors and ascitic fluid; (c) the enhanced adhesion of ovarian cancer cells to SP<sup>−/−</sup> peritoneal mesothelial cells and their augmented invasion of fibronectin in response to SP<sup>−/−</sup> ascitic fluid are mediated, in part, through the inhibitory effect of SPARC on integrin expression and activation of ovarian cancer cells, either directly or by modulation of levels and/or activity of soluble factors in the ascitic fluid; (d) SPARC inhibits VEGF- and integrin-mediated ovarian cancer cell proliferation in vitro, as well as VEGF-mediated mitogenic and tumorigenic effects in vivo; and (e) host SPARC ameliorates the peritoneal tumor–induced inflammatory response that accompanies ovarian cancer through attenuation of levels and activity of bioactive lipids, IL-6, MCP-1, and 8-isoprostane proinflammatory mediators (Fig. 9).

VEGF has been shown to activate MMP-2 in ascitic fluid during peritoneal dissemination of ovarian cancer (58). Moreover, VEGF-regulated ovarian cancer invasion and migration was recently reported to involve expression and activation of MMP-2, MMP-7, and MMP-9 (59). Conversely, MMP-2 and MMP-9 have been implicated in formation of ascites through induction of the release of VEGF by ovarian carcinoma cells (41), suggesting the existence of a positive autocrine feedback loop between VEGF and MMPs. A link between integrins and the up-regulated MMPs in SP<sup>−/−</sup> ascites may be suggested based on the earlier findings that α<sub>v</sub>β<sub>3</sub> integrin binds MMP-2 on the surface of melanoma and endothelial cells and both colocalize in caveolae and promote tumor growth and angiogenesis in vivo (60). α<sub>v</sub>β<sub>3</sub> integrin also cooperates with MMP-9 by increasing migration of metastatic breast cancer cells, as α<sub>v</sub>β<sub>3</sub> integrin does not adhere to native, intact collagen but it does adhere to collagen that has undergone proteolytic degradation due to exposure of an RGD binding site (61). Furthermore, α<sub>v</sub> and β<sub>1</sub> integrins, through “outside in” and “inside out” integrin signaling, have been reported to induce the transcription of MMP genes in a variety of cancer cell lines, including ovarian cancer (refs. 62, 63 and the references reviewed therein). This well-orchestrated interplay of VEGF-integrin-MMP axis in ascitic fluid favors the proinvasive and migratory phenotype of ovarian cancer cells after temporary ligand recognition and binding. It is therefore plausible to suggest that perturbation of this axis at the transcriptional and posttranscriptional levels by SPARC attenuates ovarian cancer cell motility, invasiveness, and metastasis.
LPA has been identified as an “ovarian cancer promoting factor” because high levels of this bioactive lipid have been reported in both plasma and ascites of ovarian cancer patients with stage I disease, suggesting that LPA promotes early events in ovarian carcinoma dissemination (64). Up-regulation of potent prosurvival, proangiogenic, as well as proinflammatory cytokines and chemokines has been reported downstream of LPA activity in vivo and in vitro (6, 7, 65, 66). LPA is constitutively produced by peritoneal mesothelial cells and has been shown to be crucial for the chemotactic activity of shed ovarian cancer cells (9, 27). Moreover, LPA enhances motility and invasiveness of ovarian cancer cells, in part, through up-regulation and activation of MMPs in ovarian cancer cells (67). Through binding to its cognate receptor, LPA has been shown to synergize with signals induced by β1 integrin and activation of EGF or platelet-derived growth factor receptors to modulate cellular migration (68). Accordingly, our results in the present study suggest that LPA- and VEGF-induced integrin activation may function via convergent signal transduction pathways to promote MMP expression and/or processing, resulting in significant augmentations in the migratory and invasive behavior of ovarian cancer cells. Moreover, the mitogenic and prosurvival effects of LPA have been attributed to its direct effect on cell cycle progression, up-regulation of VEGF, transactivation of receptor tyrosine kinases (e.g., EGFR and VEGFRs), and induction of an inflammatory response (66). Our recent report on significant attenuation of the LPA-mediated crosstalk between mesothelial cells and human ovarian cancer cell lines by exogenous addition or ectopic overexpression of SPARC (27), as well as significant diminutions in proliferation, migration, and invasion of bioactive lipids in ascites of ovarian tumor–bearing SP/C0 mice, highlights the important role of SPARC in suppression of LPA-mediated events in ovarian carcinoma.

In ovarian cancer, tumor cells are not the only source of MMPs and proinflammatory cytokines. Tumor-associated monocytes/macrophages are recruited from peripheral blood monocytes in response to chemokines produced by tumor cells, mainly through the action of MCP-1 (CCL2; refs. 69, 70). As a consequence, tumor-associated monocytes/macrophages undergone marked phenotypic changes with activation of hypoxia-inducible factor 1α, dramatically up-regulating the expression of a large number of genes encoding mitogenic, proangiogenic, and prometastatic cytokines, enzymes, and bioactive lipids (70, 71). Interestingly, in vivo and in vitro studies have shown that VEGF, MMPs, and IL-6 in the tumor microenvironment inhibit the differentiation and maturation of dendritic cells and switch their differentiation toward the macrophage lineage (72, 73). Accordingly, the infiltration of malignant ascitic fluid and solid peritoneal tumors with macrophages in our syngeneic ovarian cancer model was directly correlated with the levels of ascitic fluid VEGF, MMPs, MCP-1, and IL-6. Our results that SP/C0 tumor-bearing mice had higher ascitic fluid MCP-1 and IL-6 levels and more extensive macrophage infiltration than SP/+/+ mice further validate a negative regulatory role for host SPARC in the development of ovarian carcinomatosis. In support of these findings, we have recently shown that IL-6 and MCP-1 production by human ovarian cancer cell lines is enhanced by LPA stimulation and down-regulated by SPARC (27).

In this study, further evidence for the anti-inflammatory role of host SPARC is provided by the dramatic increase in 8-isoprostane (prostaglandin F2α) levels in SP/+/+ ascitic fluid, compared with SP/+/+ counterparts. Isoprostanes, including 8-isoprostane, are produced by the action of cyclooxygenase enzymes (Cox-1 and Cox-2) on arachidonic acid via a free radical–catalyzed mechanism of phospholipid peroxidation, and have been associated with oxidative stress and high levels of LPA (74, 75). LPA has recently been shown to up-regulate Cox-1 and Cox-2 expression in ovarian cancer, with subsequent up-regulation of MMPs and growth factors, enhancing ovarian cancer cell survival and invasiveness (66). Furthermore, in a recent study by Rask et al. (76), the expression of Cox-1 and Cox-2 in ovarian cancer, as well as their downstream prostaglandin E2, was associated with the high grade of ovarian cancer, suggesting an important role in their malignant transformation and progression. The observation that Cox-1 and Cox-2, as well as prostaglandin E2, were expressed in the tumor cells as well as in stroma led the authors to suggest a paracrine signaling mechanism mediated by growth factors, cytokines, and possibly prostaglandins due to the interactions between tumor cells and stromal cells (fibroblasts and immune cells). Moreover, cytosolic phospholipase A2 (cPLA2) has been implicated in LPA production in ascitic fluid and was associated with the invasive metastatic phenotype (77). Recently, it has been reported that under the hypoxic conditions encountered in ovarian cancer ascitic fluid, LPA up-regulates cytosolic phospholipase A2 and augments the metastatic phenotype through a hypoxia-inducible factor 1α–dependent pathway (78). Taken together, we can speculate that the up-regulation of 8-isoprostane in SP/+/+ ascitic fluid is attributed to the activation and up-regulation of cPLA2 and the downstream Cox enzymes by LPA in the hypoxic microenvironment created by the rapidly proliferating tumor cells and other infiltrating inflammatory cells. To the best of our knowledge, this is the first report implicating the involvement of 8-isoprostane (prostaglandin F2α) in the ascitic fluid of ovarian cancer and its down-regulation by host SPARC. The anti-inflammatory effects of SPARC have previously been implicated in SP/+/+ mice as evidenced by decreased leukocyte infiltration in bleomycin-induced peritonitis (79), attenuated 12-O-tetradecanoylphorbol-13-acetate–induced skin inflammation (24), as well as lung and pancreatic tumors, and have been attributed, at least in part, to abundance of less dense, immature collagen fibrils and a more malleable extracellular matrix (19, 31). Lastly, SPARC has also been shown to negatively regulate the availability and/or activity of proinflammatory/angiogenic cytokines including platelet-derived growth factor (80), fibroblast growth factor-2 (81), and VEGF (82). Collectively, these data strongly suggest a negative regulatory role for host SPARC in the inflammatory process that accompanies ovarian cancer.

In summary, we have provided evidence that SPARC, through interference with the VEGF-integrin-MMP axis as well as that of the inflammatory mediators/intermediates in the malignant ascitic fluid, plays a crucial role in the normalization of the reactive microenvironment of peritoneal ovarian carcinomatosis. These findings, in combination with the
established antiproliferative, proapoptotic, and antimetastatic properties of SPARC in ovarian cancer, underscores its therapeutic potential as a promising novel inhibitor of peritoneal ovarian carcinomatosis.

**Materials and Methods**

**Cell Culture**

ID8, a cell line that was derived from spontaneous in vitro malignant transformation of C57BL/6 mouse ovarian surface epithelial cells (83) and was stably transfected with a retroviral vector expressing green fluorescent protein (GFP) or VEGF164 plus GFP (ID8-VEGF; ref. 39), was kindly provided by Dr. George Coukos (Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA). Cells were maintained in DMEM, supplemented with 2 mmol/L L-glutamine (Sigma), 5% fetal bovine serum (Atlanta Biologicals), and 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma).

**Reagents and Antibodies**

SPARC from mouse parietal yolk sac was purchased from Sigma. Bovine osteonecin was purchased from Haematalogic Technologies. Collagen I from rat tail, human plasma fibronectin, and vitronectin were from Sigma. Integrin-activating and integrin-blocking antibodies, rabbit anti-VEGF, rabbit anti-cytokeratin 5, anti-cytokeratin 18, and anti-vimentin antibodies were from Chemicon. Polyclonal antibody, rabbit anti–cytokeratin 5, anti–cytokeratin 18, and integrin-blocking antibodies, rabbit anti-VEGF neutralizing antibody, and vitronectin were from Sigma. Integrin-activating and -inhibiting antibodies were from RayBiotech. All other chemicals were of Sigma. Recombinant murine IL-6 and anti-mouse IL-6 antibodies were from Santa Cruz Biotechnology, Inc. Rat anti-mouse CD31 was from Chemicon. MMP inhibitor and MMP inhibitor from Calbiochem. Lysophosphaticid acid (LPA) and RGD peptides were purchased from Sigma. Recombinant murine IL-6 and anti-mouse IL-6 antibodies were from RayBiotech. All other chemicals were of analytic grade and were purchased from Sigma and Fisher Scientific.

**Mice**

C57BL/6 × 129SvJ SPARC-null (SP−/−) and wild-type (SP+/+) mice (6-8 weeks old) were a kind gift of Dr. Helene Sage (Hope Heart Program, Baranaya Research Institute at Virginia Mason, Seattle, WA). Mice were backcrossed against wild-type C57BL/6 mice for at least six generations before use in tumor studies. All experimental procedures were approved by the Laboratory Animal Services of the Medical College of Georgia and were done in specific pathogen-free facilities.

**In vivo Tumor Generation**

Intraperitoneal and subcutaneous tumors were generated by injection of ID8 and ID8-VEGF cells into SP+/+ and SP−/− mice (n = 10 per group) as previously described (25, 39). Animal survival and tumor development were monitored twice weekly. At the end of the experimental period, animals were sacrificed and ascitic fluid and tumor tissues from the animals were collected and processed as previously reported (25, 39).

**RNA Isolation and Reverse Transcription-PCR**

RNA was isolated from tissues and cells with Trizol reagent (Invitrogen) and was purified with RNasey isolation kit (Qiagen). Total RNA (2 μg) was reverse transcribed using Imprrom II RT enzyme kit (Promega) and cDNA was amplified using Jumpstart Taq polymerase (Sigma) as recommended by the manufacturers. Specific oligonucleotide primers were synthesized according to published sequences (Table 1). PCR products were resolved in 2% agarose gels and were visualized with ethidium bromide staining under UV light. Gel imaging, documentation, and analysis were done with a Kodak Gel Logic 100 imaging system equipped with Kodak D 3.6 software (Eastman-Kodak).

**Immunohistochemistry**

The expression of MMP-2, MMP-9, TIMP-1, TIMP-2, and F4/80 was determined in deparaffinized tumor sections after antigen retrieval with AutoZyme (BioMeda Corp.). Sections were incubated with specific primary antibodies overnight at 4°C, washed with PBS-0.2% Tween 20, and incubated for 1 h with peroxidase-labeled appropriate secondary antibodies (Jackson ImmunoResearch laboratories, Inc.). Sections were then developed with Vectastain ABC Elite kit (Vector Laboratories) and stable 3,3' diaminobenzidine (ResGen), counterstained with hematoxylin, and mounted in Permount (Fisher Scientific). Images were acquired with Leica microscope (DM5000) equipped with a Q-Imaging digital camera. The mean vascular density in tumor sections was determined by immunostaining with anti-CD31 antibody and averaging the

<table>
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<th>Primer Sequence</th>
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<tr>
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<tr>
<td>F: 5'-TGGATATTTGCAATGACAGCC-3'</td>
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<tr>
<td>F: 5'-CCATTTCGAGCAGCGAT-3'</td>
<td>35</td>
</tr>
<tr>
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### Table 1. PCR Primers Used in Semiquantitative Reverse Transcription-PCR


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number of positively stained vessels in five microscopic fields per section of tumors from at least four different animals (n = 4).

**Adhesion Assays**

Adhesion assays were done as previously described (25). Harvested cells were preincubated with and without MMP inhibitors (40 μmol/L), VEGF inhibitors (40 μg/mL), integrin blocking antibodies (10 μg/mL), SPARC (10 μg/mL), or RGD peptides (20 μmol/L) followed by stimulation with VEGF164 (40 ng/mL), integrin activating antibody (5-10 μg/mL), MnCl₂ (1.5 mmol/L), IL-6 (5-50 ng/mL), LPA (5-50 μmol/L), or ascitic fluid. Cell suspensions were then added to ligand-coated plates. After a 4-h incubation at 37°C/5% CO₂, nonadherent cells were washed out and adherent cells were stained with Hemacolor 3 stain (Fisher Scientific) as recommended by the manufacturer. Cell adhesion was determined by measuring the absorbance at 590 nm.

**Isolation of Murine Peritoneal Mesothelial Cells**

Murine peritoneal mesothelial cells were isolated from SP+/+ and SP−/− mice by peritoneal lavage and from collagenase/dispase-digested explants from the mesentery, as previously described (84). The digested tissue was placed into collagen I–coated plates and cultured for 2 days in DMEM containing 12% fetal bovine serum and EGF (20 ng/mL). Mesothelial cells used for these studies (passages 1-2) were confirmed to be positive for the expression of cytokeratin 5, cytokeratin 18, and vimentin, as determined by immunocytochemical staining.

**Adhesion of ID8-GFP Cells to SP+/+ and SP−/− Peritoneal Explants In vitro**

Fresh peritoneal tissues were excised and prepared as previously described (85). Briefly, 6 × 6-mm² peritoneal pieces were dissected from the diaphragmatic peritoneum and the peritoneum lining the anterior abdominal wall and were placed in 24-well plates with the mesothelium! facing upward. ID8-GFP cells (5 × 10⁶/500 μL of DMEM-0.4% bovine serum albumin) were added onto peritoneal pieces in each well. Plates were then incubated for 72 h in a humidified incubator at 37°C/5% CO₂. At the end of the incubation period, peritoneal pieces were rinsed thrice in PBS to remove nonadherent cells. Adherent cells were removed by Q-tips (Fisher Scientific). Cells attached to the underside of the inserts (the invasive cells) were stained with Hemacolor 3 stain and counted under an inverted microscope as recommended by the manufacturer. Cell adhesion was determined by measuring the absorbance at 590 nm.

**Invasion Assays**

Invasion assays were done as previously described (25). Harvested cells were treated exactly as described in adhesion assays and cell suspensions were added to the upper chamber of polycarbonate inserts (8-μm pore size, Corning Costar) pre-coated with fibronectin, collagen I, or vitronectin. After a 5-h incubation at 37°C/5% CO₂, cells in the upper chamber were removed by Q-tips (Fisher Scientific). Cells attached to the underside of the inserts (the invasive cells) were stained with Hemacolor 3 stain and counted under an inverted microscope equipped with DFC 320 digital camera (Leica) under ×200 magnification.

**ELISA**

Mouse IL-6 ELISA kit (RayBiotech), MCP-1 ELISA kit (BD Biosciences), and 8-isoprostanola ELA kits (Cayman Chemical) were used to determine IL-6, MCP-1, and 8-isoprostanes levels in ascitic fluid samples, respectively, according to the manufacturer’s instructions.

**Immunoblotting**

Tumor tissues from SP+/+ and SP−/− animals (n = 4) were homogenized in lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L NaF, 0.5% sodium deoxycholate, 1% NP40, 1 mmol/L Na₂VO₄, and 1% protease inhibitor cocktail mixture] as previously described (25). One hundred micrograms of cell lysates were resolved by 10% SDS-PAGE under nonreducing conditions and transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were then incubated overnight at 4°C with antimouse specific integrin antibodies (Santa Cruz Biotechnology) and monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion) as loading control. Protein detection was carried out with horseradish peroxidase–conjugated secondary antibodies and a SuperSignal West Dura Chemiluminescence kit (Pierce).

**Nonradioactive Cell Proliferation Assay**

CellTiter96 kit (Promega) was used according to the manufacturer’s instructions. The number of proliferating cells was determined colorimetrically by measuring the absorbance at 590 nm (A590) of the dissolved formazan product after the addition of 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) for 3 h.

**Statistical Analysis**

Statistical analysis was done using GraphPad Prism version 3.1 for Windows (GraphPad Software). Survival curves were generated using the Kaplan-Meier method followed by log-rank test for comparison of curves. The concentration-dependent studies were done by one-way ANOVA followed by Newman-Keuls multiple comparison test. All other statistical analyses were determined by Student’s t test. Differences were considered significant at P < 0.05.

**Acknowledgments**

We thank Dr. George Cookos for providing ID8-GFP and ID8-VEGF-GFP cells and Dr. E. Helene Sage for providing SP−/− and SP+/− mice.

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