Macrophage-Derived SPARC Bridges Tumor Cell-Extracellular Matrix Interactions toward Metastasis

Sabina Sangaletti, Emma Di Carlo, Silvia Gariboldi, Silvia Miotti, Barbara Cappetti, Mariella Parenza, Cristiano Rumio, Rolf A. Brekken, Claudia Chiodoni, and Mario P. Colombo

Abstract

Other than genetic imprinting and epithelial to mesenchymal transition, cancer cells need interaction with the nearby stroma toward metastasis. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein known to regulate extracellular matrix (ECM) deposition and cell-ECM interaction. Gene expression profiles associate SPARC to malignant progression. Using reciprocal bone marrow chime-ras between SPARC knockout and wild-type mice, we show that SPARC produced by inflammatory cells is necessary for spontaneous, but not experimental, i.v. metastasis. Macrophage-derived SPARC induces cancer cell migration and enhances their migration to other ECM proteins at least through αvβ3 integrin. Indeed, RNA interference knockdown of β3 integrin expression reduces cell migration in vitro and metastasis in vivo. Together these results show that macrophage-derived SPARC takes part in metastasis, acting at the step of integrin-mediated migration of invasive cells. [Cancer Res 2008;68(21):9050–9]

Introduction

Metastatic cells leaving the primary tumor gain access to blood vessels through processes that require de-adhesion from the tumor mass, invasion into surrounding tissue, and intravasation. Extravasation is then required to seed the secondary organ. To successfully complete this complex cascade, tumor cells require a permissive microenvironment and productive heterotypic interactions with host cell (1, 2). Macrophages can affect the tumor microenvironment by providing trophic factors and promoting tumor cell migration and intravasation (3). Macrophages, recruited by tumor-derived colony-stimulating factor 1 (CSF-1), produce epidermal growth factor (EGF) that activates tumor cell migration (3, 4). Indeed, in many human cancers, macrophage infiltration correlates with poor prognosis (5).

Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40, is a matricellular glycoprotein involved in tissue remodeling and repair that regulates cell-extracellular matrix (ECM) interaction (6). Gene expression profiling applied to breast cancer identified SPARC as a marker of poor prognosis and metastasis (7, 8). In general, SPARC has been associated with advanced breast, head and neck, stomach, and prostate cancers, melanoma, and glioma.

Different results come from studies showing the effect of SPARC produced autonomously by cancer cells. SPARC inhibits proliferation of the MDA-MB-231 carcinoma cell line, whereas its metastatic clone LM2 requires SPARC for lung virulence (9). On the contrary, forced SPARC expression by adenoviral infection in the same cell line results in metastasis inhibition (10).

Colon carcinoma cell lines that down-regulate SPARC expressions acquire resistance to chemotherapy, and addition of recombinant SPARC restores susceptibility to chemotherapy and apoptosis via caspase-8 (11). Accordingly, colon carcinomas negative for SPARC expression are associated with a poor prognosis (12). Similarly, ovarian, lung, and pancreatic carcinomas down-regulate SPARC by promoter methylation during progression (13–15). The reason why SPARC alternatively enhances or halts tumor progression is largely unknown. Complexity may stem from the cell origin and the complex interplay between SPARC produced by tumor and nearby stroma cells. Host-derived SPARC has been described as tumor suppressor for ovarian cancer via modulation of cell surface clustering and expression of αvβ3 integrins, thus altering growth factor–stimulated survival signaling pathways including focal adhesion kinase, mitogen-activated protein kinase, and AKT (16). In this regard, a study on glioma shows that down-regulation of SPARC expression by short interfering RNA decreases tumor cell survival and invasion, reducing focal adhesion kinase and AKT activation (17). In contrast to ovarian cancer, melanoma is not influenced by host-derived SPARC (18).

No better insight comes from mouse studies. In mice lacking SPARC, Lewis lung carcinoma cells grow faster whereas N2C mammary carcinoma cells grow slower in N4 backcrosses to B6 and fully congenic BALB/c knockout (KO) mice, respectively (19, 20). Although these contrasting results have been explained on the basis of the different type of collagen whose proteolysis generates fragments favoring or inhibiting tumor angiogenesis in Lewis lung carcinoma and N2C tumors, respectively, other mechanisms are likely in place. Cell motility is related to collagen density. For example, dendritic cells can move quickly to draining lymph nodes and activate an early immune response because of scarce and loose collagen present in SPARC KO mice (21). With this premise, it might also be possible that tumor cells move easily in the SPARC-null environment, increasing their metastatic potential.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Mario P. Colombo, Immunotherapy and Gene Therapy Unit, Fondazione Istituto di Ricovero e Cura a CarattereScientifico, Istituto Nazionale dei Tumori; Department of Human Morphology, Università degli Studi di Milano, Milan, Italy; and Departments of Surgery and Pharmacology, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas.

doi:10.1158/0008-5472.CAN-08-1327
Few studies have linked SPARC to metastasis. One reported an association between SPARC expression and lung colonization after i.v. injection of breast carcinoma cells (9). Another clearly showed a role of SPARC in promoting prostate carcinoma cell growth into purified bone matrix (22). To study the function of SPARC, in metastasis, we used the 4T1 mammary carcinoma, perhaps the most compelling model of spontaneous metastasis (23). We found that i.v. injection of 4T1 cells into SPARC KO mice resulted in significant lung colonization. In contrast, spontaneous lung metastases from mammary fat pad were reduced in SPARC KO mice compared with wild-type (wt) animals. We investigated the mechanism responsible for such a difference. We report here the characterization of the cell type producing the relevant SPARC and provide evidence that SPARC promotes cell migration and metastasis by modulating integrin-ECM interaction.

**Materials and Methods**

**Cell lines, mice, and tumors.** 4T1 (CRL-2539, LGC-Promochern) is a thioguanine-resistant carcinoma cell line (24). Female BALB/cAnNCrl mice, 8 to 10 wk old, were purchased from Charles River Laboratories. Congenic BALB/c Thy 1.1 mice were kindly provided by H. Levitsky (John Hopkins University, Baltimore, MD). SPARC KO mice on a BALB/c background have been described previously (20). Mice were maintained at Istituto Nazionale Tumori under standard conditions according to institutional guidelines; experiments were authorized by the Institute Ethical Committee for animal use.

Chimeric SPARC**5/C2** (Thy 1.2) > SPARC**5/C1** (Thy 1.1) as well as SPARC**5/C1** (Thy 1.1) > SPARC**5/C2** (Thy 1.2) mice (from here and thereafter indicated as wt > KO and KO > wt) were obtained as described (20). Engraftment was verified 8 wk after bone marrow transplant by staining peripheral blood mononuclear cells with FITC-conjugated mouse anti-mouse Thy 1.1 and phycoerythrin-conjugated mouse anti-mouse Thy 1.2, as well as isotype control FITC- and phycoerythrin-conjugated mouse IgG2a.

SPARC KO and mouse chimeras were injected orthotopically or i.v. with 5 x 10^5 4T1 cells. The lungs and primary tumor were harvested 28 d after tumor cell injection (14 d if injected i.v.) and evaluated by clonogenic assay and histology, respectively. For histology, tumors were embedded in optimal cutting medium, rapidly frozen, and sectioned at 5 µm, or fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned.

**Clonogenic assay.** Lungs were removed, minced, and digested with a collagenase IV/elastase (Worthington) solution for 140 min 4 °C. The suspension was filtered with a cell strainer (BD) and centrifuged. Cells were washed and resuspended in DMEM containing thioguanine (10 mg/mL) and seeded in 100-mm^2 Petri dishes at three dilutions (1/2, 1/10, and 1/100). Colonies were allowed to grow for 2 wk and then fixed with methanol (BDH) and stained with methylene blue.

**Plasmids for short interfering RNA of β3-integrin and cell transfection.** Targets for RNA interference were selected using the prediction software available from Ambion, Inc. The following oligonucleotide sequences were annealed and cloned into the pSilencer 1.0-U6 vector (ambion): 400 forward, 5-GGACAAAATATTTAAAGTAGTTCGGGAGATTTCGAGGTAAGTTTGCCTTTTTT; 400 reverse, 5-AAAAATTAAAAAAGGCCAATCTCCATGAAATTTTCGAGGTAAGTTTGCCTTTTTT; 754 reverse, 5-GGCACTCTGTCTCCTCCTCAAGAGGAGAGAGAGGAGATGCTTTTTTTT; 754 reverse, 5-GGACATCTCTCCTTCCTCAGAGAGGAGAGAGGAGATGCTTTTTTTT; 1381 forward, 5-T CCTTCTCCTCTTCAAGAGGAGAGAGAGGAGATGCTTTTTTTT; 1381 forward, 5-T CCTTCTCCTCTTCAAGAGGAGAGAGAGGAGATGCTTTTTTTT; and 1381 reverse, 5-T CCTTCTCCTCTTCAAGAGGAGAGAGAGGAGATGCTTTTTTTT; and 1381 reverse, 5-T CCTTCTCCTCTTCAAGAGGAGAGAGAGGAGATGCTTTTTTTT. The annealed oligos were cloned into Apatil and EcoRI sites. All plasmids were sequenced to verify accuracy.

For transfection, 4T1 cells were grown to 70% confluence and transfected using Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer’s instructions. To create stable cells, the clones were selected with 600 µg/mL puromycin for 3 wk. The RNA interference effect was tested by flow cytometry for expression of β3-integrin by using a polyclonal rabbit anti-β3-integrin.

**Antibodies, histology, and immunohistochemistry.** The following anti-SPARC antibodies have been used: monoclonal antibody (mAb) 303 and mAb 293 (25), a commercial monoclonal raised in rat (clone 124/413), and a polyclonal raised in goat (R&D Systems Europe). To detect integrin β3, we used a polyclonal rabbit antibody from Abcam (ab15499) and a monoclonal antihuman antibody (cross-reacting with mouse) from e-Bioscience (clone KN52, functional grade endotoxin free) as blocking antibody in migration assays. Also from Abcam were the polyclonal rabbit antibody to N-cadherin (ab12221), the polyclonal goat anti-phosphorylated vascular endothelial growth factor receptor 2 (ab38473), and the mAb to vimentin (clone RV202). α-Smooth muscle actin (α-SMA) mAb (clone 1A4) was from Sigma. From Calbiochem (Merck KGaA) we obtained the mAb to E-cadherin (clone ECD2-2). To detect or block integrin β1 (CD29) we used a mAb from Biolegend (clone HMB1-1, functional grade). To detect integrin β3 (CD61), we used a mAb from Becton Dickinson (clone 2C9-G2). Also from Becton Dickinson was the mAb to CD31 (clone MEC 13.3). To visualize SPARC, paraffin sections were boiled for 5 min in a pressure cooker. Pan-cytokeratin, F4/80, and α-SMA immunostaining required an antigen retrieval in citrate buffer (DakoCytomation; 10 min). Immunostaining of frozen sections was done as previously described (20).

**Double immunofluorescent staining and laser scanning confocal analyses.** Acetone-fixed frozen sections were rehydrated in PBS and incubated for 30 min with the first primary antibody. The sections were washed in PBS and then incubated for 30 min with biotinylated secondary antibody, washed, and incubated with Alexa Fluor 488–conjugated streptavidin (Molecular Probes) for 20 to 30 min. After washing, sections were incubated for 30 min with the second primary antibody, washed again, and incubated for 30 min with biotinylated secondary antibody. After washing, sections were incubated with Alexa Fluor 594–conjugated streptavidin for 20 to 30 min and then washed. Cross-reaction between the first secondary antibody and Alexa Fluor 594 was prevented by saturation of all its binding sites with Alexa Fluor 488. Slides were mounted with Vectashield medium (Vector Laboratories) and examined with a Zeiss LSM 510 Meta laser scanning confocal microscope.

**Flow cytometry.** Flask-cultured 4T1 and 4T1/C2 cells were gently detached and stained for 60 min at room temperature with β3 integrin, CD61, and SPARC antibodies diluted in PBS for cell surface staining or in saponin buffer 0.2% saponin and 0.5% bovine serum albumin (Sigma–Aldrich) for intracellular staining. Isotype-matched IgGs were used as controls. Cells were analyzed with a FACSCalibur (Becton Dickinson).

**Migration assays.** The underside of the Transwell filter inserts (6.5 mm; Costar) were coated with 10 µg/mL of fibronectin (Sigma), multimeric vitronectin from bovine plasma (Oxford Biomedical Research, UK), rat tail collagen type I (Becton Dickinson), and/or 200 ng of mouse SPARC purified from parietal yolk sac (Sigma) or recombinant human SPARC (ProSci) overnight at 4 °C. The inserts were air-dried, and freshly trypsinized 4T1 or 4T1/C2 cells were added in serum-free media to the upper chamber of the transwell insert. The lower chamber was filled with 10% FCS supplemented medium. In some cases, cells were incubated with 5 µg/mL of functional grade endotoxin-free antibodies against αv (Chemicon, CD51, RMV-7 clone), β3 integrin (e-Bioscence, KN52 clone), or SPARC (mAb 303), or with RGD or RGES peptides (Sigma).

Cells were allowed to migrate overnight at 37 °C. Nonmigrated cells were gently removed from the topside of the filter by scrubbing twice with cotton swab moistened with FCS-free medium. Cells on transwell insert were stained with Diff-Quik stain kit (PBI international) and washed with distilled water. Finally filters were removed from the insert and mounted on cover glass. The number of migrated cells was counted under a microscope in 10 randomly chosen high-power fields by two different observers.

**Bone marrow macrophage preparation.** Bone marrow from wt and SPARC KO mice was harvested by flushing femurs and tibias with medium. Cells were resuspended at 2 x 10^7/mL in rCRPMI 1640 (Life Technologies, Inc.) supplemented with 5 ng/mL of macrophage colony-stimulating factor (M-CSF). On day 5 of culture, medium was replaced with fresh medium containing M-CSF. On day 6, adherent cells were harvested andwww.aacrjournals.org

9051


Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2008 American Association for Cancer Research.
phenotypically characterized by mAbs to F4/80 and CD40 at flow cytometry. For in vitro migration experiments, \(5 \times 10^5\) macrophages from wt and SPARC KO were allowed to attach for 2 h at 37°C to the underside face of a 24-mm transwell insert (Costar). 4T1cells (\(5 \times 10^5\)) were labeled with the PKH-26 vital dye, following the manufacturer's instructions (Sigma), and added in serum-free medium to the upper chamber of the transwell insert. The lower chamber was filled with serum-free DMEM added with lipopolysaccharide (LPS; 200 ng/mL; from Sigma) and IFN-γ (500 units/mL; Peprotech). 4T1 cells were allowed to migrate for 24 h, then cells were harvested with trypsin. Harvested cells (macrophages + migrated 4T1 cells) were counted with a hemocytometer. The number of migrated PKH-26+ 4T1 cells was determined as percentage of PKH-26+ cells on total migrated cells by flow cytometry.

Results

SPARC requirement for metastasis. The 4T1 mammary carcinoma cell line, once injected into the mammary fat pad, spreads to several distant organs. 4T1 cells possess 6-thioguanine resistance, and their metastases are easily detected easily by plating dissociated organs under drug selection; resistant colonies are considered representative of individual clonogenic tumor cells. We injected 4T1 carcinoma cell lines into wt and SPARC KO mice. Although the growth of the primary tumor was similar in the two mouse lines (Fig. 1A), clonogenic assay revealed numerous metastases in the lungs of wt but a paucity in the lungs of SPARC KO mice (Fig. 1B). We also observed a significant decrease of 4T1 metastases in liver, lymph nodes, and brain of SPARC KO, but not wt, mice (Supplementary Table S1). Injection of the same cells into the tail vein, however, gave rise to lung metastasis without differences between wt and KO mice either counted in a clonogenic assay at day 14 (Fig. 1C) or as macrometastases at day 28 (data not shown). These results indicate that 4T1 cells require host SPARC to leave the primary tumor but not to seed in distant organs.

Bone marrow–derived SPARC is required for metastasis. Immunohistochemical analysis of 4T1 tumors grown in either wt or SPARC KO mice shows neoplastic cells that express SPARC at low level. Accordingly, in vitro 4T1 cells produce a low amount of SPARC in comparison with other carcinoma cell lines as tested by ELISA (4T1, 20 ng/mL; CT26 colon carcinoma, 177 ng/mL; and N2C mammary carcinoma, 200 ng/mL; Peprotech). By flow cytometry.

![Figure 1](image)

**Figure 1.** SPARC is required for spontaneous metastasis. 4T1 cells were injected into the mammary fat pad (A and B) or i.v. (C) of wt (○) or SPARC KO (■) mice. A, growth of primary tumor following mammary fat pad injection. Points, mean volume \((n = 20)\); bars, SD. B, spontaneous lung metastases from tumors shown in A. Bars, median number of lung colonies. Clonogenic assay was done 28 d after mammary fat pad cell injection. ***, \(P < 0.001\), two-tailed Mann-Whitney test. C, experimental lung colonies after i.v. cell injection. Bars, median number of lung metastasis. Clonogenic assay was done 14 d after i.v. cell injection.
This pattern of expression indicates that cells at the leading edge acquire the mesenchymal phenotype regardless of whether recipient mice produce SPARC. Should the low amount of SPARC produced directly by 4T1 cells be sufficient to induce EMT (27), it is, however, insufficient to move the metastatic process forward.

Bone marrow–derived SPARC regulates fibronectin fiber deposition. A well-known SPARC activity is the regulation of ECM deposition (19, 20), an effect that we analyzed by Masson trichrome, silver staining, or immunohistochemistry. A well-developed or scant stroma characterized 4T1 tumors from wt or SPARC KO mice, respectively. Distinct collagen deposition and a robust fiber network characterized tumors from wt mice, whereas faint collagen and a thin and fragile fiber network characterized tumors from SPARC KO mice (Fig. 4A, a and b). In addition, 4T1 tumors grown in wt > KO chimeras showed a collagen content and a fiber network similar to those of tumors grown in wt mice (Fig. 4A, a and b), whereas tumors from KO > wt chimeras phenocopied those from SPARC KO mice (data not shown). In agreement to the fiber network, fibronectin staining was much more prominent in tumors from wt or wt > KO chimeras than in tumors from SPARC KO or KO > wt chimeras (Fig. 4A–c and data not shown). Additional confocal microscopy analysis shows macrophages spreading and colocalizing with fibronectin fibers in tumors from wt, but not KO, mice. These data suggest that macrophages can be a source of fibronectin in tumors (Supplementary Fig. S1), an extension of what has been shown for fibroblasts (28), which, in the absence of SPARC, cannot organize fibronectin fibers.

SPARC affects cell migration and spreading on fibronectin fibers. Cell-matrix adhesive interactions involving basement membrane components and fibronectin fibers play an active role in tumor cell migration and metastasis (29). To test whether SPARC affects cell migration to fibronectin, we combined fibronectin with different amounts of SPARC before coating them onto the underside face of transwell inserts. We found that SPARC enhanced fibronectin-induced 4T1 cell migration in a dose-dependent manner (Fig. 4B) and that such effect was inhibited by mAbs to CD29 (β₁-integrin), CD51 (α₁-integrin), and integrin β₅ (Fig. 4C), indicating that a fibronectin receptor is likely involved (30, 31). Moreover, SPARC enhanced 4T1 cell adhesion and spreading on
PKH-26+ cells on total number of cells collected form the Migration, tested 24 hours later, and expressed as percentages of labeled with PKH-26 vital dye, were added in the upper chamber. onto the underside face of a transwell insert, and 4T1 cells, In this assay, bone marrow–derived macrophages were seeded SPARC KO macrophages, enhance migration of 4T1 cells we found that macrophages producing SPARC (wt), but not + SPARC (data not shown). Thus, bone marrow–derived SPARC macrophages, in addition to matrix deposition, has a role in inducing 4T1 tumors. We tested whether SPARC produced by macrophages, in addition to matrix deposition, has a role in inducing 4T1 cell migration. By using a modified in vitro transwell assay, we found that macrophages producing SPARC (wt), but not SPARC KO macrophages, enhance migration of 4T1 cells in vitro. In this assay, bone marrow–derived macrophages were seeded onto the underside face of a transwell insert, and 4T1 cells, labeled with PKH-26 vital dye, were added in the upper chamber. Migration, tested 24 hours later, and expressed as percentages of PKH-26+ cells on total number of cells collected form the underside transwell, was significantly higher when macrophages were from wt mice, and the difference between wt and KO was even greater if macrophages were preactivated with LPS plus IFNγ (Fig. 5A), a stimulus that increases the production of SPARC as well as other proinflammatory cytokines. Accordingly to a role of SPARC in migration, a mAb to SPARC (mAb 303) significantly reduced the boost of migration induced by LPS + IFNγ on wt macrophages, but was ineffective on the same boost on KO macrophages. No migration was detected in the absence of macrophages, whereas the limited but significant migration toward SPARC KO macrophages indicates that SPARC works in addition to other macrophage-derived factors in inducing cancer cell migration (3).

To test the distinct role of SPARC in inducing 4T1 cell migration, purified murine SPARC (or rhSPARC) was coated onto the underside face of transwell or given as soluble protein. Figure 5B shows that coated, but not soluble, SPARC induces haptotactic migration of 4T1 cells in a dose-dependent manner. Haptotactic migration requires integrin engagement (32). SPARC has been described to mediate prostate cancer cell adhesion to bone matrix through αvβ3 and αvβ5 integrins (22). In our hands, SPARC-induced migration was inhibited by the addition of a specific mAb against αv integrin (CD51) or RGD peptide but not by mAb to β3 integrin (CD29) or control RGES peptide (Fig. 5C), suggesting that 4T1 cells respond to SPARC through an αv integrin that is not β3. Because SPARC does not contain a canonical RGD sequence, it is possible that such effect could be mediated through the interaction between SPARC and a β3 or β5 integrin ligand directly produced by the tumor cells, like vitronectin. Accordingly, SPARC enhanced the migration of 4T1 cells to multimeric vitronectin (Fig. 5D). Differently from fibronectin, tumors from wt and KO mice show a similar vitronectin content, thus excluding the possibility that a different vitronectin deposition determines the metastatic phenotype (data not shown).

Effect of β3-integrin silencing on 4T1 cell migration and metastasis. The above experiments suggested αvβ3 and αvβ5 integrins as candidate target for a direct SPARC activity. We analyzed their expression in 4T1 cells and tumors. Although 4T1 cells express both β3 and β5 integrins at the mRNA (data not shown) and protein (flow cytometry) levels in vitro (Fig. 6A), the expression of β3 integrin in vivo was undetectable by immunohistochemistry on tumor cells being restricted to blood vessels (Fig. 6B). On the contrary, a mAb to β3 integrin stained 4T1 cells (Fig. 6B), thus suggesting the silencing of β3 integrin as an approach to test its function in SPARC-induced migration of 4T1 cells. Expression plasmids containing three different putative β3- RNA interference were cotransfected with a neo resistance gene,
and the best stable β3-silenced 4T1 cell clones (754sil and 1381sil; Fig. 6B) were selected for further in vivo and in vitro experiments. Silenced clones were tested for migration toward SPARC (Fig. 6C) and for their metastatic capacity into wt mice (Fig. 6D). We found that both the migration toward SPARC and the number of lung metastasis of 754sil and 1381sil cells were reduced compared with control nonsilenced cells (400sil, 4T1empty, and parental 4T1 cells). In addition, migration to multimeric vitronectin, which specifically binds β5 integrin, was reduced in β3-silenced cells (754sil) but not in parental 4T1 cells (data not shown). Confirming the role of β5 integrin as a SPARC counter-receptor, a blocking antibody to β5 integrin (clone KN52) inhibited 4T1 cell migration toward wt macrophages, as well as antibodies to αv (CD51) or SPARC (mAb 303). Accordingly, the migration of 4T1β5sil cells (754sil) to wt macrophages was as low as that of parental 4T1 cells to SPARC KO macrophages, indicating that SPARC-independent macrophage-induced migration does not require β5 integrin (Fig. 6E).

Based on the above-described experiments, we reasoned that SPARC affects metastasis in two ways. One is by regulation of ECM fiber deposition and promotion of their interaction with the cognate receptors (αvβ1 and αvβ3), and the other is by a direct promigratory effect via the β3-integrin. As shown in Fig. 4, bone marrow–derived SPARC reverts the paucity of fibronectin fibers in recipient KO mice and offers the possibility of testing the role of β3 integrin in metastasis by injecting 4T1β3sil cells into wt > KO chimeras. In this setting, 4T1β3sil (754sil) produced less metastasis than parental 4T1 cells injected into the same recipient, indicating that the promigratory signals transduced by β3 integrin have a true role in metastasis (Fig. 6F).

Impaired metastasis was not because of reduced cell proliferation or primary tumor take of silenced clones because both

Figure 4. SPARC modulation of ECM deposition. A, Masson trichrome (a) and silver staining (b) of 4T1 tumors from wt, SPARC KO, and wt > KO chimeras. c, immunohistochemical analysis of fibronectin (FN) in tumors from wt, SPARC KO, and wt > KO chimeras showing a paucity of fibronectin fibers in tumors from SPARC KO mice. B, SPARC synergizes with fibronectin in inducing 4T1 cell migration (B) and such effect is inhibited by mAbs to αv, α1, and β3 integrins (C).
proliferation (in vitro) and tumor size (in vivo) were not different from those of parental cells (data not shown).

Discussion

Here we have shown that SPARC produced by host cells, most likely macrophages, is an important factor in spontaneous 4T1 mammary carcinoma cell metastasis. The lack of host SPARC has no role in experimental lung metastasis (i.v. injection) nor does it have a role on EMT. Reciprocal bone marrow chimeras between wt and SPARC KO mice and immunolocalization experiments established that macrophages of donor origin are the source of SPARC. In vitro migration assays showed that macrophages induce 4T1 cell migration especially if SPARC is produced. We provided evidence that SPARC favors fibronectin and vitronectin interaction with 4T1 cells through integrins. Silencing experiments indicate that the β5 integrin was needed for in vitro migration and partially for metastasis in vivo.

The low constitutive SPARC expression in 4T1 cells marks the difference from that produced by macrophages. A cell-autonomous function of SPARC is mostly unknown; nevertheless, SPARC can be found in the nucleus (33), and addition of exogenous SPARC results in phosphorylation of β-catenin (34). Furthermore, SPARC through the interaction with cyclin D1 can inhibit cell proliferation (35), thus explaining why many tumor cells down-modulate SPARC expression by promoter hypermethylation (15). Indeed, forced SPARC expression, by gene transduction, in 4T1 cells reduces cell proliferation in vitro and impairs tumor formation in vivo, rendering the assay on metastasis unfeasible (data not shown). Accordingly, doxycycline-induced SPARC expression in the human breast cancer cell line MDA-MB-231 results in growth inhibition (36), and adenoviral-mediated SPARC gene transfer into the same

Figure 5. SPARC induces 4T1 cell migration. A, migration of PKH-26 labeled 4T1 cells in response to macrophages from wt and SPARC KO mice treated or not with LPS plus IFNγ. Migration to both wt and KO macrophages is enhanced by LPS + IFNγ but only the increased migration to wt macrophages is inhibited, in part, by a mAb to SPARC (mAb 303). B, dose-dependent SPARC-induced 4T1 cell migration. Different doses of purified murine SPARC were coated onto the underside face of the transwell insert. 4T1 cells were added to the upper chamber of the transwell, and nonmigrated cells were removed after overnight incubation. Migrated cells were counted on the underside of filters with a microscope in 10 randomly chosen fields. Columns, mean number of cells per field; bars, SD.
Figure 6. 4T1 cells express \( \beta_3 \) integrin, which is necessary for cell migration to SPARC and for in vivo metastasis. A, the level of expression of \( \beta_3 \) integrin (CD61) and \( \beta_5 \) integrin by 4T1 cells cultured in vitro was determined by flow cytometry. Blue, binding of the isotype-matched control antibody; green, target antibody binding. B, expression of \( \beta_3 \) and \( \beta_5 \) integrins on 4T1 tumor sections. Expression of \( \beta_3 \) integrin was confined to cells with endothelial morphology that coexpress CD31 (data not shown). On the contrary, almost all tumor parenchyma stained positive for \( \beta_5 \) integrin. \( \text{Bar, } 100 \text{ µm.} \) C, migration of 4T1\( \beta_5 \)-integrin–stable silenced 4T1 clones (754\( \beta_5 \) and 1381\( \beta_5 \)), obtained from two different target sequences, in response to SPARC. Columns, mean number of migrating cells; bars, SD. D, reduced spontaneous lung metastasis by \( \beta_5 \) integrin silencing. Stable silenced 754\( \beta_5 \) and 1381\( \beta_5 \) were compared with a nonsilenced clone (400\( \beta_5 \); blue dots), empty vector–transduced (open dots), and parental 4T1 (black dots) cells. E, inhibition of 4T1 cell migration in response to wt macrophages depends on \( \beta_5 \) integrin. Addition of a mAb blocking \( \beta_5 \) integrin inhibited 4T1 cell migration to the same extent as antibodies blocking \( \alpha_v \) (CD51) or SPARC (mAb 303). Accordingly, 4T1\( \beta_5 \) migrated to wt macrophages as 4T1 cells to SPARC KO macrophages. \( \text{F, spontaneous lung metastasis of 4T1\( \beta_5 \) and parental 4T1 cells injected into wt > KO and KO > wt bone marrow chimeras. Bars, median number of lung colonies. ***, } P < 0.001, \text{two-tailed Mann-Whitney } t \text{ test. Representative of three independent experiments.} \)
cells reduced their invasive capacity and metastatic behavior (10). On the other hand, gene expression profile and RNA interference silencing pointed to SPARC as a key gene involved in the metastasis of MDA-MB-231 cells and clone variants (9). This discrepancy likely reflects the differences between cell-based (in vitro) and tissue-based (in vivo) analyses. Stroma cells are likely the most relevant source of SPARC in the tumor microenvironment; acting on the cell surface, SPARC might provide different signals to the tumor either directly or through bystander molecules (see below). Accordingly, leukocyte-derived rather than tumor-derived SPARC influenced the stroma density, composition, and outgrowth of N2C mammary carcinoma cell line (20).

Embedded in the ECM, macrophages are often the most represented cell type in tumor stroma (5). In addition to fostering tumor growth, macrophages have long been implicated in metastasis through several mechanisms (3).

4T1 tumors are largely infiltrated by SPARC-producing macrophages. Our data indicate that SPARC produced by macrophages is necessary to advance the metastatic process without affecting EMT. In vitro assay shows that macrophages from wt mice induce 4T1 cell migration more efficiently than macrophages from SPARC KO mice, a difference that is nullified by the addition of a mAb to SPARC (mAb 303). Stimulation with LPS and IFNy similarly activates macrophages from the two strains, in terms of NO production and CD40 up-regulation, as well as increases 4T1 cell migration. The increased migration to SPARC KO activated macrophages was not inhibited by the mAb 303, suggesting that SPARC works in addition to other macrophage-derived factors in inducing tumor cell migration.

The best described role of macrophages in tumor cell migration and intravasation involves a loop in which tumor cells producing CSF-1 recruit macrophages, which in turn secrete EGF, promoting carcinoma cell migration along ECM fibers (4). Here, we have described a new and alternative mechanism of macrophage-tumor cell interaction that involves a matricellular protein (SPARC) able to affect both tumor cell migration and fiber availability toward metastasis.

It is well recognized that ECM is not a merely scaffold for tumor cell but provides signals fostering cell growth, survival, and motility (37). One of the SPARC-associated functions is to promote ECM deposition. Indeed, the absence of SPARC has been associated with decreased collagen deposition (19, 20) and fibronectin production (28). Fibronectin has a role in cell migration during embryonic development, wound healing, and malignancy (29, 38). In addition to acting as a substrate, fibronectin can be cleaved to provide bioactive fragments with chemotactic activity (39). Thus, reduced metastasis in the SPARC KO host can, in part, be explained by the paucity of fibronectin.

Tumor cell migration to ECM component is multifaceted and depends primarily on the reciprocal amount of integrins, their ligands, and affinity. At low ligand concentration, cells are rounded and unable to move (40). This condition likely mimics the absence of host SPARC. On the contrary, at high ligand concentration, cells are very spread while remaining static because of excessive adhesion. In three-dimensional systems, matrix stiffness is also a critical factor. Cells with amoeboid migration, such as dendritic cells and T lymphocytes, move independently from integrin-mediated focal contact while they are affected by matrix hindrance and stiffness. Accordingly, these immune cells move faster in SPARC KO mice, characterized by a loose collagen structure (21). In sharp contrast, tumor cell movement requires a traction force generated by integrin-ECM ligand interactions (41). In this setting, SPARC has a role in regulating ECM-ligand availability (20, 28) and their interaction with cognate integrin receptors.

The complete loss of integrin-mediated cell-ECM contacts induces anoikis (42). In our hands, physiologic amounts of SPARC (nanogram range) similar to those produced by macrophages induced migration without changing the adhesion to other ECM molecules. To completely detach cells from substrate, a higher amount of SPARC (>20 µg/mL) is required (data not shown; ref. 43). This seems to be compatible with the intermediate state of adhesion that allows cell migration (44). These data indicate the importance of a tight control of SPARC expression by cancer/stroma cells. Moreover, the fact that only coated, but not soluble, SPARC supports 4T1 cell migration further confirms that this effect is mediated by integrin receptors according to the notion that an integrin ligand presented in soluble form is not able to induce the recruitment of focal adhesion kinase complexes and cytoskeleton reorganization (45). For instance, plasma-born ECM proteins fibronectin and vitronectin do not work as integrin ligands in their native soluble form but have to undergo conformational changes associated with fiber deposition and denaturation, respectively, to expose integrin binding sites (46, 47). We have identified β3 integrin as a potential SPARC receptor. In addition, SPARC binds to fibronectin present in the ECM and modulates its promigratory signal through its receptors (αvβ1 and αvβ3). RGD peptide blocks 4T1 cell migration to SPARC coated onto a transwell inserts, although SPARC has no RGD sequence. This suggests that SPARC effect requires a canonical integrin ligand, which, in this setting, might be vitronectin provided by 4T1 cells. Accordingly, cell migration induced by multimeric vitronectin is enhanced by the addition of SPARC.

Differently from breast cancer, ovarian carcinoma down-regulates SPARC expression during progression. However, data obtained by immunohistochemistry on tissue specimens indicate that SPARC is produced by the reactive stroma of invasive human ovarian carcinomas and lymph node metastases (48, 49), according to our view of stroma-derived SPARC fostering a permissive environment for cell migration.

The type and amount of integrin receptors expressed on the cell surface and their association with growth factor receptor might help to explain the different effects of SPARC in mammary versus ovarian carcinomas. It has been described that αvβ3 integrin triggers an apoptotic rather than a prosurvival signal, depending on the integrin/ligand ratio. If the ligand level is low or in the presence of ligand antagonist, αvβ3 integrin induces apoptosis (45). Moreover, accordingly to our observation of negligible β3 expression on 4T1 cells, this classic fibronectin/vitronectin receptor has been reported to be absent from most human breast cancer cells and compensated with the presence of αvβ1 and αvβ5 as alternative receptors (50).

In summary, we provide evidence that SPARC is a factor that, once produced by bone marrow host cells, creates an environment that facilitates tumor cell metastasis mainly by modulating cell-matrix adhesive properties. The presence of EMT in tumors from SPARC KO mice has suggested that SPARC effect on metastasis is after EMT and located at the step of the integrin-mediated migration of the cells. This is supported by the fact that in the absence of SPARC, metastasis failed to occur both because of the reduced deposition of fibers and because of its effect on 4T1 cell migration to fibronectin or multimeric vitronectin. We cannot
References


34. Tomanini BR, Mosher DF. Conformational states of α5β1 integrin: preferential expression of an antителopeptide when vitronectin is covalently and noncovalently complexed with thrombin-antithrombin III or coated with tissue. Blood 1989;73:903–12.


Macrophage-Derived SPARC Bridges Tumor Cell-Extracellular Matrix Interactions toward Metastasis

Sabina Sangaletti, Emma Di Carlo, Silvia Gariboldi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/21/9050

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/10/28/68.21.9050.DC1

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/21/9050.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/68/21/9050.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.