Tissue-resident stem cells promote breast cancer growth and metastasis

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Mesenchymal stem cells derived from bone marrow have recently been described to localize to breast carcinomas and to integrate into the tumor-associated stroma. In the present study, we investigated whether adipose tissue-derived stem cells (ASCs) could play a role in tumor growth and invasion. Compared with bone marrow-derived cells, ASCs as tissue-resident stem cells are locally adjacent to breast cancer cells and may interact with tumor cells directly. Here, we demonstrate that ASCs cause the cancer to grow significantly faster when added to a murine breast cancer 4T1 cell line. We further show that breast cancer cells enhance the secretion of stromal cell-derived factor-1 from ASCs, which then acts in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis. The tumor-promoting effect of ASCs was abolished by knockdown of the chemokine C-X-C receptor 4 in 4T1 tumor cells. We demonstrated that ASCs home to tumor site and promote tumor growth not only when co-injected locally but also when injected intravenously. Furthermore, we demonstrated that ASCs incorporate into tumor vessels and differentiates into endothelial cells. The tumor-promoting effect of tissue-resident stem cells was also tested and validated using a human breast cancer line MDA-MB-231 cells and human adipose tissue-derived stem cells. Our findings indicate that the interaction of local tissue-resident stem cells with tumor stem cells plays an important role in tumor growth and metastasis.

Introduction

It has been increasingly recognized that cancer cells actively recruit stromal cells into the tumors and that this recruitment is essential for the generation of a microenvironment that promotes tumor growth (1–4). The presence of a large number of myofibroblasts is apparent in the stromal compartment of invasive human breast cancers but not found in the stroma of normal breast tissue (5). Myofibroblasts are stromal fibroblasts with features of both myofibroblasts [e.g. expression of smooth muscle actin (SMA)] and fibroblasts that have been implicated in breast cancer invasion, extracellular matrix remodeling, wound healing and chronic inflammation (6). The cell type of origin of myofibroblasts is not conclusively established. Isolation of various stromal and epithelial cells from breast tumors and their coculturing

Abbreviations: ASC, adipose tissue-derived stem cell; CT, computed tomography; CXCR, chemokine C-X-C receptor; EC, endothelial cell; FBS, fetal bovine serum; GFP, green fluorescent protein; i.v., intravenously; mASC, mesenchymal stem cell; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1, stromal cell-derived factor-1; SMA, smooth muscle actin; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

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in vitro demonstrated that cancer epithelial cells can induce the expression of myofibroblast markers in a subset of fibroblasts (7). However, the finding that only a small fraction of fibroblasts were transformed into myofibroblasts (7) raises the question of whether myofibroblasts could be derived from specific stem cells that are recruited by cancer epithelial cells from either bone marrow or locally in the adjacent breast adipose tissue. Recent data both in animal models and human breast tumors support the hypothesis that at least a subset of cancer-associated myofibroblasts is derived from circulating bone marrow-derived cells (8,9). It has been shown that adipose tissue also contains pluripotent mesenchymal stem cells (10). In the present study, we set out to determine whether and how adipose tissue-derived stem cells (ASCs) could be involved in promoting tumor growth and metastasis.

Materials and methods

Cell lines and cell culture techniques

4T1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium from Cellgro (Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. 4T1 mammospheres have been selected by culturing in a defined serum-free medium (Dulbecco’s modified Eagle’s medium—F12) that is supplemented with 2% B-27 (Invitrogen, Carlsbad, CA), 40 ng/ml recombinant human fibroblast growth factor basic (bFGF) (Chemicon, Billericia, MA), 100 ng/ml throrbin (R&D Systems, Minneapolis, MN), 20 ng/ml epidermal growth factor, 1 mM 2-mercaptoethanol, 1 ng/ml leukemia inhibiting factor and 1% insulin transferrin sodium selenite (ITS) (Sigma, St. Louis, MO).

Isolation of murine adipose tissue-derived stem cell

Ten Balb/c mice were killed by cervical dislocation. Perirenal, pelvic and subcutaneous fat tissue were dissected from mice, washed in phosphate-buffered saline and immediately processed. After mincing the tissue in pieces <2 mm³, serum-free α-modified Eagle medium (1 ml/1 g tissue) and 2 U/l tissue Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, IN) was added and incubated under continuous shaking at 37°C for 45 min. The digested tissue was sequentially filtered through 100 and 40 μm filters (Fisher Scientific, Pittsburgh, PA) and centrifuged at 450g for 10 min. The supernatant containing adipocytes and debris was discarded, and the pelleted cells were washed twice with Hank’s balanced salt solution (Cellgro) and finally resuspended in growth media. Growth media contained alpha-modification of Eagle’s medium (Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro) and 100 μg/ml penicillin with 100 μg/ml streptomycin (Cellgro). Plastic-adherent cells were then grown in Nunculture cell vials (Nunc, Rochester, NY) at 37°C in a humidified atmosphere containing 5% CO₂ followed by daily washes to remove red blood cells and non-attached cells. After 80% confluence of passage 0, cells were seeded at a density of 3000 cells/cm² (passage 1).

Migration assay

The Fluoroblok Transwell Migration System (BD Biosciences, Bedford, MA) with 3 μm pore size was used for migration experiments. 4T1 cells (3 × 10⁴) were plated in the upper chamber of a 3 μm transwell system. The lower chamber was filled with 1 ml of 72 h conditioned medium of mASC. After 24 h of migration through the transwell membrane, cells were fixed and stained with calcine. For blocking of CXCR4, a neutralization antibody was used (R&D Systems) at a concentration of 30 μg/ml 1 h before and during the migration assay.

Invasion assay

The chemo invasion assay was performed using Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel in 24-well dishes (BD Biosciences) as described previously (1). The 4T1 and ASCs (3 × 10⁴ cells and 6 × 10⁴ cells, respectively) were placed in 600 μl of 5% FBS containing modified Eagle medium in the upper chamber, and 750 μl of the same medium containing 10% FBS was placed in the lower chamber. The plates were incubated for 48 h at 37°C in 5% CO₂. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with phosphate-buffered saline. Cells on the underside of the filters were

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examined and counted under a microscope. Green fluorescent cell signal of green fluorescent protein (GFP)-labeled 4T1 cells was counted in five randomly chosen view fields at a 5-fold magnification of every insert. Each experiment was repeated at least three times.

**mASC GFP labeling**

Stable GFP labeling was performed with a third-generation lentivirus system as described previously by our group (11).

**CXCR4 knockdown**

CXCR4 expression was diminished in 4T1 cells by transfection with a third-generation lentivirus containing short hairpin RNA construct against CXCR4 coding sequence as described previously by our group (12). Primers used for the detection of CXCR4 were 5'-TCCAA-CAGGAACCCGCTTTC-3' (forward primer) and 5'-TTGCGGACTAT-GCCAGTCAAG-3' (reverse primer) (amplicon 122 bp) and for GAPDH 5'-TGGCAAGTGGAGATTGTGCC-3' (forward primer) and 5'-AAGAT-GTGATGAGGCCCTCCG-3' (reverse primer) (160 bp).

**Enzyme-linked immunosorbent assay for stromal cell-derived factor-1/vascular endothelial growth factor**

The enzyme-linked immunosorbent assay was performed using the mouse CXCL12/SDF-1 and VEGF Quantikine Kit (R&D Systems) as per the manufacturer's instructions. Medium was conditioned over 72 h, centrifuged and filtered through a 0.45 μm Steriflip Filter Unit (Millipore, Billerica, MA). The amount of stromal cell-derived factor-1 (SDF-1) was normalized to DNA content (nanogram SDF-1 per microgram DNA). DNA isolation was performed using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following manual instructions.

**Western blot analysis**

Cells were lysed in radial immunoprecipitation assay lysis buffer (Upstate, Billerica, MA), including protease inhibitor cocktail (Roche Diagnostics), and western blot was performed as we have described previously (13).

**Animal experiments**

Thirty nude male Balb/c mice in the age of 6–8 weeks were injected with 5 × 10^4 4T1 spheroid-forming cells subcutaneously into the inguinal mammary fat pad number four. Tumor measurements every fifth day was carried out by caliper. At day 21, post-injection mice were scanned by high-resolution micro-computed tomography (CT) under anesthesia with 2–3% isoflurane.

For SDF-1 measurements in mASCs of cancer mice, fat tissue around the primary tumor was minced quickly with scalpels into fragments <2 mm. Digestion into single-cell suspension was performed using an enzyme mix containing 12 500 U collagenase II and IV (Sigma–Aldrich) in 20 ml serum-free media under 37°C for 35 min. Suspension was then filtered through a 70 μm nylon mesh and red blood cells were finally removed by centrifugation with Ficoll for 10 min at 300g. Fluorescence-activated cell sorting for GFP-positive cells was performed immediately afterward without any further in vitro culture.

**Flow cytometry**

Cells were incubated with antibodies against von Willebrand Factor (vWF) and alpha-SMA and the percentage of positive cells analyzed by flow cytometry as described previously by our group (14).

**MicroCT imaging**

CT scans were made using GE Healthcare Enhanced Vision System. Scan parameters: tube voltage 80 kV, anode current 470 μA, number of views 360, exposure time 600 ms, detector bin mode 2 × 2, effective pixel size 0.046 mm. Fenestra™ vascular contrast solution (ART Advanced Research Technologies, Alfred Nobel, St. Laurent, QC, Canada) was injected into tail veins 2 h prior to CT scans. Anesthesia was induced using 5% isoflurane and maintained with 1–2% isoflurane. Image review was performed at a Vitrea® workstation (Vital Images, Minnetonka, MN). The size of the primary tumor was obtained in x, y, and z axis. Calculation of the tumor volume was performed by employing the spherical volume equation \( V = \frac{4}{3} \pi r_x r_y r_z \). Vascularinity of the tumors was assessed by measuring the average Hounsfield units of a region of interest (ROI) that was always excluding the central necrosis if present. As an intraindividual reference, the density (Hounsfield unit) of a ROI of thigh musculature was obtained.

**Immunohistochemical staining and capillary density**

Immunostaining was performed using tumor sections using antibody against vWF and vascular density was determined as described previously by our group (15).

**Results**

**Isolation and characterization of 4T1 mammospheres**

Spheroid-forming cells are cancer-initiating cells. Cancer-initiating cells have been found in both breast cancer lesions and from an established breast carcinoma cell line (16–18). In an elegant study, human mammary epithelial cells were used to generate non-adherent spheroids (designated mammospheres) in cell culture and demonstrate the presence of the three mammary cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell (19). To enrich the small subpopulation of cancer-initiating cells, we used the selection method developed in our lab by culturing the 4T1 cells in a special serum-free medium (containing bFGF/epidermal growth factor/leukemia inhibiting factor) for 7 days. The selection method is based on the fact that the condition of the medium allows cancer progenitor cells (also called cancer-initiating or cancer stem cells) to form spheroids but does not allow regular cancer cells to grow. After 24 h of culture, the cells start to form sphere-like bodies that appear as non-adherent or only partially adherent (Figure 1). Normally, at least 4000 regular 4T1 cells are required to induce tumor in mice. However, the...
injection of only 100 spheroid-derived cells into eight nude Balb/c mice resulted in tumor formation in all cases (data not shown) suggesting a remarkable tumor initiation potential and thereby making our results more comparable as far as the initiation of tumor formation is concerned. Unless indicated differently, those spheroid-forming 4T1 cells (4T1 mammospheres) were used in the subsequent studies.

Adipose tissue-derived stromal cells
The stromal cells used in this study are not adipocytes. There is a growing body of experimental evidence from both in vitro and in vivo studies demonstrating the multipotency of ASCs from adipose tissue isolated from humans and other species. These include the differentiation potential into adipocyte (10,20–22), chondrocyte (10,20,23,24), hematopoietic supporting cells (25,26), hepatocyte (27,28), neuronal-like cells (20,29–31), osteoblast (10,20,32,33), pancreatic (34) and skeletal myocyte (10,20,35), vascular cell (15) and cardiomyocyte pathways (36). The phenotypes of ASCs have been characterized previously (14). Briefly, ASCs are positive for CD44 (99.36 ± 0.75%), CD90 (97.59 ± 2.45%) and CD105 (98.51 ± 1.83%) and negative for CD11b (0.33 ± 0.18%), CD14 (0.51 ± 0.11%), CD34 (1.09 ± 0.16%), CD45 (0.39 ± 0.29%) and HLA-DR (0.68 ± 0.92%). Clonal expansion studies have shown that ASCs are capable of differentiating into all three lineages (X. Bai, Y. Song, E. Alt, submitted for publication).

Migration of 4T1 breast cancer cells and 4T1 mammospheres toward ASCs
mASCs were isolated from the perirenal and pelvine fat tissue of 10 immunocompetent Balb/c mice. Conditioned medium was collected from mASCs after 72 h in culture.

Using an in vitro transwell migration assay, we demonstrated that 4T1 breast cancer cells migrate toward the conditioned medium of mASC (Figure 2A). Interestingly, 4T1 mammospheres show a significantly higher number of migrating cells (~40%) compared with unselected adherent 4T1 cells.

SDF-1/CXCR4 axis is mainly responsible for migration of 4T1 cells and 4T1 mammospheres toward mASC
It has been shown that SDF-1 plays an important role in tumor growth and metastasis (37). In this study, we found that mASC secrete elevated levels of SDF-1, whereas 4T1 cells express CXCR4 which is the receptor for SDF-1 (Figure 2B). Interestingly again, 4T1 mammosphere cells have significantly higher messenger RNA levels of the CXCR4 receptor compared with their unselected counterparts or to mASC.

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Fig. 2. (A) 4T1 cells and especially spheroid-forming 4T1 cells migrate toward the conditioned medium of mASC. 4T1 cells (3 × 10^4) were plated in the upper chamber of a 3 μm transwell system. The lower chamber was filled with 1 ml of 72 h conditioned medium of mASC. After 24 h of migration through the transwell membrane, cells were fixed and stained with calcine. The results are the mean and SD number of migrated cells per microscopic field under fluorescent microscope. Background: regular 4T1 cells on upper chamber and the lower chamber contains non-conditioned serum-free medium; 4T1: regular 4T1 cells on upper chamber and conditioned serum-free medium from the ASCs; 4T1 spheroids: 4T1 spheroid-forming cells on top and conditioned serum-free medium from ASC on the bottom. (B) mASC but not 4T1 cells produce SDF-1; 4T1 cells especially spheroid-forming 4T1 cells show higher messenger RNA levels of the specific receptor CXCR4. Secreted amounts of SDF-1 were measured by enzyme-linked immunosorbent assay and CXCR4 messenger RNA levels were determined by quantitative reverse transcription-polymerase chain reaction (RT–PCR). Reverse transcription-polymerase chain reaction results were normalized against GAPDH. (C) 4T1 cells and mammospheres migrate toward recombinant SDF-1. A total of 3 × 10^4 4T1 spheroid-forming cells were plated in the upper chamber of a 3 μm transwell system. Recombinant murine SDF-1 in serum-free medium was filled into the lower chamber in stated concentrations. CXCR4 inhibition was performed by 30 μg/ml neutralizing antibody 1 h before and during the migration assay. (D) 4T1 migration toward mASC conditioned medium is mediated mainly by SDF-1. *P < 0.05, ***P < 0.001, ****P < 0.001.
Fig. 3. (A) Tumor growth is enhanced when 4T1 spheroid-forming cells are co-injected with mASC. A total of $5 \times 10^3$ 4T1 spheroid-forming cells or spheroid-forming cells with CXCR4 knockdown, respectively, were injected or co-injected with $5 \times 10^4$ GFP-labeled mASC subcutaneously. Values represent volume measurements (mm$^3$) with scientific caliper. (B) Tumor volume 21 days post-injection is increased when 4T1 spheroid-forming cells are co-injected with mASC and decreased using 4T1 cells with CXCR4 knockdown. Columns represent tumor volumes in mm$^3$ ± SDs evaluated from microCT images at day 21. (C) The respective microCT images show sections of tumors (upper panel) correspond to photographic images of tumors in situ (lower panels), (a) $5 \times 10^3$ 4T1 only, (b) $5 \times 10^3$ 4T1 + $5 \times 10^4$ GFP-mASC and (c) $5 \times 10^3$ 4T1 CXCR4 KD + $5 \times 10^4$ GFP-mASC. (D) Macroscopic and microscopic-sized lung metastasis: microCT image (upper picture). Arrow shows lung metastasis in a mouse co-injected with 4T1 spheroid-forming cells and mASC on day 21 post-injection. Hematoxylin and eosin staining of paraffin-embedded lung section from mouse from stem cell group (lower picture). *4T1 + mASC versus 4T1, $P < 0.05$; **4T1 versus 4T1 CXCR4 KD + mASC, $P < 0.01$. 

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metastasis in the lung, suggesting that the SDF-1/CXCR4 axis is essential not only to the growth of the primary tumor but also for its ability to metastasize. In order to find out whether ASCs produce more SDF-1 under the influence of the tumor microenvironment, we injected 1.5 × 10^4 GFP-labeled 4T1 mammospheres into the mammary fat pad of five nude Balb/c mice. Another five mice have been injected with 20 μl phosphate-buffered saline as a control group. After 2 weeks, tumors have formed in mice injected with 4T1 mammospheres (average tumor diameter 7.2 mm ± 0.98 mm), mice were killed and the mASCs were isolated from fat tissue surrounding the tumor. mASC isolated from these cancer mice showed an increase of SDF-1 release (supplementary Figure 2 is available at Carcinogenesis Online).

To determine whether ASCs could differentiate into cancer-associated fibroblasts or myofibroblast, we dissected out the tumor and prepared single-cell suspension and quantified SMA-positive cells. Our data showed that the ASCs express 42.47 ± 1.42% SMD before injection and this number increased to 57.03 ± 3.01% in ASCs isolated from tumors (P < 0.01).

Tumor growth in the knockdown group resembled symmetrical spherical growth with an exact tumor margin, whereas the 4T1 group and moreover the stem cell group plus 4T1 showed distorted spherical symmetries, invasive growth patterns, coarse tumor margins and satellite structures. Hematoxylin and eosin staining of tumor margins confirms the invasive growth patterns into the surrounding abdominal skeletal muscles in the 4T1 (Figure 4Aa) and stem cell group (Figure 4Ab), whereas the CXCR4 knockdown in 4T1 cells leads to a partial encapsulation of the primary tumor (Figure 4Ac).

In order to determine whether 4T1 tumor cells become more invasive in the presence of ASCs, we performed an invasion assay in a Boyden chamber. As shown in Figure 4B, ASCs promote the invasion of 4T1 cells when mixed with tumor cells at the ratio of 2:1 (ASC:4T1).

Vascularization and capillary density are enhanced by mASC Vascularization of the tumor as determined by the Houndsfield unit value of the tumor relative to the musculature evaluated from microCT images showed higher vascularity in the 4T1/mASC group compared with the 4T1 group (Figure 5A). The 4T1 CXCR4 KD/mASC group features lower enhancement and accordingly a decreased vascularity of the corresponding tissue. These image-derived data are supported by the capillary density as assessed from sections of frozen tissue by fluorescent staining for vWF. As illustrated in Figure 5B, tumor sections out of the stem cell co-injection group show a higher number of capillaries per microscopic field (20.6 ± 1.9) than those from the 4T1 group (12.25 ± 2.2) and the CXCR4 knockdown group (8.75 ± 1.6). Our data also showed that ASCs are incorporated into tumor vessels (Figure 5C, green) and some of them colocalized with vWF staining (Figure 5C and D, yellow) indicating differentiation of mASCs into endothelial cells (ECs).

Vascular endothelial growth factor (VEGF) plays an important role in tumor-induced neoangiogenesis. Our data demonstrate that 4T1 spheroid-forming cells secrete high levels of VEGF (104.8 ± 7.4 pg/10^6 cells/24 h) that is significantly reduced in 4T1 spheroid-forming cells with CXCR4 knockdown (46.4 ± 2.53 pg/10^6 cells/24 h) (supplementary Figure 3 is available at Carcinogenesis Online).

In order to find out if ASCs could differentiate into vascular ECs to support neoangiogenesis, we dissected the tumors and dissociated into a single-cell suspension and cell populations of vWF were quantified. Our data show that before injection, ASCs that are positive for vWF is
Systemically delivered mASC home to the tumor site and promote tumor growth

In order to determine whether systemic delivery of adipose tissue-derived mesenchymal stem cells could home to tumor site, we injected 3 x 10⁶ GFP-labeled mASC into the tail veins of eight nude Balb/c mice. These mice had been injected with 3 x 10⁴ 4T1 spheroid-forming cells 12 h before mASC injections. Subcutaneous injections of 3 x 10⁴ 4T1 spheroid-forming cells alone and co-injection of 3 x 10⁴ 4T1 spheroid-forming cells together with 3 x 10⁶ GFP-labeled mASC served as control groups. As illustrated in Figure 6A, tumor growth in mice injected with mASC intravenously (i.v.) was enhanced (398 ± 103 mm³) compared with tumors without mASC injections (198 ± 20 mm³) (P < 0.03) suggesting a supportive effect of mASC following the delivery into the circulatory system. However, mASC directly co-injected with 4T1 cells augmented tumor growth even more (698 ± 60 mm³).

After killing of mice at day 20 post-injection, tumors were dissected and sectioned. An i.v. injected mASC could be detected inside the tumor by immunostaining for GFP on paraffin-embedded sections. Furthermore, we were able to show differentiation of our i.v. injected mASC into an endothelial lineage by co-staining for GFP (Figure 6B). Additionally, we were able to show differentiation of our i.v. injected mASC into an endothelial lineage by co-staining for GFP and vWF also in this in vivo model (Figure 6C).

In order to assess the number of i.v. injected ASCs recruited into the tumor mass, GFP-positive cells were counted on paraffin sections of tumors i.v. injected with GFP-ASCs and compared with the number of GFP-positive cells in the tumors where GFP-ASC and tumor cells were co-injected by fluorescent microscopy. Tumor samples of comparable size of each group were analyzed in order to minimize possible errors due to different tumor size. As shown in supplementary Figure 4 (available at Carcinogenesis Online), approximately one-fifth of i.v. injected ASCs (19.4 ± 2.5%) are found at the tumor site compared with GFP-positive cells in tumors when tumor and ASC cells are co-injected together, suggesting that an active recruitment of systemically delivered ASCs and is in line with tumor growth data (Figure 3A).

In order to determine whether the tumor-promoting effects of adipose tissue-derived stem cells in murine 4T1 models are also valid in other tumor models, we performed a similar study using the MDA-MB-231 human breast cancer model. We injected 5 x 10⁴ MDA-MB-231 cells into a group of 10 nude Balb/c mice and 5 x 10⁴ MDA-MB-231 cells plus 5 x 10⁴ human ASC into another group of 10 mice subcutaneously into the inguinal mammary fat pad. There was no tumor formation observable in the group injected with MDA-MB-231 cells alone after three months; whereas six mice in the group co-injected with ASC showed tumor formation within 30 days (supplementary Table 1 is available at Carcinogenesis Online), suggesting that the tumor-promoting effect of tissue-resident stem cells is a general phenomenon.

Weinberg’s group has recently shown that regulated on activation, normal T-cell expressed and secreted (RANTES) is highly involved in human breast cancer metastasis. We performed additional experiment to investigate whether ASCs also produce RANTES. We were not able to detect RANTES in ASCs or MDA-MB-231 cells when cultured alone; however, we detected significant amount of RANTES in conditioned medium from ASC and MDA-MB-231 cocultures. In order to determine the cellular source of RANTES, we added conditioned medium from MDA-MB-231 cells to ASCs and found significant amount of RANTES produced by ASCs. We also added conditioned medium from ASCs to MDA-MB-231 cells and found no RANTES production. These data suggest that ASCs produce RANTES under the influence of tumor cells.

Discussion

While interactions between adipocytes and breast cancer cells have been described previously (38,39), the involvement of stem cells regarding in adipose tissue has not been investigated yet. In this study, we report for the first time that ASCs incorporated into tumor vessels and lead to enhanced tumor growth when co-injected with 4T1 cancer cells. We demonstrated that ASCs home to tumor site when injected i.v. Furthermore, we demonstrate that the SDF-1/CXCR4 axis plays an important role in mediating ASC’s tumor-promoting effect.

Our findings that ASCs promote the growth of breast tumor are of special importance since ASCs are derived from adipose tissue that is particularly abundant in breast tissue. Although ASCs used in the present study were harvested from perirenal and pelves fat tissue, it has been shown that tissue-resident stem cells are multipotent cells associated with microvessels and are ubiquitously distributed throughout all tissues (40). The results of the present study suggest that in addition to bone marrow-derived cells, local stem cells adjacent to breast cancer cells are involved in tumor growth and metastasis.

SDF-1 has been shown to direct the intratissue localization of tumor cells and to induce metastasis through direct effects on tumor cell migration (41). In line with previous findings (42), our in vitro assay showed that 4T1 tumor cells migrate toward conditioned medium of ASCs and that this migration is significantly reduced when the CXCR4 receptor in 4T1 cells is knocked down.

The mechanisms on how SDF-1 stimulates the outgrowth of established tumors and metastasis are not fully understood yet. In a mouse model of established extrapleural colorectal metastasis, SDF-1 promoted tumor cell migration in vivo and tumor growth of established extrapleural metastasis in vivo due to angiogenesis-dependent induction of tumor cell proliferation and inhibition of apoptotic cell death (43). Furthermore, Brand et al. (44) have demonstrated in the colorectal cancer cell line HT-29 that SDF-1 stimulation induces a significant increase in VEGF protein levels. Consistent with these findings, our data show that VEGF level is reduced in 4T1 cells with CXCR4 knocked down. In separate studies, we found that ASCs produce significant amount of insulin-like growth factor-1 (12) and interleukin-6 (S.Pinilla, Y.Song, F.Muehlberg and E.Alt, submitted for publication) that are also involved in tumor growth and metastasis.

Using the Luminex-based Bio-Plex suspension array system, Karnoub et al. (4) found that breast cancer cells stimulate de novo secretion of RANTES from mesenchymal stem cells, which then acts in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis. This enhanced metastatic ability is reversible and dependent on RANTES signaling through the chemokine receptor CCR5. We demonstrated that ASCs also produce significant amount of RANTES under the influence of cancer cells. These novel findings opened up the possibility of targeting the RANTES-signaling pathway in treating metastatic diseases.

Expression of alpha-HEMA is a defining marker for myofibroblast. Orimo et al. have shown that fibroblast differentiate into myofibroblast under the influence of the tumor microenvironment. They further demonstrated that myofibroblast but not regular fibroblast produce SDF-1 and promote tumor growth when co-injected with tumor cells. We therefore quantified SAMA-positive cells and after injection. Our data show that 42.47 ± 1.42% of our ASCs express SAMA before injection and this number increased to 57.03 ± 3.01% (P < 0.01) in ASCs isolated from tumors. Our findings are in line with that of Mishra et al. (45) that 17% of bone marrow-derived mesenchymal stem cells express SAMA at basal level and this number increased to 89% after exposure to conditioned medium from cancer cells. Our data confirmed the finding by Mishra et al. (45) that myofibroblast originate from mesenchymal stem cells; furthermore, our data suggest that tissue resident ASCs are an important source for myofibroblast.

We show that ASCs were not only unable to promote tumor growth when the CXCR4 receptor in 4T1 cells was knocked down but also that the overall tumor size is smaller. Our findings are in line with previous reports. It has been shown that endogenous CXCR4 expression on carcinoma cells correlate with a poor prognosis for several types of carcinomas (46). It is also known that CXCR4 ectopically expressed on carcinoma cells enhances primary tumor growth in a mouse xenograft model (47) and that the knockdown of CXCR4...
Fig. 5. Vascularity and capillary density are enhanced by mASC. (A) Increase of vascularization in stem cell group evaluated from microCT images. Contrast dye injection (Fenestra VC®) 2 h prior to microCT scans on day 21 post-injection facilitated software-supported measuring of vascularization in Hounsfield units by independent radiological core lab (University Magdeburg, Germany). (B) Frozen tumor sections were stained for vWF and stained vessels counted under fluorescent microscopy. Each specimen was examined in eight different microscopic fields. Columns represent mean number and SD of capillaries per field. (C and D): Co-injected GFP-labeled mASC differentiated into ECs. Paraffin-embedded tumor sections from mice injected with 4T1 spheroid-forming cells and GFP-labeled mASC were co-stained with GFP (green) and vWF (red) antibodies. *P < 0.05, **P < 0.01.

Fig. 6. mASC home to and engraft at tumor site when injected i.v. (A) 3 × 10⁴ 4T1 spheroid-forming cells were injected into 12 nude Balb/c mice. Eight of those mice were injected with 3 × 10⁵ GFP-mASC i.v. 12 h later. Another group of eight mice was co-injected with 3 × 10⁴ 4T1 spheroid-forming cells and 3 × 10⁵ GFP-mASC subcutaneously. Values represent tumor volumes over time evaluated by scientific caliper. (B) Paraffin-embedded tumor sections of mice injected with 4T1 spheroid-forming cells and GFP-mASC i.v. stained for GFP and nuclei with 4’,6-diamidino-2-phenylindole (blue) (C) Immunostaining of paraffin sections for vWF (red) and GFP (green). 4’,6-Diamidino-2-phenylindole (blue) was used for nucleus staining. **P < 0.01.
expression in breast carcinoma cells abrogates the tumor growth (48). Moreover, a small-molecule inhibitor of CXCR4 reduces primary brain tumor growth (49). Thus, it is probably that SDF-1 secreted by ASCs significantly affects CXCR4-expressing 4T1 tumors through direct paracrine stimulation.

Based on observations made nearly 100 years ago that blood vessels grow around tumors (50) and the pioneering work of Folkman (51), it is now widely accepted that tumors must induce angiogenesis in order to grow beyond a size of 1–2 mm in diameter, as well as to metastasize to distant organ sites. The exact mechanisms underlying this tumor-induced neoangiogenesis are still not fully understood. Our findings that GFP-labeled ASCs incorporate into blood vessels within the tumor via direct differentiation into ECs indicate that ASCs contribute to tumor angiogenesis. This observation mirrors previous findings that implanted ASCs differentiated into ECs and SMCs, which incorporated into newly formed vessels in an ischemic injury model (52). We have shown previously that ASCs are multipotent stem cells that have extraordinary proangiogenic potential (11).

However, other mechanisms may also be involved. It has been shown that myofibroblast-derived SDF-1 recruits endothelial progenitor cells into carcinomas, enhancing angiogenesis and thus tumor growth (38). Therefore, ASC-derived SDF-1 may be partially responsible for the increased vascular density observed in our study. Moreover, our finding that ASCs release higher levels of SDF-1 after coculture is in agreement with that of previous reports that myofibroblasts extracted from human mammary tumors express increased levels of the SDF-1 messenger RNA (38). Together, these findings highlight the similarities between tumor stroma formation and wound-healing response.

The aim of our experimental design was to better understand the mechanisms relating to breast cancer growth. Recent studies by others have shown that cancer stem cells were found in both breast cancer lesions and from an established breast carcinoma cell line (18). In an elegant study, human mammary epithelial cells were used to generate non-adherent spheroids (designated mammospheres) in cell culture and demonstrate the presence of the three mammary cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell (19). However, the true role of stem cells in cancer in general were clonally derived, providing evidence for a single pluripotent cell lineages. More importantly, the cells in the mammospheres were clonally derived, providing evidence for a single pluripotent stem cell (19). However, the true role of stem cells in cancer in general has been less elucidated. In order to shed more light specifically on interactions of normal stem cells and cancer stem cells as a possible mechanism of cancer initiation and progression, the current design of our study was selected. Although an experimental model is never completely consistent with the complex mechanisms in nature, our results support the concept that cancer stem cells initiate the tumor and the interaction with regular tissue-resident stem cells contribute to tumor growth.

Studies from Dr Chia-Cheng Chang’s group clearly demonstrate that there exist breast adult stem cells residing in the breast tissue that are capable of differentiating into breast tissue and to give rise to breast carcinoma cells (53–62). These breast adult stem cells express Oct4 (17) and have the ability to differentiate into other cell types and to form budding/ductal structures on Matrigel (63). More interestingly, these cells are more susceptible to telomerase activation (63) and immortalization by SV40 large T antigen (59). Using complementary DNA microarray analysis, 148 genes have been identified as being either upregulated or downregulated related to immortalization (64). These studies provided enhanced understanding of breast stem cell and tumor initiation.

Limitations

Otsu et al. investigated on the effect of bone marrow-derived stem cells on angiogenesis in an in vitro Matrigel angiogenesis assay and an in vivo melanoma model. They found concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. Therefore, future studies should try different condition for a general phenomenon (65).
Tissue-resident stem cell and breast cancer


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