Identification of Cancer-Associated Fibroblasts in Circulating Blood from Patients with Metastatic Breast Cancer

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Abstract

Metastasis is facilitated by cancer-associated fibroblasts (CAF) in the tumor microenvironment through mechanisms yet to be elucidated. In this study, we used a size-based microfilter technology developed by our group to examine whether circulating CAF identified by FAP and α-SMA co-expression (cCAF) could be distinguished in the peripheral blood of patients with metastatic breast cancer. In a pilot study of patients with breast cancer, we detected the presence of cCAFs in 30/34 (88%) patients with metastatic disease (MET group) and in 3/13 (23%) patients with localized breast cancer (LOC group) with long-term disease-free survival. No cCAFs as defined were detected in healthy donors. Further, both cCAF and circulating tumor cells (CTC) were significantly greater in the MET group compared with the LOC group. Thus, the presence of cCAF was associated with clinical metastasis, suggesting that cCAF may complement CTC as a clinically relevant biomarker in metastatic breast cancer. Cancer Res; 75(22); 4681–7. ©2015 AACR.

Introduction

A human tumor is a complex tissue composed of malignant cells and noncancer-associated cells, including stromal cells, inflammatory cells, and immune cells. Circulating tumor cells (CTC) are tumor cells found in the peripheral blood of patients with cancer. Studies of CTCs demonstrate that they have promising value as a prognostic biomarker in several cancers, including breast cancer, colorectal cancer, and prostate cancer (1). The existence of clusters of CTCs has recently been reported, and several groups have described the clinical relevance of CTC clusters (1). Although the prognostic value of CTCs has been well validated, there are limitations preventing the use of CTC enumeration in routine clinical practice (1), in particular, regarding the use of CTCs as a clinical marker for early cancer detection or as a surrogate endpoint in interventional studies (1). These limitations include uncertainty regarding the specificity of CTC detection assays and justifiable concerns that detection of CTCs alone may be misleading or inadequate, especially when applied in the early detection of metastasis. Additional biomarker assays may enhance the specificity and broaden the application of "liquid biopsies" in early cancer detection, in monitoring disease progression, and in determining response to therapy.

Tumor-associated stromal cells, or cells of the tumor microenvironment, are made up of various types of cells including fibroblasts, endothelial cells, immune cells, adipocytes, pericytes, and extracellular matrix (ECM) components (2). Moreover, there is substantial evidence highlighting the contributing role of all these stromal cell types to tumor progression and metastasis (2, 3).

In response to wounding or tissue damage, epithelial cells and immune cells (including monocytes and macrophages) induce an activated phenotype in fibroblasts by secreting growth factors and chemokines, such as TGFβ, EGF, PDGF, FGF2, MCP-1, reactive oxygen species, and ECM proteases (4). Activated fibroblasts can be identified by expression of various markers, such as α-smooth muscle actin (α-SMA), fibroblast-specific protein, vimentin, prolyl 4-hydroxylase, and fibroblast activation protein (FAP). In the context of a solid tumor, fibroblast activation is sustained constitutively, and these activated fibroblasts are called cancer-associated fibroblasts (CAF; ref. 4). Currently, the most definitive method that is agreed upon for the identification of CAFs is double positivity for both FAP and α-SMA (4). FAP is a type II transmembrane serine protease (5); although the specific function of FAP is unknown, its enzymatic activity has been implicated in tumor progression, ECM remodeling, and metastasis (5).

CAF-secreted cytokines and growth factors can act in a paracrine fashion to regulate a multitude of critical steps in tumor metastasis (4). CAFs are involved in breast cancer initiation by producing TGFβ and hepatocyte growth factor (4). CAFs promote tumor progression and invasion by secreting matrix metalloproteinases, proteases that contribute to ECM degradation (4). CAFs promote tumor angiogenesis through secretion of VEGF, FGF2, and SDF-1.
CAFs are known to induce epithelial-to-mesenchymal transition (EMT) in cancer cells through secretion of TGFβ and hepatocyte growth factor, a process that is thought to be critical to tumor metastasis (4). CAF-secreted cytokines confer a survival advantage to tumor cells, and facilitate cancer-cell evasion of the immune system (6). CAFs collectively serve as an "incubator" for cancer cells by providing a favorable "soil" that supports growth and proliferation of cancer cells at distant sites (4, 7). Depletion of CAFs decreases tumor metastasis by altering production of pro-tumor cytokines, repressing angiogenesis, and interfering with the recruitment of immunosuppressive cells (6). These data underline the importance of CAFs in the process of metastasis.

Because CTCs can be detected in the peripheral blood of patients with cancer, it follows that a "liquid biopsy" to detect tumor components in blood will not only contain tumor cells but will also contain other cellular components from the tumor microenvironment. In support of this notion, Duda and colleagues demonstrated that CTCs can carry stromal cells, including fibroblasts, as "soil" to facilitate metastasis formation in a mouse model of lung cancer metastasis (7). In addition, Hjerpe and colleagues demonstrated the existence of CAFs in ascites fluid from patients with ovarian cancer, as indicated by α-SMA expression (8). In spite of these studies demonstrating the presence of CAFs outside of primary tumor site or metastatic lesions, there has been little direct evidence showing the presence of CAFs in the circulation of patients with cancer in a clinical setting. Fibroblast progenitor cells were reported by Ishii and colleagues in the circulation of patients with lung cancer (9), and fibroblast-like cells were reported by Jones and colleagues in the circulation of patients with metastatic prostate cancer (10); both studies used vimentin as a marker for the fibroblastic nature of these cells. Vimentin, however, is not a fibroblast-specific marker, and is expressed on lymphocytes (11), breast cancer cells that have undergone EMT, and CTCs that have undergone EMT (12). Here, we employed the most widely accepted markers for defining populations of CAFs (FAP+/α-SMA+/Cytokeratin+/CD45−) and we report circulating CAFs (cCAFs) in the peripheral blood of patients with breast cancer with metastatic disease. This is, to our knowledge, the first time that cCAFs have been reported in the context of metastatic breast cancer. Moreover, using our novel cell-size-based microfilter technology, initially developed for CTC capture (13), and employing methods to discriminate CAFs from CTCs by evaluating FAP/α-SMA co-expression, we show for the first time the presence of CAFs along with CTCs in the peripheral blood of patients with breast, colorectal, and prostate cancer. In addition, we report that, in a pilot study involving patients with breast cancer with metastatic disease (MET group) or with local breast cancer and no evidence of disease for 5 years (LOC group), cCAFs are significantly elevated in patients with breast cancer with overt metastases. Moreover, CAFs can be detected in peripheral blood and easily quantified following a minimally invasive liquid biopsy. These findings establish a significant association of CAFs with breast cancer metastasis and suggest the potential of cCAFs as a promising biomarker for disease metastasis.

Materials and Methods

Tissue culture

MCF-7 cells were obtained from the ATCC and maintained in phenol red DMEM supplemented with heat-inactivated 10% FBS. Primary CAF cell lines were previously isolated and characterized (14) and maintained in phenol red Gibco’s Improved Minimum Essential Medium (IMEM) A10489-01 supplemented with heat-inactivated 10% FBS. All cells were grown in 37°C, 5% CO2, in a forced air incubator, and passaged continuously by detachment using TrypLE Express (Gibco-Life Technologies). Cell cultures were checked routinely for adventitious pathogens using MycoAlert Mycoplasma Detection Kit (Lonza).

Blood collection and processing

Blood samples were collected from patients with breast cancer under a protocol approved by the University of Miami (Miami, FL) Institutional Review Board (20130312), and from patients with prostate cancer under (protocol 20100635 and 20101056), following informed consent. Blood samples from patients with colorectal cancer were obtained from the University of Miami Tissue Bank Core Facility. These colorectal cancer patient samples as well as healthy donors’ blood samples were determined as nonhuman subject research by the Institutional Review Board. Blood was collected via venipuncture into EDTA tubes (BD Biosciences). The first tube for blood was discarded or used for other analysis in order to avoid potential contamination of epithelial cells and stromal cells during venipuncture. A total of 7.5 mL blood was diluted 1:1 with 1× PBS (Gibco) and fixed with a final concentration of 1% formalin (Sigma Aldrich) for 10 minutes. After fixation, blood was processed through our microfilter at a flow rate of 200 mL/hour using the same protocol reported previously for CTC capture (13).

cCAF culture

To maintain the viability of cCAFs during the filtration process, we employed a slot pore microfilter that enables capture of viable cells (15). Three milliliters of blood was diluted 1:1 with 1× PBS (Gibco) and processed through our slot pore microfilter at a flow rate of 75 mL/hour. After capture, cCAFs are released from the filter and maintained in culture in a 24-well plate (Greiner Bio-One) using IMEM medium supplemented with heat-inactivated 10% FBS. A transwell insert (Corning) seeded with 5,000 CAF-23 cells was inserted on top of the well to facilitate cCAF growth in vitro.

Immunofluorescence staining

To enumerate both CTCs and cCAFs on the same microfilter, samples were subjected to triple staining with pan-cytokeratin, CD45, and FAP. Samples were blocked with blocking buffer consisting of 5% normal goat serum (Life Technologies) and 0.3% Triton X-100 (Sigma Aldrich) at room temperature for 1 hour. Samples were then incubated with primary antibodies: mouse anti-human CD45 (Dako) and rabbit anti-human FAP (Millipore) at 4°C overnight. Samples were then incubated with secondary antibodies: goat anti-mouse Alexa 680 and goat anti-rabbit Alexa 594 (Life Technologies) at room temperature for 1 hour. Finally, samples are mounted on coverslips with ProLong Gold Antifade mounting media with DAPI (Life Technologies).

For the parallel sample collected from patients with breast cancer at the same time point, double staining of α-SMA and FAP was performed by incubation with primary antibodies consisting of mouse anti-human α-SMA (Santa Cruz Biotechnology) and maintained following a minimally invasive liquid biopsy. These findings establish a significant association of CAFs with breast cancer metastasis and suggest the potential of cCAFs as a promising biomarker for disease metastasis.
and rabbit anti-human FAP (Millipore) at 4°C overnight after the same blocking procedure as described above, followed by incubation with goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (Life Technologies) incubation at room temperature for 1 hour. Samples were then mounted on coverslips and stained with DAPI as described above.

Data analysis
Microsoft Excel 2010 and R Statistical Software (v 3.0.3) were used for data analysis and visualization. Comparisons between proportions of H, LOC, and MET patients with breast cancer groups based on cCAF or CTC counts were performed by Fisher exact test. Differences in enumeration of cCAF and CTCs between LOC and MET patients with breast cancer groups were assessed by the one-tailed unpaired Wilcoxon Rank Sum test.

Results
Validation of cCAF enumeration using microfilter technology
We hypothesized that cCAFs would be a rare cellular population in the patients’ peripheral blood, therefore we anticipated facing similar technical challenges as in the capture and study of CTCs and we explored the possibility of employing our novel cell-size-based CTC isolation microfilter (13) to isolate cCAFs, as CAFs are also larger than hematopoietic cells. To test the possibility of this notion in a model system, we used CAF23 cells, a primary CAF cell line, which was established from a triple negative primary breast tumor, and which is FAP+ and α-SMA+, as well as vimentin+ and pan-cytokeratin~ (14). Equal numbers of CAF23 and MCF-7 breast cancer cells (3,000 of each) were resuspended in 20 mL 1× PBS. The sample was then processed through our microfilter using the same protocol as we previously reported for CTC capture (16). Following cell capture, the microfilter was subjected to a double immunofluorescence staining for pan-cytokeratin and FAP to identify epithelial MCF-7 and fibroblastic CAF23 cells, respectively. These proof-of-principle experiments showed that CAF23 cells can be captured on our filter based on their larger size (~20 to 40 μm; Fig. 1A). Further evaluation of CAF capture efficiency was performed by spiking 100 CAF-23 cells into 7.5 mL of healthy donor’s blood. An average capture efficiency of 95.0 ± 2.8% was demonstrated (Fig. 1B).

cCAF identification and enumeration from human patients
Having demonstrated that CAFs could be captured from human blood with a very high efficiency, we employed the microfilter capture process to attempt identification of cCAFs in the patients with cancer peripheral blood. Seven and a half milliliters of peripheral blood samples were collected from a total of 47 patients with breast cancer: 34 patients with metastatic breast cancer (stage IV, MET group) and 13 patients with localized breast cancer with >5 years of long-term disease-free survival (stage I patients treated with curative therapy, LOC group). This latter group of patients is predominantly composed of individuals who will not recur although a small number of these patients will develop recurrences late. Triple-staining for pan-CK, FAP, and the lymphocyte marker CD45 was used to identify CTCs, cCAFs, and lymphocytes: cCAFs are identified as CK+, FAP+, and CD45− cells, whereas CTCs are identified as CK+, CD45+ cells (Fig. 2A and B). FAP/α-SMA double staining in parallel clinical samples demonstrated that

![Figure 1](https://example.com/figure1.jpg)

Validation of microfilter capture of CAF cells. **A**, 3,000 CAF-23 and 3,000 MCF-7 cells were spiked into 5 mL of PBS, fixed with formalin, processed through microfilter device, and immunofluorescently stained with anti-FAP-Alexa 488 and anti-CK-Alexa 594 antibodies. Top, merged picture. Bottom, picture split into individual channels. **B**, 100 CAF-23 cells were spiked into 7.5 mL of blood, processed through microfilter, and immunofluorescently stained for FAP. The samples were enumerated under fluorescent microscope.
these FAP+ cCAFs are also α-SMA positive, confirming their identification as CAFs (Fig. 2C). In addition, we maintained viably captured cCAFs from parallel samples in in vitro culture; these cells showed a spindle-like morphology, typical of cells of mesenchymal origin (Fig. 2D).

As shown in Fig. 3A, cCAFs were detected in 30 of 34 patients (88.2%) from the MET group but in only 3 of 13 patients (23.1%) from the LOC group; in the LOC group, cCAFs were detected at very low numbers (<2 cCAFs maximum; Fig. 3D). Summary statistics for enumeration of cCAFs and CTCs from the LOC and MET groups are shown in Fig. 3D and E. The enumeration of cCAFs indicates that patients with breast cancer with metastatic disease not only have a higher incidence of cCAFs that can be detected in their circulation, but also that the absolute counts of these cCAFs are significantly increased, as compared with patients from the LOC group (Fig. 3B; \( P = 0.000017 \)). Binary classification of patients by cCAF count (\( n < 5 \) or \( n \geq 5 \)) does not indicate a higher proportion of patients in the MET group with elevated CTC counts than patients in the LOC group, likely due to the low number of patients in the LOC group (Fisher exact test \( P \) value = 0.18; Fig. 3E). These data raise the question of the use of CTCs as a standalone biomarker for cancer metastasis and highlight the potential importance of cCAFs as a clinically relevant cancer biomarker, either in addition to or independent of CTCs.

We also analyzed cCAFs in peripheral blood from patients with metastatic colorectal cancer as well as patients with localized prostate cancer. We demonstrated that cCAFs are detectable in the peripheral blood of patients with both colorectal and prostate cancer, and not detectable in healthy donor’s blood (Fig. 3A). We observed that cCAFs are detected at high levels in patients with metastatic colorectal cancer and at low levels in patients with localized prostate cancer, although further validation with comparable controls is needed. It is also worth mentioning that in blood samples from one patient with metastatic breast cancer, from two patients with metastatic colorectal cancer, and from one patient with localized prostate cancer, we detected cCAFs, but did not detect CTCs. Moreover, during analysis of CTCs and cCAFs in patient with breast cancer from the MET and LOC groups, we identified clusters of cells captured by our microfilter. We observed CTCs clustering with CTCs, CTCs forming clusters with...
Figure 3.
CTC and cCAF enumeration from patients with cancer. A, table showing cCAF and CTC enumeration from patients with metastatic breast cancer (MET group), patients with localized breast cancer >5 years disease-free survival (LOC group), patients with metastatic colorectal cancer with metastasis to liver, patients with localized prostate cancer, and healthy donors. B and C, swarm plots indicating the enumeration of cCAFs (B) and CTCs (C) from blood samples of healthy donors (H; n = 7), patients from the LOC group (C; n = 13), and patients from the MET group (M; n = 34); P values for Wilcoxon rank sum test are indicated for comparison of counts of cCAFs (B) and CTCs (C) between patients from the LOC and MET groups. D and E, summary statistics and contingency tables are shown for enumeration of cCAFs (D) and CTCs (E); P values for Fisher exact test comparing CTC and cCAF counts within groups are indicated.

Identification of cCAFs from Metastatic Cancer Patients
cCAFs, CTCs clustering with leukocytes, and clusters of cCAFs alone (Fig. 4). Importantly, these clusters of cCAFs and CTCs were only detected in the peripheral blood from patients with metastatic breast cancer, and not in the blood from patients from the LOC group or from healthy donors.

Discussion
Metastasis is the underlying cause for most cancer-related deaths. In order for metastases to successfully occur, the tumor cells have to overcome various hurdles (17), including invading the basement membrane to reach the circulation, surviving in circulation, extravasating, and finally seeding and growth at the distant site (17). The microenvironment plays a critical role in determining if the cancer cells will undergo senescence, apoptosis, dormancy, or if they will form metastasis at the distant site (17). There is extensive data demonstrating the involvement of numerous cellular and molecular players within the tumor microenvironment and their role in metastasis (2, 3). Recent studies have also shown that stromal cells, like macrophages, cluster with CTCs and can promote tumor metastasis (18).

Previously, two groups have observed vimentin-positive fibroblast-like cells in the blood samples from patients with lung and prostate cancer (9, 10), and the fibroblast-like cells in the blood samples from patients with lung cancer were identified as fibroblast progenitor cells. In the context of breast cancer, the use of vimentin for identifying CAFs is limited, as vimentin is not specific to CAFs, nor even to fibroblasts, and can be expressed by both lymphocytes (11) and, notably for this study, by breast cancer cells, including breast CTCs that have undergone EMT (12). Tchou and colleagues described a subpopulation of FAP⁺ cells in breast cancer stroma that were also positive for CD45 (19), suggesting FAP can be expressed in monocytes/macrophages. Here, we have used double positivity for FAP and α-SMA and double negativity for CK and CD45 to define CCAFs, as these are the currently the most definitively accepted markers for CAFs (4). Moreover, the cells we identified as CAFs (FAP⁺/α-SMA⁺/CK⁻/CD45⁻) showed spindle-like morphology in culture and were of the appropriate size for mesenchymal/epithelial cells. We show direct evidence of cCAFs present with CTCs in the peripheral blood from patients with breast cancer with metastatic disease. Our findings support previous observations indicating the role of CAFs in tumor metastasis, such as reports of CTCs carrying CAFs as their own “soil” during circulation, thus evading cell death in circulation and facilitating the establishment of a metastatic niche at distant sites (7), as well as the finding of fibroblast-like cells in the circulation of patients with lung and prostate cancer (9, 10). These data establish a significant association between the presence of cCAFs in patients’ blood with breast cancer and the presence of metastatic disease.

To date, it has been widely documented that CTCs are of important prognostic value in certain cancer types (20–22). However, using CTCs for early detection of solid tumors is not established. This is primarily due to the “false-positive” detection of circulating epithelial cells in circulation caused by other diseases, such as benign colon disease (23). In addition, CTCs are detected in both early stage and metastatic breast cancer (24) making it difficult to use their presence or number as a standalone biomarker for metastasis. A companion biomarker, such as cCAFs, could enhance the early detection of solid tumors and prove to be an efficient biomarker for disease metastasis. In our pilot study, cCAFs were only detected in 3 of the 13 samples analyzed from the LOC group, and in these three samples the cCAF number was two or fewer, although in the MET group, CCAFs were detected in 30 of 34 samples, with only 12 of the 30 having two or fewer. Similarly, CCAF number was significantly higher in metastatic colorectal cancer samples compared with localized prostate cancer samples. Also, in one patient with metastatic breast cancer, two patients with metastatic colorectal cancer, and one localized patient with prostate cancer, we detected cCAFs but not CTCs, thus further highlighting the potential importance of this newly discovered cCAF population.

As our study examined the presence of cCAFs in stage I (LOC) and stage IV (MET) patients, further investigation of the presence of cCAFs in stage II/III patients at high risk for metastases, but without overt metastases, and in a larger cohort is necessary to validate the robustness of cCAFs as a potential biomarker for the detection of cancer metastasis. Additional investigation in larger cohorts is also necessary to determine both the sensitivity of cCAF detection, as well as a potential threshold of cCAF number for a minimally invasive liquid biopsy biomarker.

These findings will lead to further studies on this novel cCAF population in the circulation of patients with breast cancer. With the ability to establish the cCAFs in culture, further characterization of cCAFs both in in vitro assays and in vivo co-xenografts of cCAFs together with tumor cells would define and validate their role in facilitating metastasis. Our findings also support the rationale for therapeutically targeting CAFs as a single-target population in the treatment of multiple cancer types. A FAP-targeting DNA vaccine has recently been shown to specifically eliminate CAF populations and to mitigate tumor metastasis in a mouse model of breast cancer (6). Sibrotuzumab, an anti-human FAP antibody, has shown remarkable properties targeting the tumor microenvironment in humans (5). Though phase I/II clinical trials results of sibrotuzumab failed to show therapeutic activity, FAP radioimmunoconjugates and antibody drug...
Disclosure of Potential Conflicts of Interest

R.J. Cote and R.H. Datar have ownership interests in patented parylene microfilter technology. No potential conflicts of interest were disclosed by the other authors.

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