Phosphorylation of FAK, PI-3K, and Impaired Actin Organization in CK-positive Micrometastatic Breast Cancer Cells

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INTRODUCTION

The development of metastasis in cancer is one of the most important factors determining the long-term outcome of the disease. Theoretically, tumor cells deriving from the original tumor and possessing advantageous biological characteristics could acquire the capability of generating metastasis. Recent studies have shown that many “early-stage” breast cancers present a poor prognosis gene expression signature (1–3), suggesting that these tumors may be predestined to become or to be metastatic from the very beginning (4,5). In otherwise metastasis-free patients, metastasis is associated with the presence of circulating tumor cells (CTC) and disseminated tumor cells (DTC) in peripheral blood and bone marrow aspirates, respectively (6,7). Several studies have shown that the detection of CTC and DTC represents a strong and independent predictive and prognostic factor for a decreased disease-free period and overall survival, respectively (8–10).

Several markers have been used to detect occult tumor cells in the peripheral blood of patients with breast cancer. However, analysis of activated signaling kinases in CTC implicated in cellular transformation, migration, and survival has not been addressed so far. In the present study, we focused on the phenotypic profile of micrometastatic cells in peripheral blood mononuclear cells (PBMC) preparations from 45 breast cancer patients. PBMC cytospins from 28 cytokeratin (CK)-positive and 17 CK-negative samples were assessed for the expression of phosphorylated FAK (p-FAK), phosphorylated PI-3 kinase (p-PI-3K), and HER2 using confocal laser scanning microscopy. The expression of p-FAK was documented in all 28 CK-positive samples, while all 17 CK-negative samples were tested negative for p-FAK. Immunomagnetic separation using EpCAM antibody fully confirmed these findings, implying a sound correlation for the co-expression of the two molecules. Interestingly, 15 of 28 CK- and p-FAK-positive samples also expressed the HER2 oncoprotein, p-PI-3K was documented in 15 of 17 CK- and p-FAK-positive samples. Immunoblot analysis of micrometastatic cells in coculture with PBMC confirmed the specific expression of both p-FAK and p-PI-3K. Finally, impaired actin organization was apparent in CK- and p-FAK/p-PI-3K-positive samples, comparable to that observed in MCF-7 human breast cancer cells. Our findings provide strong evidence that micrometastatic cells express activated signaling kinases, which may regulate migration mechanisms, supporting the presumption of their malignant and metastatic nature.

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ment, by modifying cell-substratum adhesion, controls many cell functions such as motility, division, and secretion, and is involved in a large number of human diseases (20). Initial signals controlling these rearrangements may include modification of focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that is localized in focal adhesions (21). FAK is thought to be a potential oncogene because it has been implicated in the progression of cancer by promoting invasion and metastasis (22). In a previous study, we have reported the identification of a distinct signaling cascade in MCF-7 human breast cancer cells, involving FAK/PI3 kinase/Cdc42/Rac1 activation. Stimulation of this pathway triggers actin reorganization and regulates both cell proliferation and motility (23). These findings imply that activation of FAK and PI3 kinase via phosphorylation, followed by actin reorganization, may be correlated to the metastatic potential of breast cancer cells. However, the potential impact of these observations in vivo remains unknown.

In the present study, we have used immunofluorescence microscopy to analyze the expression of activated FAK and PI3K in breast cancer patients’ CTC. Findings were correlated with that of CK-19 and HER2 expression. In addition, we report the setting up of micrometastatic and PBMC co-cultures followed by immunoblot analysis for the study of the specific expression of p-FAK and p-PI3K in CTC. Finally, the morphology of actin cytoskeleton organization in CTC was analyzed and compared with that of MCF-7 cells. Our findings provide strong evidence that phosphorylated FAK and, to a lesser extent, PI3 kinase are predominantly and specifically expressed in CTC of otherwise metastasis-free breast cancer patients.

MATERIALS AND METHODS

Patient Samples and Cytospin Preparation

Blood samples from 45 women with operable (stage I and II) breast cancer, of whom 28 were CK-19 mRNA-positive and 17 CK-19 mRNA-negative by real-time PCR (24) were analyzed. Peripheral blood (10 mL in EDTA) was obtained before initiation of adjuvant treatment (usually 3–4 wk after primary surgery). All blood samples were obtained at the middle of vein puncture after the first 5 mL blood were discarded. This precaution was undertaken to avoid contamination of the blood sample with epithelial cells from the skin during sample collection. Before primary surgery, a complete baseline diagnostic evaluation for distant metastases was performed, including chest X-rays, mammography, ultrasound of the liver, and whole-body bone scan. Further imaging studies (CT scans or MRIs) were performed if clinically indicated. No patient included in the present analysis showed any evidence of distant metastasis. All patients gave their informed consent to participate in the study, which has been approved by the Ethics and Scientific Committees of our institution.

Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Hypaque (d = 1.077 gr/mol) density as described above and 30×10^6 PBMC were seeded in culture flasks coated with extracellular matrix (Biological Industries, Kibbutz Beit Haemek, Israel). The culture medium contained RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 10 μg/mL transferrin, 5 μg/mL insulin, 2 mM glutamine, 10 ng/mL Fibroblast Growth Factor, and 10 ng/mL Epidermal Growth Factor in a humidified atmosphere of 5% CO2 and reduced 5%-10% oxygen. Subcultivation was performed with 0.25% trypsin and 5 mM EDTA.

Confocal Laser Scanning Microscopy

The expression of cytokeratins in PBMC cytosplains was double-checked using two different antibodies: A45-B/B3 anti-mouse (detecting CK8, CK18, and CK19) and pancytokeratin anti-rabbit. Cytospins were also double stained with anti-CD45 (common leukocyte antigen) antibody to exclude possible ectopic expression of cytokeratins by hematopoietic cells. In cytosplains of the same patients, we further investigated phospho-FAK, HER2, and phosho-PI3 kinase expression, with dou-
Immunomagnetic Separation of CTC

PBMC cytospins were fixed with cold acetone: methanol 9:1 for 20 min and stained for cytokeratin with a pancytokeratin antibody as mentioned above. Subsequently, the same slide was stained with either phospho-FAK, HER2, or phospho-PI-3 kinase antibodies for 45 min. Cells were then incubated with the corresponding secondary antibodies for 45 min. Slides were analyzed using a confocal laser scanning microscope module (Leica Lasertechnik, Heidelberg, Germany) and images were analyzed with the respective software. In pFAK-positive/CK-positive cytopsins double staining control experiments were performed for FAK and pFAK with anti-rabbit polyclonal and anti-mouse monoclonal antibodies, respectively. Direct fluorescence microscopy of F-actin was accomplished by rhodamine-phalloidin in PBMC cytopsins. Tumor cells were double stained with A45-B/B3 anti-mouse antibody.

Immunomagnetic Separation of CTC

PBMC (2×10^7) isolated with Ficoll-Hypaque density gradient (d = 1.077g/ml) as described before were placed in 1 mL PBS/20% FCS. Fifty microliters of CELLection beads (coated with EpCAM monoclonal antibody via a DNA linker to provide a cleavable site for cell detachment) were added to the PBMC. After 30-min incubation at 4°C, cells were washed three times with RPMI/1%FCS. Supernatant was removed and 4 μL of releasing buffer in 200 μL RPMI/1%FCS was added to the beads. After 15 min of incubation at room temperature, samples were placed in a magnetic device and the released cells were transferred into a different tube. Isolated cells were centrifuged at 2000 rpm for 2 min on glass slides. Double staining microscopy experiments were performed as described before.

Immunoblot Analysis

Micrometastatic cells co-cultured with PBMC for 4 wk were lysed using 500 μL cold lysis buffer (50 mM Tris/HCl, 1% TritonX-100, pH 7.4, 1% Sodium deoxycholate, 0.1% SDS, 0.15% NaCl, 1 mM EDTA, 1 mM sodium orthovanadate) at 4°C and flasks were scrapped off. The remaining insoluble material was removed by centrifugation. Protein concentration of the samples was determined using the Bio-Rad protein kit. Equal amounts of protein (100 μg) were immunoprecipitated with (7 μg/500 μg total protein) monoclonal phosphotyrosine antibody overnight at 4°C. Antigen-antibody complexes were then bound to 100 μL agarose-conjugated protein A. Samples were resuspended in 100 μL SDS sample buffer, separated with SDS electrophoresis and blotted onto nitrocellulose membrane. Proteins were incubated with anti-phosphotyrosine, anti-FAK, and anti-PI-3 kinase antibodies, respectively, for 1 h at room temperature and then with the appropriate secondary antibody. Detection of protein bands was succeeded using the ECL kit. All the proteins were quantified using a PC-based image analysis system (Image Analysis Inc, Ontario, Canada).

Materials

RPMI 1640 and DMEM/Ham’s F12 culture media, trypsin, EDTA, and fetal bovine serum (FBS) were from Gibco-BRL. Rhodamine-phalloidin and Alexa Fluor 555 anti-rabbit IgG were obtained from Molecular Probes Inc. (Eugene, OR, USA). Pancytoketatin, FAK, CD45 PI3-kinase anti-rabbit, phospho-tyrosine antimouse, and phospho-PI-3 kinase anti-goat antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-FAK anti-mouse antibody, rhodamine anti-goat IgG, and FITC anti-mouse IgG were obtained from Chemicon International Inc (Temecula, CA, USA). CD45 anti-mouse antibody was supplied from DAKO Corporation (Carpinteria, CA, USA). HER2 anti-mouse antibody was from Oncogene (Dermstadt, Germany). A45-B/B3 anti-mouse antibody was obtained from Micromet (Munich, Germany). Immunomagnetic beads (Dynabeads) were from Dynal, Inc (Wirral, UK). DAPI was from Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from usual commercial sources at the purest grade available.

RESULTS

Expression of Phosphorylated FAK in CK-positive CTC

The presence of CTC was initially investigated in cytopsins of PBMC from 45 breast cancer patients, of whom 28 were already known to be CK-19 mRNA-positive and 17 CK-19 mRNA-negative by real-time PCR (data not shown). Double staining immunofluorescence experiments with two different anti-cytokeratin antibodies (polyclonal or A45-B/B3) for the detection of epithelial cells and an anti-CD45 antibody for the recognition of hematopoietic cells were used. CK-positive/CD45-negative cells were detected in all 28 samples already known to be CK-19 mRNA-positive (Figure 1A). The median number of CTC detected in each sample was 3 (range, 1-10). On the contrary, no CK-positive cells were identified by immunofluorescence microscopy in the 17 samples previously tested CK-19 mRNA-negative. These findings indicate a complete concordance between the immunofluorescence detection and the previously performed real-time RT-PCR analysis for CK-expression (See Figure 1A, right panel), implying that both techniques provide comparable and reliable results.

To investigate whether activated signaling kinases can be identified in micrometastatic cells, all 45 samples were double stained for p-FAK and cytokeratin. The intracellular co-expression of p-FAK (green staining) and CK (red staining) could be clearly demonstrated in CTC’s using immunofluorescence microscopy, while mononuclear cells (blue staining) do not express these proteins (See Figure 1B). Confocal laser scanning microscopy confirmed these findings (see Figure 1C). p-FAK staining was documented in all 28 CK-positive samples, while p-FAK was not observed in the 17
CK-negative samples (not shown), implying a high correlation in the expression patterns of both molecules in CTC (see Figure 1C, right panel). However, it should be noted that in samples from two patients, both CK-positive/p-FAK-positive and CK-positive/p-FAK-negative cells could be identified in the same blood sample (data not shown).

Interestingly, the presence of CK-negative but p-FAK-positive cells could not be documented. In an effort to provide additional evidence for the specific expression of p-FAK in CTC but not in PBMC, CK-positive samples were double stained with specific antibodies recognizing FAK and p-FAK, respectively. As shown in Figure 1D, while FAK expression (red) was found to be unanimous in all PBMC’s, p-FAK staining (green) was evident only in distinct cells, indicating specificity of p-FAK expression. In addition, because all CK-positive cells were CD45-negative (see Figure 1A) and all p-FAK-positive are CK-positive as well (see Figure 1B) it is assumed that p-FAK is expressed in micrometastatic cells.

Figure 1. Cytokeratin and p-FAK expression in CTC from breast cancer patients. (A) Representative micrograph of cytospin, double stained with CD45 monoclonal antibody (green) for hematopoietic cells and with pancytokeratins polyclonal antibody (red) for circulating tumor cells. Magnification, ×400. Right panel: Quantification of CK-positive samples by immunofluorescence microscopy and RT-PCR analysis. (B) Representative micrograph of PBMC cytospin stained with pancytokeratins polyclonal antibody (red), p-FAK monoclonal antibody (green), and DAPI (blue). Magnification, ×400. (C) Confocal laser scanning microscopy analysis of PBMC cytospins stained with p-FAK monoclonal antibody (green) and pancytokeratins polyclonal antibody (red). Magnification, ×600. Right panel: Quantification of p-FAK and CK co-expression by immunofluorescence microscopy in CTC. (D) Representative micrograph of PBMC cytospins double stained with FAK polyclonal antibody (red) for total protein and with p-FAK monoclonal antibody (green) for activated kinase. Note that FAK is expressed in all the cells, while pFAK is expressed only in a distinct cell. Magnification, ×200. (E) p-FAK expression in CTC after immunomagnetic separation of PBMC with Ep-CAM antibody coated beads and indirect immunofluorescence with pancytokeratins polyclonal antibody (red), p-FAK monoclonal antibody (green), and DAPI (blue). Magnification, ×600.
Finally, additional indication for the specific expression of p-FAK in circulating tumor cells was provided by experiments applying immunomagnetic separation with EpCAM-coated antibody in peripheral blood from two different breast cancer patients. As shown in Figure 1E, double staining of the cytospins with pancytokeratin and pFAK antibodies confirmed the expression of p-FAK in CTC.

Expression of HER2 and Phosphorylated PI-3 Kinase in CK-positive CTC

To evaluate whether CK- and p-FAK-positive cells also express HER2, we analyzed the 28 CK-positive/p-FAK-positive CTC samples by using double-staining immunofluorescence microscopy. HER2 was co-expressed in 15 of 28 (53.6%) CK-positive/p-FAK-positive samples (Figure 2A), implying differential phenotypic patterns for HER2 expression in CTC. Conversely, no HER2-positive cells could be identified in the 17 CK-negative samples (data not shown). As controls, we have examined PBMC cytospin preparations from 20 normal donors for cytokeratin (with A45-B/B3 and polyclonal pan-cytokeratin), HER2, and p-FAK expression. No positive cells were identified in all samples examined.

The identification of p-FAK in all CK-positive CTC prompted us to analyze whether PI-3 kinase, a signaling molecule described to be an up- or downstream effector of FAK (26-28), may also be activated. For this, 17 of 28 CK-positive/p-FAK-positive samples, for which PBMC slide preparations were still available, were further analyzed for the co-expression of CK and p-PI-3 kinase, by using double-staining and confocal laser scanning microscopy. Figure 2B demonstrates a representative experiment of CK and phosphorylated PI-3 kinase co-expression in cytospin preparations. Overall, 15 of 17 samples (88.2%)...
The identity of the phosphorylated proteins shown in CTC co-cultured with PBMC was addressed by immunoblot analysis of these preparations. We confirmed the expression of phosphorylated FAK and PI-3 kinase (Figure 4A,B left panels), indicating that bands with apparent MW 85 and 125 KDa (see Figure 3) most probably correspond to these kinases. In addition, microscopic analysis of cultured PBMC using double staining confocal laser scanning experiments supported these observations. Indeed, using pancytokeratin-phospho-FAK- and phospho-PI-3 kinase antibodies we showed that p-FAK and p-PI-3 kinase are co-expressed with CK and located mainly under cell membrane (see Figure 4A, 4B, right panels). Interestingly, no staining for CK/p-FAK and CK/p-PI-3 kinase could be identified in cell preparations from normal donors (data not shown).

These experiments confirm the expression of activated FAK and PI-3 kinase predominantly in CTC, co-cultured with PBMC from breast cancer patients.

Finally, in an effort to provide experimental evidence that these kinases are exclusively activated in micrometastatic cells, we performed immunomagnetic separation using EpCAM antibodies to divorce epithelial tumor cells from cultured PBMC. Western blot analysis showed clearly that FAK is phosphorylated in cells of epithelial origin expressing CK but not CD45 (see Figure 4C).

**DISCUSSION**

The phenotypic analysis of micrometastatic cells in breast cancer patients may provide valuable information in understanding their biological heterogeneity and, thus, facilitating their effective targeting and eventual elimination (29). In the present study, we have used PBMC cytospins from 45 breast cancer patients, some of whom were known to harbor CTC by previous real-time RT-PCR analysis, to carry out systematic phenotypic analysis of CK-positive CTC preparations by focusing on signaling kinases, implicated in regulating both cell phenotype and, thus, facilitating their effective targeting and eventual elimination.
due to the relatively small number of samples analyzed. Because the samples were selected so that 28 were already known to be CK-19 mRNA-positive by real-time PCR, multiple slides (usually 4-5) were analyzed from each sample to detect CK-positive cells. The primary and secondary antibodies used in double-staining experiments were specifically chosen to be from different species, for example, anti-rabbit, anti-mouse, anti-goat antibodies, to avoid non-specific cross reactivity between the antibody systems. Moreover, single staining experiments further confirmed the presence of the specific markers in the studied cell population.

First, we studied whether phosphorylated FAK may be expressed in CK-positive micrometastatic cell preparations. FAK is a protein tyrosine kinase that is activated by a variety of stimuli, including integrins, growth factors, steroid hormones, cytokines, and neuropeptides (21,30,31). FAK is involved in tumor development, and it has been shown that primary human cancer cells or cell lines overexpress the protein in its phosphorylated form (32,33). In the present study, it was clearly demonstrated...
that all CK-positive but not CK-negative samples express p-FAK. This was evident with immunofluorescence and confocal laser scanning microscopy experiments. The expression of p-FAK in cells that were isolated on the basis of the EpCAM expression by immunomagnetic separation further confirms the epithelial origin of these cells. An interesting observation was the identification of both CK-positive/p-FAK-positive, and CK-positive/p-FAK-negative cells in samples derived from two different patients. This finding underlines the heterogeneity of CTC, thus supporting similar data from previously published reports (34,35). FAK transmits signals by activating downstream signaling molecules such as PI-3 kinase (26,28,31). In line with these reports, it was demonstrated here that 15 of 17 available CK-positive/p-FAK-positive samples co-expressed CK and p-PI-3 kinase, indicating a 88.2% correlation in the expression patterns of p-PI-3K and CK, as compared with 100% for p-FAK.

In addition to the microscopic analysis of breast cancer patients’ CTC, we report here the successful elaboration of micrometastatic cells and PBMC co-cultures from two independent patient samples, both tested CK-19-positive. Immunoblot analysis of the cell extracts following four week cultures revealed the specific expression of p-FAK and p-PI-3K in micrometastatic cells. The almost unanimous specific expression of p-FAK in CK-positive CTC shown in this study supports a pivotal role for this kinase in the biologic behavior of these cells. Furthermore, the co-expression of p-PI-3 kinase in the majority of CK-positive/p-FAK-positive samples corroborates that a signaling pathway involving FAK and PI-3 kinase may be activated in micrometastatic breast cancer cells. However, this assumption needs to be further investigated.

Recent reports indicate a potentially important role of actin organization and dynamics in regulating cell phenotype during malignant transformation (19,36,37,38). In line with these reports, the morphological analysis of actin cytoskeleton organization in CK-positive micrometastatic cells presented in this study showed limited actin staining at the cell periphery and impaired actin organization, reminiscent of that observed in malignant MCF-7 human breast cancer cells (23). These findings add another line of evidence for the malignant cell phenotype of these cells.

The HER2/neu oncoprotein is a transmembrane glycoprotein receptor sharing sequence homology with the epidermal growth factor receptor (39), and its amplification in primary tumor cells is associated with a higher probability of disease relapse (40). In a study where 60% of patients had occult bone marrow cells co-expressing cytokeratin-18 (CK-18) and HER2/neu molecules, the detection of HER-2-positive DTC was an independent prognostic factor for overall survival (11). Furthermore, in a study by Wulfing et al. (41), the presence of HER2-positive CTC correlated with a significantly de-

Figure 5. Actin cytoskeleton organization in CTC. (A) Representative micrographs of confocal laser scanning microscopic sections of PBMC cytospins showing the actin staining with rhodamine-phalloidin (red, a-c), CK-staining with pancytokeratin A45 B/B3 monoclonal antibody (green, p-d-f), and overlay sections (g-i). Magnification, ×600. (B) Actin organization in malignant (MCF7) and non-malignant (MCF12A) human breast cancer cells stained with rhodamine-phalloidin.
creased disease-free survival and overall survival. The finding of the present study showing HER2 co-expression in 54% CK-positive/p-FAK-positive samples further confirms the reported heterogeneity for HER2 expression in micrometastatic cells (11,29). The biological importance of HER2 expression for the survival of micrometastatic cells has been demonstrated in a previous study by our group, where the administration of anti-HER2 specific antibody trastuzumab successfully eliminated the chemotherapy-resistant DTC and DTC in the majority of heavily pretreated breast cancer patients (29).

To our knowledge, this is the first report demonstrating expression of activated FAK and PI-3K in micrometastatic breast cancer cells. Although the present study has certain limitations, primarily due to the relatively small number of samples studied and the low numbers of micrometastatic cells present in each sample, our findings suggest that analysis of p-FAK in peripheral blood samples may represent a new marker for the detection of micrometastatic cells in breast cancer patients. Our findings of the activation of these signaling molecules may also have clinical implications. Novel targeted therapies using biological agents, specific for certain activated kinases, may be used successfully in eliminating treatment-resistant micrometastatic cells (42,43).

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