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

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Several markers have been used to detect circulating tumor cells (CTC) 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 co-culture 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.

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 dis-seminated 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 bone marrow or the peripheral blood of patients with breast cancer. These markers usually represent proteins encoded by genes, which are thought to be tissue specific and are expressed on epithelial but not on hematopoietic cells. The intermediate filament cytokeratin 19 (CK-19), which is stably and abundantly expressed in the majority of epithelial tumor cells is one of the most frequently used markers. Phenotypic analysis of the CK-positive DTC has demonstrated that they frequently express HER2/c-neu (11,12) as well as EpCAM (13) molecules. Furthermore, the genetic heterogeneity at the level of single DTC has been documented by single cell genomic hybridization (14). These observations further support the biologic heterogeneity of DTC as has been previously shown (15-17). However, phenotypic analysis of CK-positive CTC to evaluate expression of activated signaling kinases implicated in cellular transformation, migration, and survival pathways has not been addressed so far.

Recent studies suggest that the dynamic state of actin cytoskeleton is strongly correlated with the expression of malignant cell phenotype (18,19). In addition, actin cytoskeleton rearrange-

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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/PI-3 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 PI-3 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 PI-3K 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-PI-3K 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, PI-3 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 density gradient (d = 1.077gr/mol) centrifugation at 1800 rpm for 30 min. Mononuclear cells (MNCs) were washed three times with PBS and centrifuged at 1500 rpm for 10 min. Aliquots of 200,000 cells were centrifuged at 2000 rpm for 2 min on glass slides. Cytospins were dried and stored at –80°C before using. Four to five slides from each patient were used for staining experiments. In addition to double staining experiments, all additional stains for other markers were carried out using different slides from the same patient's blood sample.

Cell Cultures

The MCF7 mammary adenocarcinoma cells (obtained from ATCC; American Type Culture Collection) were cultured in v/v 1:1 Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 30 mM $NaHCOB_{3B}$, 16 ng/mL insulin, and 50 mg/mL penicillin/streptomycin. The MCF12A, nontumorigenic epithelial cells (obtained from ATCC) were cultured in v/v 1:1 DMEM/F12/Ham's F12 medium supplemented with 5% horse serum, 30 mM $NaHCO_3$, 10 mg/mL insulin, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, 20 ng/mL Epidermal Growth Factor (EGF), 50 mg/mL penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% COB_2-95% air. Subcultivation for all cell lines was performed with 0.25% trypsin and 5 mM EDTA. All experiments were performed during the logarithmic growth phase. Fifteen to 20 h prior to the experiments, cells were transferred to serum-starved medium containing only L-glutamine, $NaHCOB_3$, and penicillin/streptomycin.

Micrometastatic cell culture was performed according to Solakoglu et al. (16) with the following adjustment for PBMC: peripheral blood mononuclear cells were isolated with Ficoll-Hypaque (d = 1.077 gr/mol) density as described above and $30 \times 10P^6$ 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) fatal 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% COB_2 $_B$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 cytospins 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 cytospins of the same patients, we further investigated phospho-FAK, HER2, and phosho-PI-3 kinase expression, with dou-

ble staining experiments and confocal laser scanning microscopy as previously described (25). 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 cytospins 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 cytospins. 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 = 1.077gr/mol) 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 deoxy-cholate, 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 supplied from Molecular Probes Inc. (Eugene, OR, USA). Pancytoketatins, FAK, CD45 PI3-kinase anti-rabbit, phospho-tyrosine anti-mouse, 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). Immuno-magnetic 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 cytospins 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
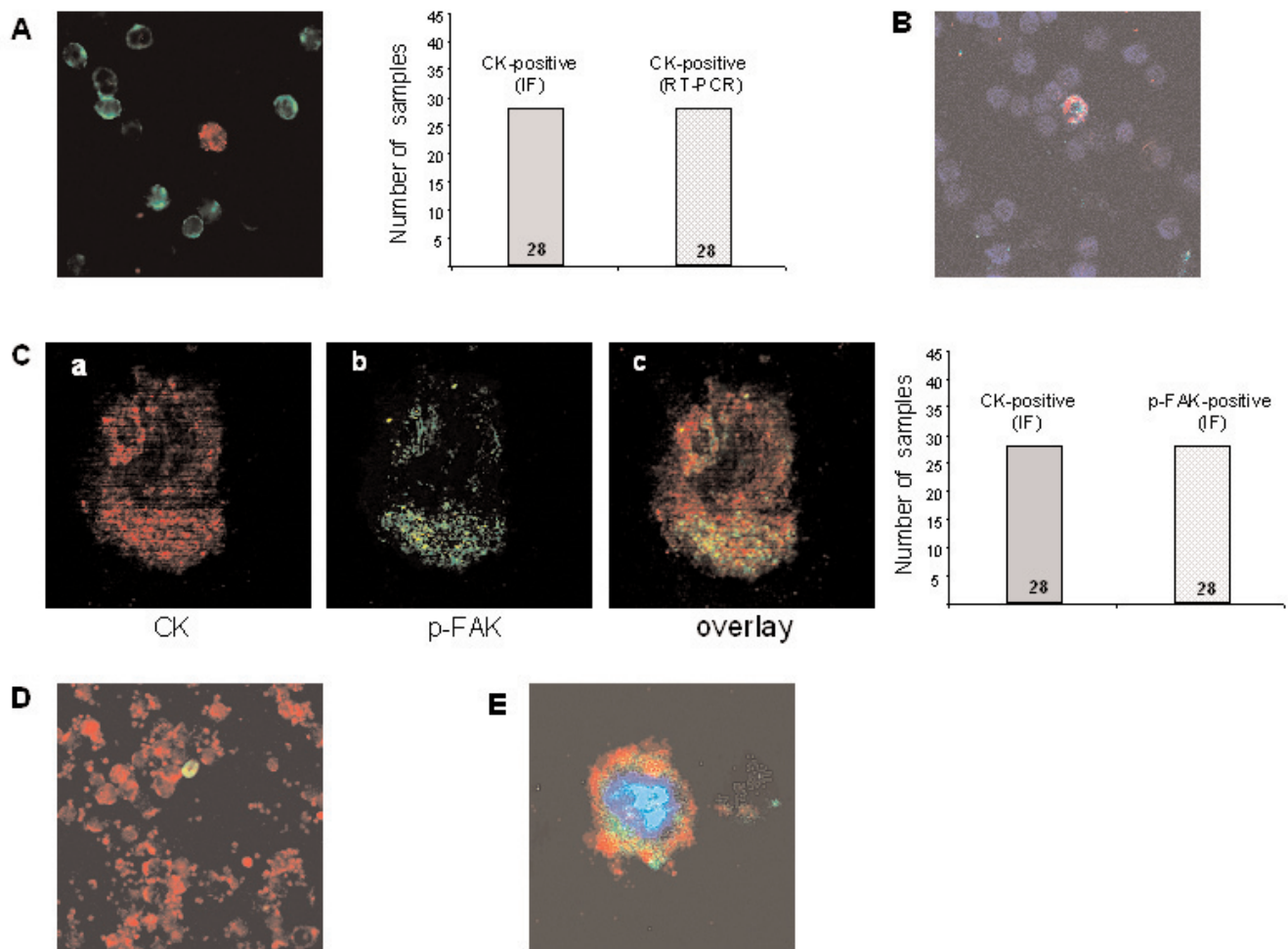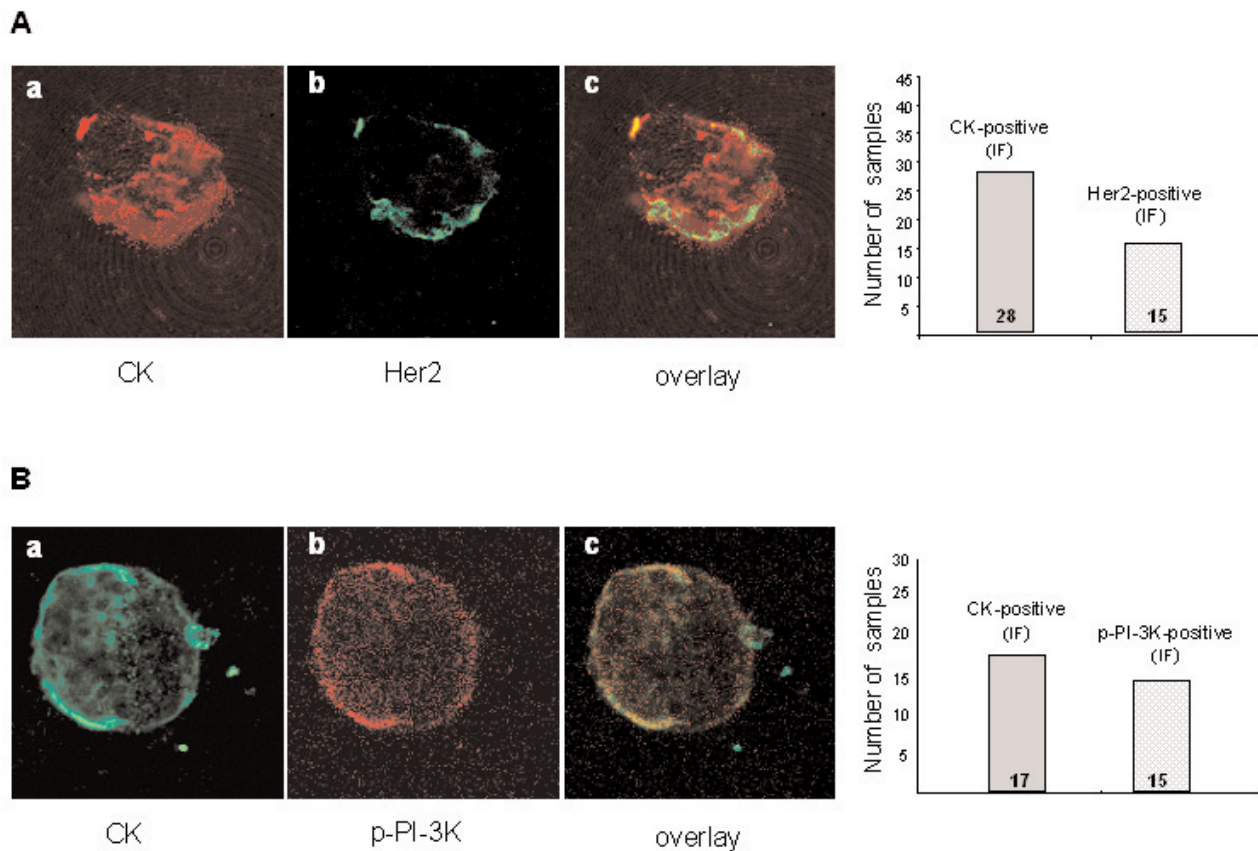
Figure 1. Cytokeratin and p-FAK expression in CTC from breast cancer patients. (A) Representative micrograph of cytospin, double stained with CD45 monoclonal antibody (green) for hematopoietic cells and with pancytokeratins polyclonal antibody (red) for circulating tumor cells. Magnification, ×400. Right 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.

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 2. Her2 and p-PI-3K expression in CTC from breast cancer patients.
(A) Representative micrographs of confocal laser scanning microscopic sections of PBMC cytospins stained with pancytokeratins poly-clonal antibody (red, a), with Her2 monoclonal antibody (green, b), and overlay section (c). Magnification, ×600. Right panel: Quantification of Her2 and CK co-expression by immunofluorescence microscopy in CTC. (B) Representative micrographs of confocal laser-scanning microscopic sections of PBMC cytospins stained with pancytokeratins monoclonal antibody A45 B/B3 (green, a), with p-PI-3 kinase polyclonal antibody (red, b), and overlay section (c). Magnification, ×600. Right panel: Quantification of p-PI-3 kinase and CK co-expression by immunofluorescence microscopy in CTC.

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 ana-lyzed 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 pheno-typic 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 poly-conal 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 mole-cule described to be an up- or down-stream 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 ki-nase, by using double-staining and con-focal laser scanning microscopy. Figure 2B demonstrates a representative experi-ment of CK and phosphorylated PI-3 ki-nase co-expression in cytospin prepara-tions. Overall, 15 of 17 samples (88.2%)

Normal PBMCs | Breast cancer PBMCs

IB:P-Tyr

250 →
150 →
100 →
75 →
50 →
37 →

IP:P-Tyr
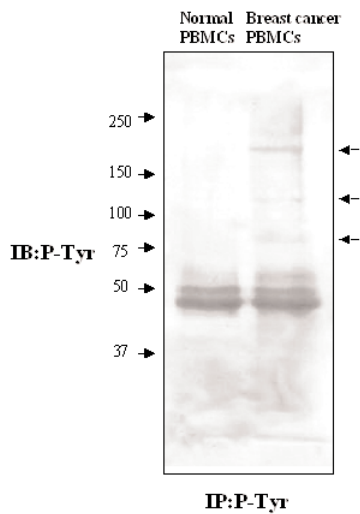
Figure 3. Tyrosine phosphorylation in cultured CTC extracts from breast cancer patients. Cells from breast cancer patients and normal donors cultured for four weeks were lysed, and equal protein amounts were immunoprecipitated (IP) with a phosphotyrosine (p-Tyr) antibody. The tyrosine-phosphorylated proteins were detected by immunoblotting (IB) using a p-Tyr antibody.

were tested positive for p-PI-3 kinase (see Figure 2B, right panel).

Detection of phosphorylated FAK and PI-3 kinase in cultured micrometastatic cells

So far, our results indicate specific expression of phosphorylated -FAK and in a lesser extent PI-3 kinase in micrometastatic cells. To further confirm these findings, we assessed the tyrosine phosphorylation imprint in protein extracts of CTC cultures, established from two breast cancer patients PBMC, tested positive for CK-19 by real-time PCR. Cells cultured for four weeks as described in materials and methods were lysed and extracted proteins were immunoprecipitated using an anti-phosphotyrosine monoclonal antibody. Representative result from these experiments is shown in Figure 3. Expression of various phosphorylated proteins was evident, three of them being more prominent at the

ranges of KDa 85, 120, and 180, respectively. Interestingly, these bands were absent in cultured PBMC from normal female blood donors (Figure 3, left panel).

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).

Actin Cytoskeleton Morphology in Micrometastatic Cells Is Reminiscent of Malignant Cell Phenotype.

Activation of FAK followed by PI-3 kinase phosphorylation has been reported to induce reorganization of actin cytoskeleton morphology in human breast or prostate cancer cells (23,28). Accordingly, in a total of five CK-positive/p-FAK-positive/p-PI-3K-positive samples, we evaluated whether actin organization of micrometastatic cells has morphological

characteristics reminiscent of malignant phenotype by studying actin cytoskeleton distribution in CK-positive/p-FAK-positive samples. By using confocal laser scanning microscopy after double staining of the samples with rhodamine-phalloidin for actin and A45-B/B3 for cytokeratins, we found that the actin network (red) was distributed at the cell periphery without obvious existence of structural elements for example, stress fibers, lamelipodia, or filopodia (Figure 5A,a-c). Figure 5 (A,d-f) shows the cytokeratin distribution (green), supporting the epithelial origin of these cells. Interestingly, a partial co-localization of actin and cytokeratin elements was evident (see Figure 5A,g-i) (yellow), indicating possible interactions between these cytoskeletal proteins. The observed actin cytoskeleton morphology is reminiscent of that seen in MCF7 human breast cancer cells, rather than the structural organization showing a well-formed microfilament network including stress fibers that were observed in non-malignant MCF12A cells (see Figure 5B). It should be noted that almost identical morphological patterns, as shown in Figure 5A, were observed in all the five samples analyzed (data not shown). From these findings, it was concluded that actin organization in micrometastatic cells is very similar to that described for the malignant cell phenotype.

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 motility and survival. We found a high concordance rate between the results of RT-PCR and CK staining, which may be
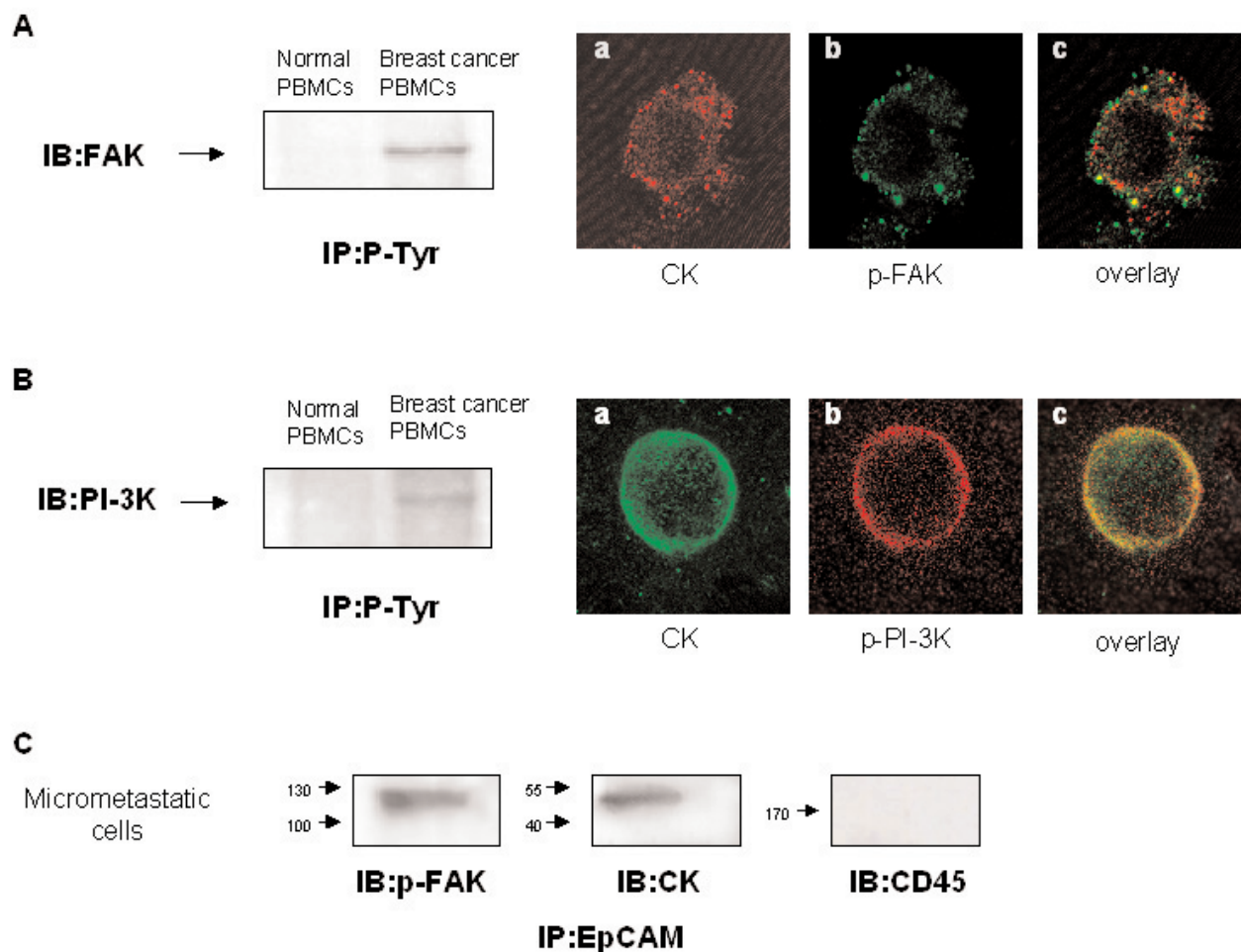
Figure 4. P-FAK and p-PI-3K expression in cultured CTC from breast cancer patients. (A) Proteins obtained from breast cancer patients and normal female blood donors cultured cells were immunoprecipitated (IP) with a phosphotyrosine (p-Tyr) antibody. FAK was identified by western blot analysis using an anti-FAK polyclonal antibody. Right panels: Representative micrographs of confocal laser scanning microscopic sections of cultured PBMC stained with pancytokeratins polyclonal antibody (red a), p-FAK monoclonal antibody (green b), and overlay section (c). Magnification, ×600. (B) As described in for section A. PI-3 kinase was identified by western blot analysis using an anti-PI-3 kinase polyclonal antibody. Right panel: Representative micrographs of confocal laser scanning microscopic sections of cultured PBMC stained with pancytokeratins monoclonal antibody (green a), p-PI-3K polyclonal antibody (red b), and overlay section (c). Magnification, ×600. (C) p-FAK and CK expression in cultured CTC after immunomagnetic separation with EpCAM antibody and western blot analysis with p-FAK and CK antibodies respectively. Note that CD45 is not expressed.

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 prepara-tions. 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
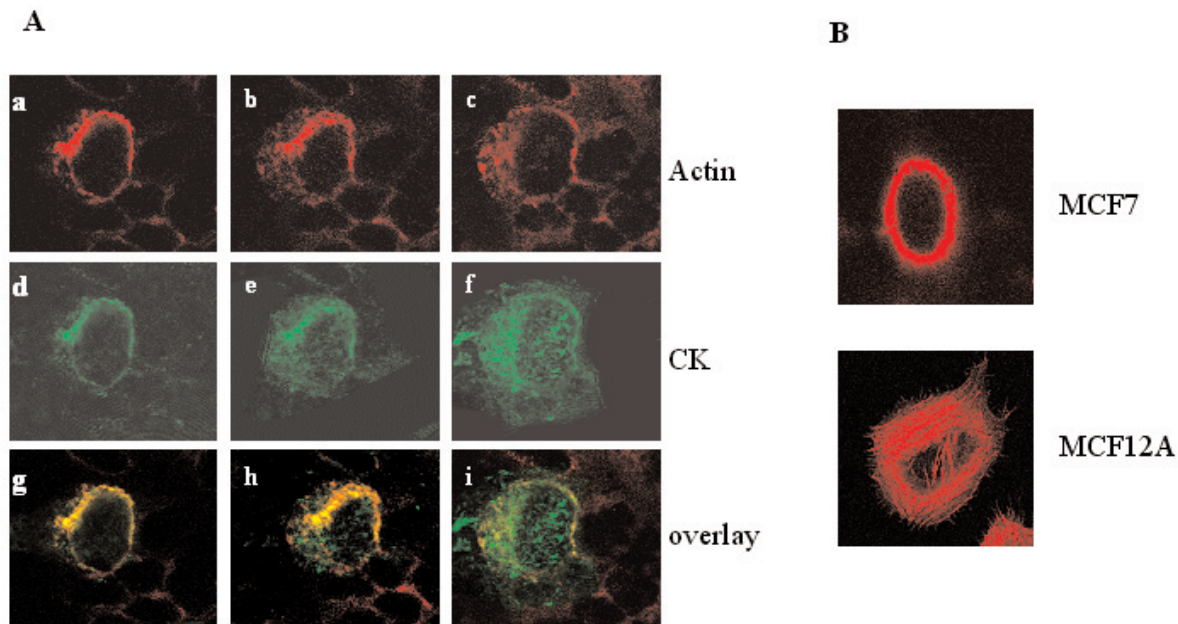
Figure 5. Actin cytoskeleton organization in CTC. (A) Representative micrographs of confocal laser scanning microscopic sections of PBMC cytospins showing the actin staining with rhodamine-phalloidin (red, a-c), CK-staining with pancytokeratin A45 B/B3 monoclonal antibody (green, d-f), and overlay sections (g-i). Magnification, ×600. (B) Actin organization in malignant (MCF7) and non-malignant (MCF12A) human breast cancer cells stained with rhodamine-phalloidin.

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 and 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-

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 CTC 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).

ACKNOWLEDGMENTS

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