Relationship Between Circulating Tumor Cells and Peripheral T-Cells in Patients with Primary Breast Cancer

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Abstract. *Background: Detection of circulating tumor cells (CTCs) in the peripheral blood of patients with primary breast cancer is associated with poor clinical outcome. Recent studies have found evidence for immunological influence on tumor cell dormancy. We therefore investigated the relationship between peripheral T-cells and CTCs, as immunological factors may contribute to the fate of CTCs. Materials and Methods: The peripheral blood immune status of 116 patients with primary breast cancer was analyzed by flow cytometry. Results were correlated with the presence of CTCs and clinicopathological parameters of these patients. Results: Appearance of CTCs was significantly associated with grade III tumors ($p<0.05$). Interestingly, CTC-positive patients presented with a significant increase of peripheral CD95(FAS)-positive T-helper cells. As immune response is regulated by CD95(APO-1/FAS)-CD95ligand interaction and tumor cells induce apoptosis via the CD95/CD95L (ligand) pathway, this might lead to tumor cell escape by apoptotic T-helper cells. Conclusion: Absence of T-cell help at the time of priming may result in a loss of long-term antigen-activation of CD8 lymphocytes and could lead to an ineffective anti-tumor cell response. This might contribute to systemic immunosuppression and open the door for tumor cell dormancy.*

In breast cancer, it has been demonstrated that presence of circulating tumor cells (CTCs) at first diagnosis is associated with reduced disease-free and overall survival (1). Years after diagnosis, persistent dormant tumor cells may return to an active state and initiate metastatic replapse. However, the mechanisms which contribute to this activation of dormant tumor cells and metastatic disease are not fully-understood. Besides evidence for genetic predisposition of dormant tumor cells (2, 3) and mechanisms of angiogenesis (4, 5), immunological factors that trigger tumor escape (6-8) are also discussed. Immunosurveillance is impaired by functional impairment of T-cells, which extend into the tumor microenvironment and can also be seen in peripheral blood lymphocytes (PBLs) (9, 10). The aim of this study was to analyze the effect of CTCs on the quantitative distribution of T-cell subsets in order to offer further insight into possible tumor-induced immune suppression.

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Key Words: Breast cancer, circulating tumor cell, immunosurveillance, tumor cell dormancy, T-cell, FAS.

Materials and Methods

A total of 116 patients with primary breast cancer were recruited routinely from the Department of Obstetrics and Gynecology of the University Hospital of Tübingen between 2006-2007. Heparinized venous blood (15 ml) was drawn from each patient before surgery. Exclusion criteria of this study were previous simultaneous secondary malignant disease, breast cancer recurrence, neo-adjuvant chemotherapy or hormone therapy, serious functional disorders of liver and kidney, and metabolic disease. For retrospective analyses, approval of the local Ethical Committee (University of Tübingen) was obtained (59/2007V).

Tumor cell selection and detection by AdnaTestBreastCancer™ assay. Blood samples were processed not later than four hours after blood withdrawal. Prior to analysis, samples were stored at 4˚C. For selection of CTCs, commercially available AdnaTestBreast CancerSelect (AdnaGen AG, Langenhagen, Germany), which enables for immunomagnetic enrichment of tumor cells *via* epithelial and tumor-associated antigens, was used according to the manufacturer's instructions (11). After extraction of labeled cells by magnetic particle concentrator, mRNA isolation from lysed tumor cells was performed by the Dynabeads mRNA DIRECT™ Micro Kit (Dynal Biotech GmbH, Hamburg, Germany), which is part of the AdnaTestBreastCancerDetect (AdnaGen AG, Langenhagen, Germany). Subsequently cDNA was obtained by reverse transcription and transcripts of mucin-1, cell surface-associated

(MUC1), human epidermal growth factor receptor-2 (Her2) and gastrointestinal tumor-associated antigen-2 (GA733-2) were amplified by multiplex PCR (12 -13), used the HotStarTaq Master Mix (QIAGEN Hamburg GmbH, Hamburg, Germany). Details of this system have been described elsewhere (14).

Cell isolation and staining for flow cytometry. After collection, blood samples were obtained on ice and stained/analysed not later than five hours. FACS-Lyse-Reagent (Becton Dickinson, Heidelberg, Germany) of 0.5 ml was added to 100 μl EDTA blood in order to recover peripheral blood mononuclear cells (PBMCs) by erythrocytic and thrombocytic lysis. After incubation (10 min), cells were washed in Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies, Inc., Grand Island, NY, USA) by centrifugation. Aliquots of 5×10^5 PBMCs were stained with 10 μl directly conjugated mouse anti-human monoclonal antibodies CD3-PerCP, CD4-APC, CD8-APC, CD95-FITC (Becton Dickinson, Heidelberg, Germany) and CD28-RPE (Dako, Hamburg, Germany). Intracellular staining with 10 μl TCR-ζFITC (Biozol, Munich, Germany) was enabled by addition of 10 μl/ml digitonin working solution (Sigma, Deisendorf, Germany). All cells were incubated with the antibodies for up to 60 min on ice, then washed twice in PBS and fixed with 0.5% paraformaldehyde in PBS prior to flow cytometric analysis. Propidium iodide-negative viable cells were detected by three color flow cytometry using a FACSCalibur with CELLQuest software (Becton Dickinson). Typically, 20,000 events were collected and the data were expressed as dot plots.

Statistical analysis. Cell populations from 116 patients with primary breast cancer were analyzed by their absolute number of cells. The statistical significance of differences between T-cell populations were determined by a nonparametric Mann-Whitney *U*-test for the unpaired analysis. To evaluate the statistical difference of the clinicopathological features, Fisher's exact test was used. Statistical analysis was performed using SPSS for Windows (version 11.5; Chicago, IL, USA). *p*-Values <0.05 were considered to be significant.

Results

Patients' characteristics. A total of 116 patients were included in the study. Their clinicopathological data are summarized in Table I.

Correlation of CTCs with established prognostic markers. There was no significant correlation between CTC positivity and tumor stage, lymph nodal status, hormone receptor status, HER2 status, menopausal status and tumor size. However patients with CTCs presented more often with tumor histological grade III than CTC-negative cases (Table I; $p=0.048$).

Quantitative distribution of peripheral T-cells in patients with and without CTCs. CTCs had no significant correlation with the quantitative distribution of peripheral T-cells (Table II). Patients with CTCs have a higher number of $CD3^+$cells/μl compared to patients without CTCs ($p=0.858$). Concerning cytotoxic T-cells, we found a similar number of $CD3^+/CD8^+$cells/μl in CTC-positive and CTC-negative cases ($p=0.889$). The number of T-helper cells (CD3+/CD4+) was also not significantly associated with the number of CTCs and did not differ in CTC-positive and CTC-negative cases ($p=0.739$).

Table I. *Clinicopathological characteristics of patients with breast cancer in this study.*

		CTC-positive n (%)	CTC-negative n (%)	*p*-Value
	n (%)	n (%)	n (%)	
Total	116	13 (11)	103 (89)	
Tumor stage				0.627
T1*	80 (69)	9 (69)	71 (69)	
T2-4	36 (31)	4 (31)	32 (31)	
Nodal status				0.148
N0	77 (66)	6 (46)	71 (69)	
N+	37 (32)	6 (46)	31 (30)	
Nx	2 (2)	1 (8)	1 (1)	
Distant metastasis				0.061
M0	112 (97)	11 (85)	101 (98)	
M1	4 (3)	2 (15)	2 (2)	
Estrogen receptor status				0.435
Positive	100 (86)	12 (92)	88 (85)	
Negative	16 (14)	1 (8)	15 (15)	
Progesteron receptor				0.527
Positive	101 (87)	11 (85)	90 (87)	
Negative	15 (13)	2 (15)	13 (13)	
Menopausal status				0.221
Premenopausal	23 (20)	1 (7)	22 (21)	
Postmenopausal	93 (80)	12 (93)	81 (79)	
Grading**				0.048
G1+G2	91 (78)	7 (54)	84 (82)	
G3	15 (13)	4 (31)	11 (11)	
HER2neu status				0.618
Positive	12 (10)	1 (7)	11 (11)	
Negative	99 (85)	11 (85)	88 (85)	
Not evaluated	5 (4)	1 (7)	4 (4)	

*Includes 10 cases with additional occurrence of ductal carcinoma *in situ*, **includes cases of invasive carcinoma.

Effect of the presence of CTCs on the expression of CD95 (FAS). The number $CD3^+/CD95^+$cells was significantly increased in CTC-positive cases ($p=0.042$). This significant upregulation of CD95 is only seen in the T-helper sub-population (Table II; $p=0.025$). Flow cytometry distinguishes between $CD95^{pos}/CD3^+/CD4^+$ and $CD95^{neg}/CD3^+/CD4^+$T-helper populations (Figure 1). Cytotoxic T-cells also exhibited an increase of CD95 expression, but not significantly ($p=0.108$).

Expression of TCR-ζ chains and CD28 in patients with primary breast cancer with and without CTCs. The number of $CD3^+/CD28^+$ cells/μl did not differ significantly between CTC-negative and positive cases. A quantitative change of

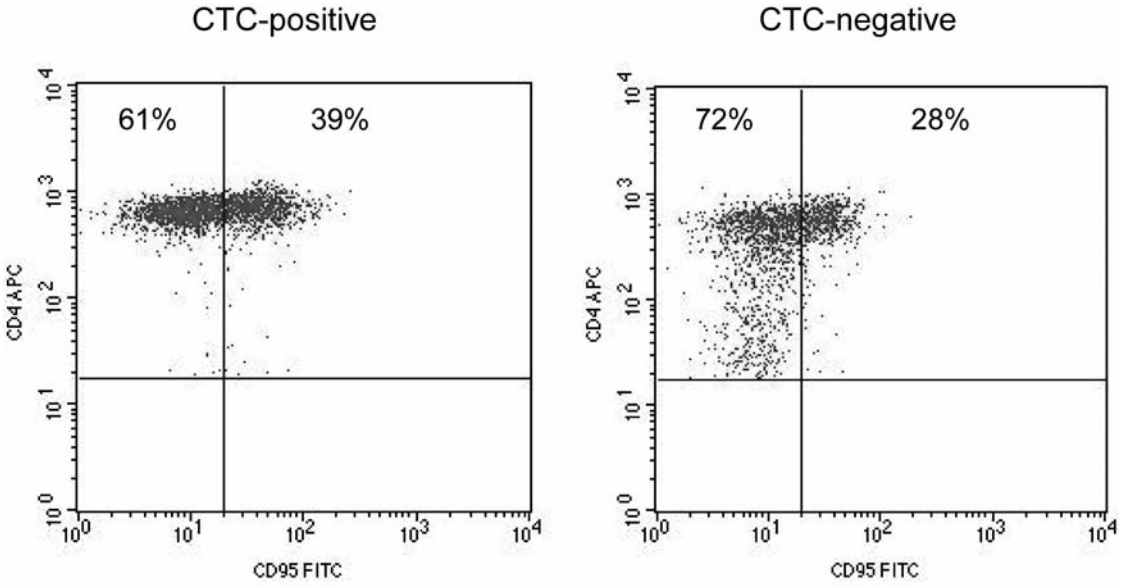

Figure 1. *Detection of CD95-positive T-helper cells by flow cytometry in circulating tumor cell (CTC)-positive and CTC-negative breast cancer cases.*

Table II. *Quantitative distribution of peripheral T-cells in patients with breast cancer with and without circulating tumor cells (CTCs).*

Phenotype	Description	Total number of viable eells/μI mean±SD		*p*-Value
		CTC-positive (n=13)	CTC-negative (n=103)	
$CD3^+$	T-Cell	1202±311	1189±446	0.858
$CD3^+/CD28^+$	Activated naive T-cell	1023±296	999±377	0.871
$CD3^+/TCR\text{-}\zeta^+$	ζ-Chain expression of T-cell	1195±313	1123±444	0.468
$CD3^+/CD95^+$	FAS-positive T-cell	502±348	315±285	0.042
$CD3^+/CD8^+$	T-Cytotoxic(Tc)cell	360±222	365±194	0.889
$CD3^+/CD8^+/CD28^+$	Activated naive Tc-cell	231±76	237±112	0.955
$CD3^+/CD8^+/TCR\text{-}\zeta^+$	ζ-Chain expression of Te-cell	358±222	353±190	0.997
$CD3^+/CD8^+/CD95^+$	FAS-positive cytotoxic T-cell	156±165	95±105	0.108
$CD3^+/CD4^+$	T-Helper(Th)cell	842±243	822±329	0.739
$CD3^+/CD4^+/CD28^+$	Activated naive Th-cell	807±264	781±322	0.615
$CD3^+/CD4^+/TCR\text{-}\zeta^+$	ζ-Chain expression of Th-cell	838±244	775±334	0.403
$CD3^+/CD4^+/CD95^+$	FAS-positive T-helpercell	340±184	220±192	0.025

TCR-ζ (p=0.468) and CD28 (p=0.871)-expresssing T-cells was not associated with the number of CTCs, as was also seen for $CD4^+$ and $CD8^+$ sub-population (Table II).

Discussion

Immunosurveillance is not always effective in controlling tumor growth. One reason can be attributed to functional impairment of T-cells. Studies have described the depressed functional potential of T-lymphocytes as a link between ineffective TCR signaling and spontaneous apoptosis of circulating lymphocytes (15-19). In addition the TCR signal is complemented by CD28-mediated co-stimulation (10). Compared to healthy controls, patients with breast cancer present with a significant down-regulation of CD28 and TCR-ζ chains in the peripheral cytotoxic T-cell population (9). Moreover, patients with breast cancer have significantly more CD95 (FAS)-expressing cytotoxic T-cells than their healthy counterparts (9). As immune response is regulated by CD95 (APO-1/FAS)-CD95L (ligand) interaction, breast tumor cells could directly induce apoptosis of activated $CD95^+$ T-cells *via* the CD95/CD95L pathway (18).

Relationship between CTCs and FAS(CD95)$^+$ T-helper cells. In our study, patients with CTCs presented a significant increase of peripheral FAS(CD 95)$^+$ T-helper cells. Müschen *et al*. (18) proposed that *CD95L* mRNA expression in breast cancer is closely-correlated with depletion of peripheral blood T-lymphocytes. Moreover, Rabinovich *et al*. (15) demonstrated the link between tumor-associated CD95L expression and the reduction of distinct T-cell subsets in ovarian carcinoma. Therefore it has been postulated that tumor cells can evade immune attack by expression of CD95L and induction of T-cell apoptosis *via* the CD95/CD95L pathway (20, 21). CD4$^+$ T-cells also play a relevant role in tumor immunity and an increased presence of CD4$^+$/CD95$^+$ T-helper cells may have a negative impact on anti-tumor T-cell response. It was shown that CD4$^+$ T-cells not only support the activation and expansion of cytotoxic T-lymphocytes (22), but their presence is also essential at the time of T-cell priming in order to generate effective CD8$^+$ T-cell memory (23, 24). Additionally, it was demonstrated that the absence of T-cell help at the time of priming results in a loss of long-term antigen-activation of CD8$^+$ lymphocytes (25). Therefore, function of CD4$^+$ T-cells directly relates to effective anti-tumor response. Significant up-regulation of CD95 in peripheral T-helper cells of patients who were CTC-positive may open the door to tumor cell escape and eventually have further influence on the primary and metastatic setting of the disease, as prognostic potential of CTCs has been shown (26, 27).

TCR signaling and CD28 mediated co-stimulation of T-cells in CTC-positive cases. Studies have shown that peripheral T-cells from patients with primary breast cancer, exhibit abnormally low levels of the TCR signaling molecule, such as TCR-ζ for development of an antigen-specific T-cell response (9, 28). Moreover, it has been seen that CD28, an essential signal for T-cell activation and differentiation, is down-regulated on peripheral cytotoxic T-cells in breast cancer patients (9, 29). This implies less activated potent effector cells for tumor defense because of ineffective T-cell priming and reduced T-cell proliferation (9, 30, 31).

In our study, patients with CTC-positive breast cancer had similar numbers of TCR-ζ$^+$ and CD28$^+$ cytotoxic T-cells, as described before in patients with primary breast cancer (9). Concerning the expression of TCR-ζ and CD28 on T-cells there was no statistical significant difference between CTC-negative and CTC-positive breast cancer cases. Therefore, the TCR-ζ and CD28 signal on peripheral cytotoxic T-cells does not appear to be affected by CTCs. This contributes to the hypothesis of immuno-tolerance with the option of tumor cell dormancy, as interaction of cytotoxic T-cells with tumor cells might fail.

Association of CTCs with histological grade of the tumor. Histological grade III of the primary tumor was significantly associated with the presence of CTCs in our analysis. Several other studies have also shown that CTC-positive breast cancer is associated with a biologically aggressive phenotype (32-37). In this case, tumor cells may easily shed from the primary tumor and enter the blood circulation, where a subset of these disseminated tumor cells may persist in a state of dormancy for years (37).

Conclusion

Results of our study allow the hypothesis that the significant increase of peripheral CD95(FAS)-positive T-helper cells in patients with CTC-positive breast cancer might lead to tumor cells escape. Moreover, systemic immunosuppression and tumor cell dormancy might be associated with the fact, that appearance of CTCs do not affect the TCR-ζ and CD28 signal on peripheral cytotoxic T-cells and that presence of CTCs is linked to the biologically aggressive phenotype of the tumor. Further ongoing studies will contribute to verify the influence of CTCs on the adaptive immune system.

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