Short communication

Clinical relevance and current challenges of research on disseminating tumor cells in cancer patients

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Introduction

Despite complete resection of their primary tumors, breast cancer patients still harbor a considerable risk of metastatic relapse caused by minimal residual disease (MRD). To identify single disseminated tumor cells (DTC) in the bone marrow (BM) and circulating tumor cells (CTC) in the peripheral blood undetectable even by high-resolution imaging technologies, sensitive and specific assays have been developed [1,2].

BM plays the most prominent role among the distant organs as an indicator organ for MRD. Moreover, BM appears to be a common homing organ for DTC derived from various types of epithelial tumors [3]. Although BM is easily accessible by aspiration through the iliac crest and is a routine diagnostic method for patients with leukemias and lymphomas, BM analysis is an invasive procedure not yet introduced in the clinical management of solid tumor patients. In contrast, peripheral blood analyses are more convenient and less stressful for patients than BM analyses, and many research groups are currently evaluating the clinical utility of CTC for assessment of prognosis and monitoring of systemic therapy. Previous findings suggest that DTC/CTC are capable of surviving chemotherapy probably by persisting in a dormant nonproliferating state for many years [4,5]. Detection and characterization of DTC/CTC provide the potential to monitor systemic tumor cell dissemination in BM and blood, and to identify therapeutic targets on DTC/CTC that might contribute to an improved individualized targeted treatment of cancer patients.

Detection of disseminated tumor cells/circulating tumor cells

Several methods for the detection of DTC/CTC from BM aspirates and blood have been developed to enrich these rare cells [6,7]. Methods to screen BM aspirates or peripheral blood for DTC/CTC after enrichment can be classified into cytometric/immunological approaches and molecular approaches [1,8,9]. Detection by immunocytochemistry [3,8] enables the characterization of both cell size and shape as well as the nucleus–cytoplasm relationship of each individual event, thereby excluding BM-derived or blood cells with weak expression of the protein of interest. Because of the absence of tumor-specific target antigens, epithelial-specific antigens such as cytoskeleton-associated cytokeratins, surface adhesion molecules, or growth factor receptors are still the markers of choice for the detection of DTC/CTC [1,8]. To overcome the problem of strongly varying detection rates of DTC/CTC in BM and blood from nonmetastatic breast cancer patients (for review see [2,10]), a concept for the detection and enrichment of DTC in BM has been proposed that includes criteria to evaluate morphology and staining results after automatic microscopic screening [11-14].

Viable DTC/CTC defined by their ability to secrete individual proteins after short-term culture can be detected by the EPISPOT (epithelial immunospot) technique. Using this approach, Muc-1-secreting and/or CK19-secreting DTC could be demonstrated in BM samples of breast cancer patients with (90%) and without (50%) overt metastases [15]. Furthermore, in a small series of 45 breast cancer patients, the detection of full-length CK19 released by cells using this technique correlated with the presence of overt metastasis and a reduced survival [16].

Progress towards a standardized method for CTC detection in peripheral blood was reached through the introduction of the CellSearch system (Veridex, Warren, NJ, USA), an automated enrichment and immunostaining device that has been cleared by the US Food and Drug Administration for the detection of CTC in patients with metastatic breast cancer, colon cancer and prostate cancer [17-24]. Furthermore, with

BM = bone marrow; CTC = circulating tumor cells; DTC = disseminated tumor cells; MRD = minimal residual disease; PCR = polymerase chain reaction; RT = reverse transcriptase.

the recently presented CTC-chip – a microfluid platform consisting of anti-EpCAM antibody-coated microposts capable of capturing CTC from unfractionated blood under precisely controlled laminar flow conditions – Nagrath and colleagues detected CTC in almost all cancer patients independent of the stage of the disease [25]. CTC counts 2 to 3 log units higher than those obtained by the US Food and Drug Administration-approved method were reported by Pachmann and colleagues in nearly 100% of breast cancer patients using the MAINTRAC™ assay. In this analysis, after erythrocyte lysis, fluorescently labeled EpCAM-positive/CD45-negative cells are counted by laser scanning cytometry [26]. Furthermore, ultraspeed automated digital microscopy fiberoptic array scanning technology and laser printing techniques allow ultrafast evaluation of images [27-29]. Very recently, Talasaz and colleagues presented a new sample preparation technology, the MagSweeper – an automated immunomagnetic separation technology [30].

RT-PCR to detect epithelium-specific or rather organ-specific transcripts such as CK19 and CK20, HER2 or mammaglobin has also been proven promising to detect DTC/CTC [31-38]. Very recently, the AdnaTest Breast Cancer Detect for analyzing tumor-associated mRNA for HER2, MUC1 or GA 733-2 was described for the detection of CTC in blood from primary breast cancer patients [39]. The main drawback of RT-PCR approaches are false positive results due to a low level of epithelial-related or tissue-related transcription in normal cells [40-42]. Moreover, heterogeneity in the expression levels of a particular target transcript between individual DTC/CTC cannot be predicted by RT-PCR. Nevertheless, several studies provided evidence for a clinical relevance of DTC/CTC detected by RT-PCR (for a review see [2,10]). The advantages and disadvantages of the mentioned detection approaches have been recently discussed elsewhere [7].

Characterization of disseminated tumor cells/circulating tumor cells

DTC are likely to be genomically unstable and heterogeneous [43] as well as capable of disseminating in a less progressed genomic state, acquiring genomic alterations typical for fully metastatic cells later [44]. Furthermore, DTC are heterogeneous regarding the expression of growth factor receptors, adhesion molecules, proteases and their inducer and receptors, major histocompatibility complex antigens, signaling kinases, melanoma-associated antigens or telomerase activity [1,3,45-51].

Of particular importance is the epidermal growth factor receptor HER2, the expression of which in primary tumors is the basis for trastuzumab treatment decisions of breast cancer patients [52,53]. HER2 overexpression on DTC in BM was predictive for a poor clinical outcome of stage I to stage III breast cancer patients [54]. The study of Vincent-Salomon and colleagues demonstrated that in the majority of cases the HER2 status remained stable between DTC and the corresponding primary tumors [55]. There is also evidence, however, for discrepancies between the HER2 status of primary tumors and that of DTC in BM [54,56], or CTC in blood [57-59]. This discrepancy might be due to the presence of a small subclone of HER2-amplified cells missed by routine fluorescence *in situ* hybridization analysis of the primary tumor or an acquisition of HER2 gene amplification in DTC/CTC after dissemination [57].

For CTC from patients with metastatic breast cancer, global gene expression profiles have been defined and a list of CTC-specific genes obtained, which might be useful to distinguish a normal control phenotype from cancer patients [60]. TWIST1, a transcription factor pivotal for metastasis by promoting epithelial–mesenchymal transition [61-64], was part of the gene expression signature identified in EpCAM-enriched cells from BM of breast cancer patients after chemotherapy. Its expression was associated with distant metastasis and local progression [65]. The first hints for stem cell features of DTC in BM were provided by Balic and colleagues and by Alix-Panabieres and colleagues, who demonstrated a significant number of DTC from BM of breast cancer patients with either $CD44^{+}/CD24^{-/low}$ or $CK19^{+}/Muc\text{-}1^{-}$ stem cell-like phenotypes [15,66]. With the AdnaTest Tumor Stem Cell/The AdnaTest EMT RT-PCT assay, Aktas and colleagues found that a major proportion of CTC from metastatic breast cancer patients showed features characteristic for stem cells and epithelial–mesenchymal transition [67]. Resistance to systemic chemotherapy and long-term persistence of DTC in BM of cancer patients is also indicative for a putative stem cell phenotype [68-70].

To perform functional studies for the validation of the descriptive findings observed in cancer patients, the development of appropriate mouse models mimicking MRD is pivotal.

Clinical relevance of disseminated tumor cells/circulating tumor cells

The meta-analysis published by Braun and colleagues, including data for 4,703 breast cancer patients, demonstrated that the presence of DTC in BM was not only predictive of the development of skeletal metastases but also predicted the development of metastases in other organs [71].

Based on these data and other results showing clinical relevance of DTC/CTC detection in breast cancer patients [7,10], DTC and CTC were mentioned for the first time in the American Society of Oncology recommendations on tumor markers in 2007 [72].

The ability of DTC to survive chemotherapy and hormonal therapy [5,68] and the persistence in BM over many years post surgery, linked to an increased risk of late metastatic relapse, have been described previously [69,70,73,74]. A European pooled analysis involving 696 breast cancer patients revealed that the persistence of DTC in 16% of

breast cancer patients was an independent prognostic factor for subsequent reduced breast cancer survival [75,76].

Bidard and colleagues recently reported about the prognostic relevance of DTC detection in stage I to stage III breast cancer patients [77]. As also published by Bidard and colleagues, the presence of DTC in BM was associated with a different pattern of loco-regional cancer cell dissemination and might influence loco-regional recurrence-free survival. Hormonal therapy and radiotherapy could help to prevent reseeding of the primary tumor area by DTC [78].

Sequential peripheral blood analyses are more convenient than BM analyses. Only few studies have thus far directly compared BM and blood analyses in the same patients [5,79-82], and only studies on larger cohorts of patients may help to clarify whether BM analysis can be replaced by a blood test.

CTC analyses for therapy monitoring have provided significant prognostic information in metastatic breast cancer. Cristofanilli and colleagues showed that the number of CTC before treatment and at the first follow-up visit after initiation of therapy is an independent predictor of progression-free survival and overall survival [17]. Moreover, results by Hayes and colleagues indicated that CTC in peripheral blood of metastatic cancer patients at any time during therapy directly reflect the patient's response, or lack of response, respectively, to therapy [20] – and CTC seem to be superior or additive to conventional imaging methods such as radiologic assessment, including computed tomograms [83,84]. Despite these promising data, however, the recent American Society of Oncology guidelines have stated that even the use of the US Food and Drug Administration-cleared CellSearch assay in patients with metastatic breast cancer cannot be recommended until further validation confirms the clinical value of this test [72]. The clinical utility of CTC measurements in metastatic breast cancer patients is therefore now being prospectively addressed in the randomized trial SWOG S0500 led by the Southwest Oncology group [85], expecting to enroll 500 patients with metastatic breast cancer.

To monitor MRD in nonmetastatic patients is the most important challenge of new DTC/CTC technologies. In a phase II trial (REMAGUS 02), Pierga and colleagues monitored CTC in 118 patients with large operable or locally advanced breast cancer before and after neoadjuvant chemotherapy, and showed that the presence of CTC after a short follow-up time of 18 months was an independent prognostic factor for reduced metastasis-free survival [86]. Interestingly, they did not find a significant correlation for response of the primary tumor to chemotherapy. In contrast, follow-up analyses of two German trials using the CellSearch technology – the GEPARQuattro trial on neoadjuvant chemotherapy and additionally, if indicated, trastuzumab treatment, and the SUCCESS trial on adjuvant chemotherapy – are still ongoing and will show whether the observed decreases in CTC rates will be associated with an improved survival rate [87,88]. Very recently, Xenidis and colleagues described patients with detectable CK19 mRNA post chemotherapy having significantly reduced overall survival and disease-free survival [38].

Conclusions

Detection of DTC in BM and of CTC in blood of breast cancer patients years before the occurrence of distant overt metastases is facilitated by several rare cell detection techniques. The resulting information may be used to assess the individual prognosis of cancer patients and to stratify the patients at risk to systemic therapies aimed to prevent recurrences and metastatic relapses. Nevertheless, the detection of DTC/CTC is still not part of the routine tumor staging in clinical practice. DTC/CTC measurements within clinical trials, however, might provide an important biomarker for real-time monitoring of the efficacy of systemic therapies in individual cancer patients, which may accelerate drug development, help to define subpopulations of patients with the highest treatment benefit, and open a new avenue for investigating drug resistance.

Recent data support the hypothesis that DTC are able to persist in the BM in a dormant state and that the BM provides a reservoir out of which DTC might disseminate also in other organs, such as the lung, the liver and the brain [89]. To understand molecular mechanisms involved in the regulation of the dormant stage together with the identification of those DTC/CTC that have the potential to initiate metastases are the most challenging topics of basic research on the biology of these cells.

Competing interests

The authors declare that they have no competing interests.

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