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REVIEW

Bone marrow as a reservoir for disseminated tumor cells: a special source for liquid biopsy in cancer patients

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Besides circulating tumor cells, disseminated tumor cells (DTCs) in bone marrow (BM) might be used as a 'liquid biopsy' to obtain information helpful to steer therapies in individual patients. Moreover, the molecular characterization of DTCs may provide important insight into the biology of cancer metastasis. BM is a frequent site of metastasis in breast, prostate and lung cancer, and it might represent a sanctuary site for DTCs derived from various additional types of epithelial tumors. Highly sensitive and specific immunocytological and molecular methods enable the detection of DTCs in BM of cancer patients at the single-cell level years before the occurrence of metastases. This information might be useful to assess individual prognosis and stratify patients at risk to systemic adjuvant anti-cancer therapies. Although most data on the prognostic value of DTCs are available for breast cancer, several single institution studies including patients with colon, lung, prostate, esophageal, gastric, pancreatic, ovarian and head and neck carcinomas have also documented an association between the presence of DTCs at primary surgery and subsequent metastatic relapse. Most DTCs are in a dormant (that is, non-proliferative) stage, frequently express HER2 and display a cancer stem cell and immune escape phenotype. Here, we summarize the current knowledge about specific biological properties of DTCs in BM, and discuss the clinical relevance of DTC detection in cancer patients with regard to an improved individualized therapeutic management. This will stimulate further technical developments that may make BM sampling more acceptable for the clinical management of patients with solid tumors.

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Introduction

Early spread of tumor cells is usually undetected even by high-resolution imaging technologies, preventing potentially effective early intervention. However, sensitive immunocytochemical and molecular assays now enable the specific detection of 'occult' metastatic tumor cells even at the single-cell stage.[1,2] These technologies provide the potential to track systemic tumor cell dissemination in the blood and homing to the bone marrow (BM) as one of the first crucial steps in the metastatic cascade.[1,3,4]

Various clinical studies have provided evidence for an association between the presence of disseminated tumor cells (DTCs) detected in BM at the time of initial tumor resection and post-operative metastatic relapse in patients with cancers of the breast,[1] prostate,[5] lung,[6] colon[7] and other epithelial organs.[8,9] This work paved the way for the introduction of circulating tumor cells (CTCs) and DTCs in international tumor staging systems.[10] Over the past years, several reviews have focused on CTCs.[11–13] In this review, we will therefore focus on the biology and clinical relevance of DTCs in the BM.

Molecular Determinants of Metastatic Spread to BM

Cytokeratins are currently the standard markers for detection of epithelial tumor cells in mesenchymal organs such BM, blood or lymph nodes.[2,14] Hematopoietic cells and BM stroma cells can be a source of false-positive findings, but it appears that most cytokeratin-positive cells in BM and blood samples are of epithelial origin, as indicated by the analysis of large cohorts of non-cancer control patients.[15] The most important question, whether these cytokeratin-positive cells are indeed tumor cells,

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was answered using whole genome amplification and comparative genomic hybridization of single DTCs.[16] Most cytokeratin-positive cells show genetic changes, clearly indicating that the cells are tumor cells.[4,17,18]

However, DTCs in patients with breast cancer and other solid tumors (for example, esophageal cancer) did not usually contain the same genetic changes as the primary tumor,[4,17,18] suggesting that DTCs that disseminate early from their primary tumor may undergo a parallel genetic progression independent from the primary tumor.[16] However, this parallel progression theory is based on the genomic analyses of CTCs and primary tumors using low-resolution technologies and small sample sizes (that is, small pieces of the primary tumor and few DTCs out of millions present in the BM). Thus, it cannot be excluded that a small metastatic subclone might already exist in the primary tumor and further genomic aberrations are not required for metastatic colonization, which would explain the failure to identify metastasis-specific mutations.[19] Consistent with this view loss of heterozygosity analyses of specific genomic regions showed that genetic aberrations of CTC in early-stage prostate cancer patients are identical to those in distinct, even small, areas of the primary tumor.[20] A similar finding was recently observed in colorectal and prostate cancer patients using next-generation sequencing; most CTC mutations were also revealed in small subclones of the corresponding primary tumors and metastases.[21,22] Thus, the parallel progression theory needs to be revisited in future studies using new technologies to capture larger amounts of DTCs and state-of-the-art sequencing technologies for genomic analyses.[23]

The Role of the BM in Clinical Cancer Dormancy

The dormancy issue is fascinating, studied mainly in breast cancer where the evidence lies for dormant DTCs heralding disease relapse decades later. Thus far, it is unclear how this concept may relate to more aggressive cancer types such as pancreatic cancers.[24] Is this biology not appreciated because of the late detection of fast moving diseases? Is parallel progression and dormancy relevant here but simply unexplored, or is linear progression with showers of invasive cells leaving the primary more likely?

Further molecular and functional analyses of DTCs may help to unravel the puzzling phenomenon of 'cancer dormancy' (that is, latency period between resection of the primary tumor and metastatic relapse, which can take >10 years in breast cancer).[25,26] This latency period referred to as 'cancer dormancy' is characterized by the presence of minimal residual disease over many years before overt metastases may eventually arise. It cannot be even excluded that many 'cured' cancer patients may harbor dormant tumor cells.[27]

Cancer dormancy may represent a steady state characterized either by DTCs that are unable to proliferate (for example, important growth factors are not provided by the microenvironment) or by a balance between cell proliferation and death of DTCs. This state might be disturbed by both changes in the DTCs (for example, additional mutations or epigenetic modifications in genes controlling cell proliferation and apoptosis) and the surrounding microenvironment (for example, release of growth factors, angiogenic factors and cytokines).[27,28] The BM microenvironment has features important for the maintenance of tumor dormancy of DTC such as the balance between osteoblast and osteoclast activity,[29,30] homing of DTCs in the hematopoietic stem cell niche[31] and immunological T-cell memory.[32] However, the role of the immune system as a potentially important host component for controlling metastatic progression is still under debate. Certain subsets of macrophages can even support metastatic spread by facilitating angiogenesis and extracellular matrix breakdown and remodeling.[33] An in-depth discussion of the influence of the microenvironment is beyond the scope of this article and we refer to the recent review from Hanahan and Coussens.[34]

Although the ability to induce angiogenesis is thought to be important for the escape from cancer dormancy and the subsequent formation of metastases,[27] the information on the expression of angiogenic factors in DTCs is sparse. Mature blood vessels produce signals that sustain tumor cell quiescence, whereas sprouting microvasculature provides stimuli that reactivate DTCs, leading to metastatic relapse.[35] Besides angiogenesis, other micro-environmental processes may also influence the dormant state of DTCs and micrometastases. For example, in prostate cancer, GAS6 receptor status is associated with dormancy and bone metastatic tumor formation.[36]

Moreover, during inflammation and wound healing a plethora of cytokines is being released, and some of these factors can induce the migration and growth of epithelial tumor cells.[27] Thus, it cannot be excluded that accidental bone fractures in cancer patients with minimal residual disease might affect the escape from dormancy.

It is still unclear whether the BM is only one of the many homing sites of cancer cells or represents a special reservoir. A recent experimental study indicated that dormant DTCs within the BM are highly malignant upon injection into the mammary fat pad, with the accelerated development of metastatic lesions within the lung, liver and kidney. These data suggest that disseminated breast cancer cells might acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone or, even more likely, that the change in the microenvironment might induce a proliferative switch in DTCs.[37]

Search for Metastasis Initiating-Cells

The cancer stem cell concept has received great attention over the past decade, but the hierarchical model is currently being controversially discussed[38] 'Stemness' or the ability to initiate tumor growth at the primary or distant site might be rather a state of tumor cells than the characteristic of a fixed population of 'stem cells'.

With regard to metastasis, it is assumed that cancer cells with stem cell-like phenotype can disseminate from the primary tumor to distant sites.[27] Furthermore, primary tumor stem cells show an expression profile associated with metastatic relapse in breast cancer patients.[39] Moreover, expression of the breast stem cell marker aldehyde dehydrogenase 1 (ALDH1) was associated with poor clinical outcome and only ALDH1-positive cells were able to form metastasis in mice.[40] Previously, it has been shown that cancer stem cells may have a particular capacity to undergo an epithelial-to-mesenchymal transition (EMT),[41] which increases their mobility and invasiveness and allows them to survive their stressful passage through the blood stream to distant organs.

There is also some evidence that the founder cells of overt metastases (that is, 'metastatic stem cells') might be among the DTCs detected by the current methods in cancer patients[27]: (i) the presence of DTCs in BM is significantly correlated to metastatic relapse; (ii) most CTCs are non-proliferatingthat is, Ki-67 negative) and resistant to chemotherapy, as postulated for cancer stem cells; and (iii) subsets of DTCs have a breast cancer stem cell phenotype (for example, $CD44^{+}CD24^{-/low}$, $CK19^{+}MUC1^{-}$, $EpCAM^{+}$). Moreover, we have generated cell lines from DTC of patients with non-metastatic cancer (breast, prostate and lung carcinomas) and recent proteomic analyses showed that these cell lines express cancer stem cell ($CD44^{high}$, $CD24^{low}$) and EMT (cytokeratin^{low}, $EpCAM^{low}$, vimentin^{high}) phenotypes with the expression of stress proteins that allow DTCs to survive hypoxia and chemotherapy.[42] Interestingly, DTCs seem to lodge in the hematopoietic stem cell niches,[31] which are located in the most hypoxic areas of the BM. Thus, these cell lines might serve as interesting models for the in-depth investigation of DTCs.

Moreover, a transcriptome analysis of osteotropic breast tumor cells found in BM has revealed an osteoblast-like phenotype: these tumor cells underwent an osteomimetism in the bone by expressing a pool of genes normally expressed by osteoclasts or osteoblasts.[43] We can speculate that such an adaptation may take place each time a CTC reaches a new niche. It is, therefore, conceivable that DTC may acquire an 'organ-mimetic phenotype'.

The reversal of EMT designated 'mesenchymal–epithelial transition (MET)' has a crucial role for the ability of DTCs to establish solid (micro-)metastasis. The exact mechanisms and interplay of EMT and MET are only partially understood.[41] However, it is now increasingly accepted that tumor cells with the highest epithelial–mesenchymal plasticity are probably the founder cells of metastases in distant organs including the bone.

Relation Between CTCs in Blood and DTCs in BM

Numerous investigations have provided evidence that CTCs are a promising prognostic marker in breast cancer[44] and other tumor types.[45] However, it is still unclear whether they provide complementary or redundant clinical information as DTCs. To the best of our knowledge, comparative analyses of CTCs and DTCs have been performed in only a limited number of studies.[46]

In breast cancer, the yield of CTCs was estimated to be lower than that of DTCs,[47] but this depends on the techniques used for CTC/DTC detection. CTCs and DTCs are detected in individual patients at the same time with variable concordance rates. Differences observed in some studies might be explained to some extent by the different technologies used to detect CTCs and DTCs. Interestingly, higher overall discordance of CTC and DTC counts was found in patients after rather than before adjuvant therapy,[48] which suggests differential sensitivity of DTCs and CTCs to chemotherapy. In this context, it might be noteworthy that DTCs may express a set of stress response proteins that may help them to survive chemotherapy.[42]

Controversy also remains regarding the clinical relevance of CTCs versus DTCs. Some authors showed superior performance of DTCs in predicting overall survival in both non-metastatic and metastatic breast cancer patients.[47,49] In contrast, others reported that CTCs predict overall survival in metastatic patients, whereas DTCs do not.[50] CTCs and DTCs have identical impact on overall survival of non-metastatic and metastatic patients, but disease-free survival is predicted only by DTCs. Simultaneous detection of CTCs and DTCs was shown to be associated with an especially poor prognosis and increased incidence of disease-related deaths in non-metastatic breast cancer patients.[46]

Of note, it has recently been reported that counts of disseminating tumor cells vary not only between BM and peripheral blood but probably depend also on the vascular compartment from which blood is being collected.[51] Substantially higher numbers of CTCs were counted in central veins than in peripheral veins. It might be speculated that the numbers of DTCs/CTCs might be site specific because the microvascular system of different organs might have a variable potency for filtering CTCs/DTCs.

In principle, CTCs might provide information on metastatic cells derived from various distant sites, whereas the DTCs reflect only the situation in the BM. However, the BM is a reservoir for tumor cells and DTCs might reflect the 'history' of tumor cell dissemination over an extended period of time, whereas CTCs represent only a snapshot of tumor cell dissemination.

Clinical Relevance of DTCs in Breast Cancer

Numerous studies have been published investigating the presence and clinical relevance of DTCs in BM of breast cancer patients. A pooled analysis consisting of 4703 breast cancer patients showed that the presence of DTCs in the BM was not only predictive of the development of skeletal metastases but was also predictive for the development of metastases in other organs.[1] More recently, similar data were obtained by a large single-center cohort study ($n = 1378$ patients) with long-term observation time (median 82 months).[52]

Besides their presence at primary diagnosis and surgery, DTCs have been described to survive chemotherapy and hormonal therapy, and they can persist in BM over many years post surgery.[27] This persistence is also linked to an increased risk of late-metastatic relapse. For example, in high-risk breast cancer patients (>3 involved axillary lymph nodes or extensive invasion of cutaneous lymph vessels), the presence of tumor cells after therapy was associated with an extremely poor prognosis.

Besides CTCs, DTCs in the BM might be used as a liquid biopsy to obtain information helpful to steer therapies in individual patients. As most DTCs are in a non-cycling state, chemotherapy might have rather limited effects on these cancer cells.[27] Thus, the use of targeted therapies in addition to chemotherapy and radiotherapy has started a new era in clinical oncology.[53]

The HER2 proto-oncogene is currently the most predominant biological target for systemic therapy. Several groups reported a striking discrepancy between the detection of HER2-positive DTCs and the HER2 score of the corresponding primary tumor,[54–56] suggesting that a small subclone of HER2-overexpressing cancer cells easily missed by routine primary tumor analysis may have the potential to disseminate. The detection of HER2-positive DTCs was correlated to an unfavorable clinical

outcome in breast and esophageal cancer.[17,54] Thus, the assessment of the HER2 status on DTCs might add important information for the clinical management of cancer patients.

The assessment of other therapeutic targets, in particular those involved in the complex interactions between DTCs and the bone microenvironment (for example, RANK), might also be important for the design of future clinical trials.[53]

DTCs can be used as a monitoring tool for adjuvant therapy in patients with primary cancer.[57] For example, a majority of initially DTC-positive primary breast cancer patients turned negative during adjuvant treatment. As DTC-persistence predicted an adverse outcome, serial DTC determination can identify patients that will probably benefit from additional or a switch of adjuvant therapy.

Presence of DTCs before primary systemic therapy was found in a significant number of patients with localized advanced breast cancer.[58] DTCs were found to be a significant prognostic factor for cancer-related death and could be an additional surrogate predictor of response to primary systemic therapy.

Clinical Relevance of DTCs in Colorectal Cancer

In colorectal cancer, a positive association between DTCs in BM and an increased recurrence rate and reduced overall survival has been so far reported.[59] In contrast, reports on smaller patient cohorts could not detect any association between prognostic factors and the presence of DTCs, and the largest study, including 275 patients and 206 non-cancer control patients, presented no clinical follow up.[60] Using reverse transcriptase–PCR (RT-PCR) analysis with cytokeratin 20 (CK20) mRNA as marker transcript, two groups reported no association to survival, whereas four groups found an association between the presence of CK20 transcripts and worse overall survival.[59] However, both negative reports were performed on metastatic patients only. A more recent study analyzed 75 colorectal cancer patients treated with radical resection using a nested RT-PCR for carcinoembryonic antigen and survivin. They found that CTCs and/or DTCs were independent unfavorable prognostic factors apart from lymph metastasis and adjuvant chemotherapy.[61]

Clinical Relevance of DTCs in Lung Cancer

In non-small cell lung carcinomas, several immunocytochemical studies investigated the prognostic relevance of DTCs using the monoclonal antibody CK2 against CK18 or different pan-cytokeratin antibodies.[59] The rate of CK^{pos} cells in the different studies ranged between 22 and 60%. Interestingly, a higher frequency of DTCs was found in BM aspirations from the rib of lung cancer patients[6] when compared with those taken from the iliac crest. Irrespective of the localization of the BM puncture, several studies have shown a correlation between DTCs in BM and worse clinical outcome.[59] However, the largest study conducted so far on 296 patients could not find a significant association between DTCs and clinical outcome.[62]

Using RT-PCR-based assays, the follow-up database is small thus far. Analyzing a small cohort of 50 patients free of overt distant metastases, the presence of MAGE-A was associated with poor prognosis.[63]

Clinical Relevance of DTCs in Prostate Cancer

In prostate cancer, BM is the most prominent metastatic site, and several research groups have focused on the detection of DTCs in this organ over the past 10 years. However, most of the studies included a relatively small number of patients and/or follow-up information, and data about the influence of hormonal treatment on prognosis are sparse or lacking. Using immunocytochemistry, DTC detection rates ranged between 10 and 90% and some evidence for a correlation of DTCs to clinically established risk factors such as histological differentiation of the primary tumor was found.[59] Although a DTC-positive BM status was associated with grading and increased risk of metastasis, a previous study on 266 patients did not find a correlation of DTC detection and survival.[64] In contrast, a significant prognostic relevance of DTCs in BM was found in 86/193 (44.6%) patients with clinically localized prostate cancer submitted to neoadjuvant hormonal therapy followed by radical prostatectomy.[65] Recently, DTCs detected in non-metastatic prostate cancer patients before the onset of therapy were significantly associated with clinical outcome.[66]

There are also studies that used RT-PCR for DTC detection, mostly amplifying prostate specific antigen (PSA)- or MAGE-specific cDNAs as markers with strongly varying detection rates.[59] DTC detection correlated to PSA serum levels and some evidence for the prognostic relevance of these findings has been reported.

In conclusion, there is some evidence that the detection of DTCs in the BM of prostate cancer patients might represent a prognostic parameter, but larger multi-center studies followed by nomogram testing against the established risk parameters are required to introduce DTC detection into the future clinical management of prostate cancer patients.

Here, we have focused on the main types of solid tumors (that is, breast, colorectal, non-small lung and prostate carcinomas). However, it should be mentioned that additional studies have been performed in patients with other epithelial tumor entities, such as gastric cancer,[67] esophageal cancer,[9,68] pancreatic cancer,[8] gynecological cancers[69,70] and head and neck carcinomas.[71]

Conclusions

Figure 1 summarizes the technical, biological and clinical aspects of DTC detection. DTCs can be found in the BM of patients from various epithelial tumors, including breast, prostate, lung and colon cancer. Although DTCs may be also present in other organs at the same time, it can be envisaged that BM might serve as a reservoir of DTCs from where they may re-circulate into other distant organs such as liver or lungs where better growth conditions may exist. Thus, BM aspiration, a routine diagnostic procedure in patients with leukemia, may be used as a liquid biopsy in patients with solid tumors. At present, the analysis of peripheral blood is considered a liquid biopsy. However, the additional analysis of the BM may provide complementary clinical information. The invasive procedure might be improved by future technical developments that may allow a gentler BM sampling.

The observed correlation between DTCs in the BM and local relapse in breast cancer[72] suggests that these cells might even circulate back to the primary tumor site. This hypothesis is supported by recent experimental findings in a mouse model,

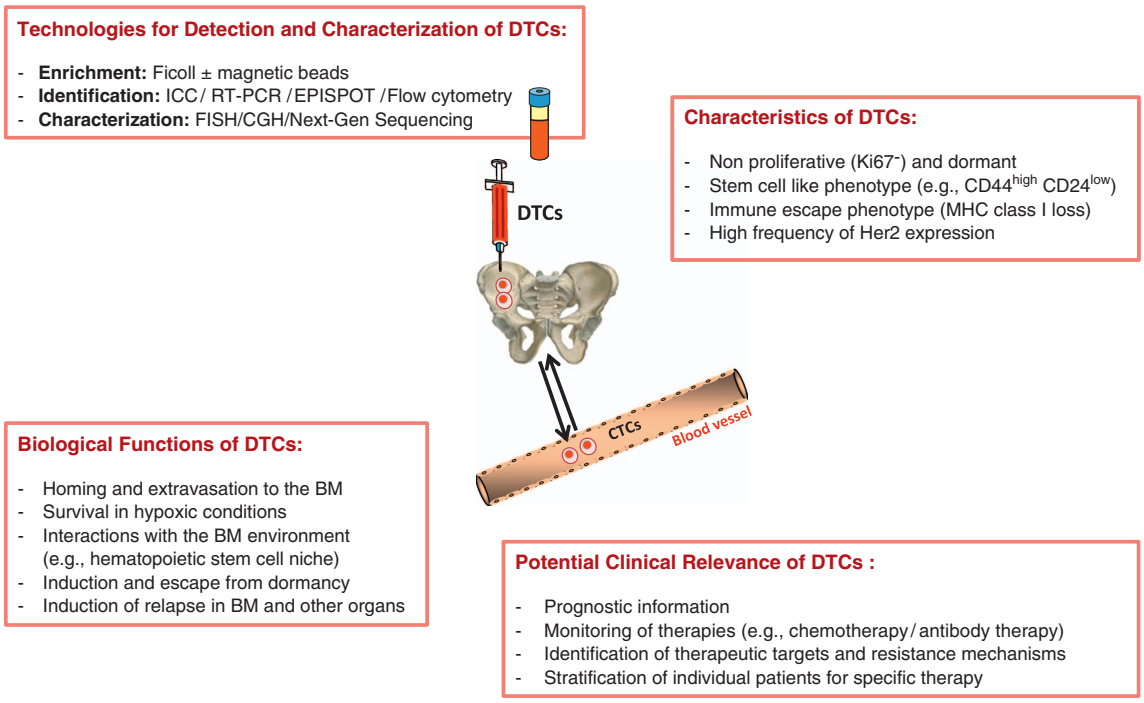

Figure 1. Detection, biology and clinical relevance of disseminated tumor cells (DTCs) in BM. The figure summarizes (i) the current technologies for detection and characterization of DTCs, (ii) the characteristics of DTCs, (iii) the biological functions of DTCs and (iv) the potential clinical relevance of DTCs. The scheme indicates that tumor cells can be exchanged between the bone marrow and blood. Tumor cells in the blood are denoted CTCs. BM, bone marrow; CGH, comparative genomic hybridization; FISH, fluorescent *in situ* hybridization; ICC, immunocytochemistry; MHC, major histocompatibility class; RT-PCR, reverse transcriptase–PCR.

demonstrating that breast cancer cells can circulate back from distant site for example, BM) to the primary site and contribute to primary tumor growth.[73] If BM is a particular sanctuary site for DTCs, drugs targeting the BM-tumor interaction (for example, biphosphonates or antibodies to the RANK ligand) may be efficient to prevent metastatic or even local relapse. In this context, it has been shown that treatment with biphosphonates in breast cancer patients reduced the DTC counts in BM.[74]

Whether DTCs use the BM environment as a niche to persist in a dormant state over many years before they disseminate into other organs is subject of current investigations. To understand this stage of hibernation and the conditions enabling DTCs to reactivate growth as well as to identify the founder cells of overt metastases ('metastases initiator cells') are some of the most important and challenging areas of research.

Conflict of Interest

The authors declare no conflict of interest.

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