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The Role of the Microenvironment – Dormant Prostate Disseminated Tumor Cells in the Bone Marrow

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Abstract

Disseminated tumor cells (DTC) leave the primary tumor and reside in distant sites (e.g. bone) early in prostate cancer. Patients may harbor dormant DTC which develop into clinically overt metastasis years after radical prostatectomy. We will describe recent evidence suggesting high p38/ERK ratio, bone morphogenetic proteins, and tumor growth factor-beta 2 promote dormancy in solid tumors. Furthermore, we will discuss the possible regulation of dormancy by hematopoietic stem cell and vascular niches, and describe novel models recapitulating bone marrow metastatic latency and outgrowth, 3D microvascular networks, and 3D biomatrix supportive niches in the studies of tumor cell dormancy.

Keywords

Disseminated tumor cells; dormancy; microenvironment; bone marrow; prostate cancer; metastasis

Introduction

Cancer dormancy refers to the prolonged clinical disease-free time between removal of the primary tumor and disease recurrence, which is common in prostate cancer (PCa), breast cancer (BCa), esophageal cancer, B-cell lymphoma, and melanoma. PCa cells disseminate before radical prostatectomy (RP) [1–5], and reside in distant organs including bone, lymph nodes, liver, and lung. These disseminated tumor cells (DTC) can remain dormant in the distant organs for a prolonged period of time (e.g. >10 years) until in some patients clinical

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Conflict of interest

The authors declare no conflict of interest.

metastases develop. Dormant DTC retain the capability to proliferate but by definition they are currently not dividing, therefore they are resistant to chemotherapies targeting cell division. To this end, understanding the molecular and cellular nature of DTC will allow the identification of novel drug targets to prevent overt cancer metastases from dormant DTC. This review summarizes the biological and technical advances in the last couple of years contributing to our understanding of the role of the microenvironment in PCa dormancy in the bone. We will highlight (i) the relevance of DTC in prostate cancer, (ii) key elements that have been proposed to regulate cell dormancy in the bone marrow, (iii) technological advances in characterizing and modeling dormancy, and (iv) potential dormant DTC targeting strategies.

DTC in PCa patients

Patients with localized PCa undergo surgery to remove the prostate, so any tumor cells that eventually gives rise to metastatic outgrowths have to disseminate prior to surgery. Clinically, PCa metastasizes to lymph node, bone, liver, lung, and the adrenal glands. From our rapid autopsy program at University of Washington, approximately 90% of patients with advanced disease have bone metastases [6]. DTC could exist individually or as a cluster in the bone marrow (BM) of PCa patients and some can express prostate-specific antigen (PSA) while others do not (Fig. 1).

In the clinic, it is relatively easy to acquire BM aspirates, when compared to other tissues, to isolate DTC for molecular analyses. DTC have been shown to be present in the BM of 13–72% of PCa patients prior to RP and 20–57% of patients with no evidence of disease >5 years after surgery [2;5]. The wide range of divergence in DTC detection may be partly attributable to the different detection methods used in different laboratories (please refer to the section Technical Advances – Methods). However, to our knowledge, no systematic quantification of DTC has been reported in PCa patients. The current literature invariably categorize PCa patients as either positive (+) or negative for DTC [2;5;7].

What does a positive DTC mean?

Our group has been attempting to identify, isolate and characterize DTC in BM of PCa patients since 2003 [1]. The results correlating DTC and clinical outcomes have been largely inconclusive. Studies from Weckermann et al. and Lilleby et al. demonstrated that pre-operative or pre-treatment DTC predicted disease recurrence while our group did not observe such a correlation; in contrast our group reported the detection of post-operative DTC predicted poor prognosis but their results did not show such a trend [2;5;8]. Nevertheless, the bottom line is that if a PCa patient has DTC, it does not necessarily mean that he will develop an overt metastasis.

DTC vs. primary PCa

While DTC disseminate from the primary PCa, their genomic aberrations appear to largely differ from those of the primary tumor. Specifically, comparative genomic hybridization of a pool of DTC (10–20 cells) isolated from the BM of patients with advanced disease showed considerable difference in genomic aberrations when compared with their paired local

tumors [9]. Single cell array comparative genomic hybridization further revealed only 0–25% shared chromosome aberrations between an individual DTC and the corresponding primary PCa [5]. Undoubtedly, more chromosomal aberrations were detected in DTC from patients with active metastasis than the primary tumor, suggesting chromosomal changes needed for metastatic outgrowth may be selected in the BM microenvironment [5]. However, one point to keep in mind is that this study compares single DTC to multiple cells in the primary PCa sample, in which array quality may result in discrepancies in data interpretation. At the gene expression level, a recent study in BCa patients showed that ERBB2 was detected in DTC in the BM while the primary tumor was HER2/ERBB2 negative, suggesting these patients may be eligible for trastuzumab therapy for the recurrent disease [10]. Collectively, DTC are likely different from the primary tumor at both the genomic and gene expression level, suggesting treatments for DTC should not be based on the features of the primary tumor, despite the fact that they originate from the primary tumor. Using tissue microarrays of primary PCa and metastases, we have shown repeatedly that the protein expression of biomarkers are often quite different between these tissue types and among bone metastases in a given patient [11;12].

Despite the genomic and gene expression difference detected between DTC and their primary tumor, the primary tumor microenvironment (e.g. hypoxia, collagen dense matrix) may influence the gene expression of disseminating cells. Bragado and Aguirre-Ghiso have suggested that DTC before leaving the primary site could develop a high or low dormancy score signature, hence predisposing the cell to enter prolonged dormancy or proliferate after a brief quiescence at the distant site, respectively [13]. In PCa, even after 5 years of no evidence of disease, Aho et al. showed that more aggressive primary tumor can still be predictive of recurrence [14].

To date, important research efforts focus on understanding the regulation of DTC dormancy by the microenvironment. However, characterization of the gene and protein expression patterns in dormant DTC in relation to the disease prognosis in patient samples is still lacking. Combining the results from model systems and the characterization of DTC in patient samples is required to determine the intrinsic characteristics of a DTC that predisposes how long it is going to remain dormant.

Bone microenvironment

‘Cellular dormancy’ refers to DTC surviving as individual cells and ‘tumor dormancy’ or micrometastasis refers to DTC existing as a group of cells. Tumor dormancy is represented by a group of tumor cells that cannot grow beyond a certain size. Factors underlying this phenomenon including balanced proliferation and apoptosis, angiogenic suppression, and immunosurveillance have been discussed in multiple reviews [15–18]. Herein, we will focus on cellular signals and the bone microenvironment that contribute to cellular dormancy, highlighting the latest discoveries.

Cellular dormancy and stress signaling

Aguirre-Ghiso’s work in stress signal-associated cellular dormancy in other cancers has been comprehensively summarized recently [13;19]. Briefly, activation of p38 and

inactivation of ERK signaling (i.e. increased p38/ERK ratio) promotes DTC dormancy (Fig. 2a). However, this process is plastic – DTC interactions with a favorable microenvironment at the distant site and activation of tumor cell surface receptors such as the urokinase receptor (uPAR)– $\alpha 5\beta 1$ –integrin complex and the epidermal growth factor receptor (EGFR) will increase the ERK/p38 ratio, resulting in DTC proliferation. In the scenario that high p38/ERK ratio is maintained, prolonged dormancy can be achieved. Activation of p38 elevates the expression of BHLHB3, NR2F1 and p53 in squamous cell cancer dormancy models [13]. Interestingly, our gene expression profiling results of individual DTC also suggested that a p38-associated signature is altered in DTC acquired from PCa patients with no evidence of disease (dormant disease) when compared to those with active metastasis (unpublished data). Recent advances along this line showed that BM-derived transforming growth factor-beta2 (TGF- $\beta 2$) activates p38, resulting in a high p38/ERK ratio and dormancy of DTC in a head and neck squamous cell carcinoma model (Fig. 2a) [20]. TGF- $\beta 2$ -induced dormancy required TGF- β receptor-I (TGF- β -RI), TGF- β -RIII and SMAD1/5 activation to induce cell growth arrest, and systemic inhibition of TGF- β -RI or p38 activities awakened dormant DTC, leading to metastasis [20]. Additionally, Kobayashi et al. have reported that in PCa, bone morphogenetic protein 7 (BMP7) secreted from bone stromal cells induces senescence in PCa stem-like cells by activating p38 and increasing expression of the cell cycle inhibitor p21 [21]. Along the same line in BCa, Gao et al. have suggested that in animal models Coco, a TGF- β and BMP ligand antagonist, induces dormant BCa cells to undergo reactivation in the lung [22]. However, the authors point out that Coco induces a discrete gene expression signature that is strongly associated with BCa metastasis to the lung but not to the bone.

Hematopoietic stem cell (HSC) niche

Taichman's group has demonstrated that PCa cells target the HSC niche during metastasis and replace the occupant HSCs [23]. The mechanisms by which DTC act as molecular parasites of the HSC niche and the possibility of the HSC niche promoting tumor dormancy has been reviewed [24]. Recent data have showed that osteoblast-derived growth-arrest specific 6 (GAS6) ligands induced PCa cells that express a high GAS6 receptor Axl /Tyro3 ratio to a largely dormant phenotype, whereas PCa cells that express a low Axl /Tyro3 ratio to escape from dormancy (Fig. 2b) [25]. Another new study showed that human PCa cells binding to mouse niche osteoblasts induce a higher percentage of Ki67-negative cells ('dormancy-enriched' population) and increase the chemoresistance of cancer cells to taxotere [26] when compared with PCa cells growing alone, suggesting at least a subset of PCa cells are under osteoblast-derived growth suppression [26]. In this study, the 'dormancy-enriched' population expressed a higher TANK binding kinase 1 (TBK1) level, and abolishing TBK1 in these bone-homing PCa cells sensitize the cells to taxotere – this result reveals an important clue that suppressing TBK1 may allow DTC to be targeted by chemotherapy [26]. Future studies establishing the causal link between a particular Ki67-negative (dormant) cell, the high TBK1 level, and chemoresistance may uncover the role of TBK1 in dormant DTC.

Perivascular niche

BCa DTC have recently been demonstrated by Ghajar et al. to reside adjacent to the blood vessels in the lung, BM, and brain [27]. Mature blood vessels produce local endothelial-derived factors such as thrombospondin-1 (TSP-1) to sustain tumor cell dormancy, whereas sprouting microvasculature secretes periostin (POSTN) and tumor growth factor-beta 1 (TGF- β 1) to stimulate the growth of dormant DTC (Fig. 2c) [27]. Whether the same mechanism applies to PCa DTC is worth exploring. If sprouting vasculature is conducive to dormant DTC reactivation, disturbance of stable vasculature including wound healing, trauma, and oxidative stress may contribute to PCa metastatic recurrence.

Immune environment

Both adaptive and innate immunity controlling cancer initiation have been extensively reviewed, but a consensus on their role for controlling DTC dormancy has not been reached [15;16;28]. Earlier studies by Pantel and colleagues attempted to explain why similar rates of DTC were detected in the BM aspirates of patients with colon and BCa, even though bone metastasis is rare in colon cancer patients. Immunocytochemistry results showed that the histocompatibility leukocyte antigen class I molecule that is required for presenting antigen to T cells, was expressed at a lower level in DTC of patients with BCa than those in colon cancer, suggesting the BCa DTC may escape from T cell-induced cytotoxicity and hence are available for subsequent outgrowth [29]. In addition, the activity of T cells may be suppressed in the bone microenvironment in PCa [30]. However, possibly due to the highly inconclusive results, the lack of animal models, and the difficulty to identify relevant clinical specimens, little advance has been made in the dormancy-immunosurveillance topic in the past two years.

Technical advances

Methods of isolation and characterization of DTC from the BM

There are different methods of DTC isolation and detection in the BM. Generally, the BM aspirate is subjected to density-gradient centrifugation to enrich for mononuclear cells containing DTC. These cells are negatively selected against leukocytes and platelets (e.g. anti-CD45 and anti-CD61) and positively selected for epithelial cells (e.g. anti-human epithelial antigen (HEA)) by immunobeads to obtain an epithelial cell-enriched population. Enriched cells are then subjected to a second round of positive selection using fluorescently-labeled antibody for the target of interest (e.g. anti-EpCAM), and then the labeled PCa-DTC are either identified and isolated using a micro-manipulator under a microscope or sorted through a fluorescence-activated cell sorting procedure [4;5;9;31]. Alternatively, mononuclear cells are spun down onto a slide and then DTC are directly detected by positive pancytokeratin staining (e.g. A45B/B3 or AE1/AE3) [8;32].

The reverse transcriptase polymerase chain reaction is another detection technique for DTC that was reported as long as a decade ago [1;3;4]. Owing to high reaction sensitivity, no cell enrichment is required. PSA and telomerase have been valid targets for detection of PCa DTC [3;4]. However, some PCa DTC do not express PSA as shown in Fig. 1, therefore the presence of DTC may be underestimated. In view of the risk of missing a subset of DTC

based on one selection marker, a new Nanostring nCounter™ platform has been developed based on 38 markers for classification of DTC in the BM of BCa patients [10]. Tumor cell-specific gene expression by nCounter™ was detected with a sensitivity of one cancer cell per 1×10^6 nucleated BM cells [10].

In our attempt to characterize individual DTC, we recently reported a single cell transcriptomic analysis of PCa DTC isolated from the BM using commercially available technologies to directly amplify RNA for gene expression analysis [31]. While a transcriptomic profile can be reliably obtained from a single cell, fewer amplified genes are detected from a single-cell than from a pooled-cell sample [31]. DTC heterogeneity within an individual becomes evident when a number of patients are analyzed (unpublished).

Models of dormancy

Generally, mouse models attempt to recapitulate clinical dormancy by resecting an established primary PCa (either subcutaneous or orthotopic) and analyzing DTC in distant organs (e.g. bone) after a period of time. A very interesting *in vivo* model was newly established to test whether dormant DTC from the BM can develop cancer: BCa tumorspheres were implanted in the mammary fat pad and then resected. Total BM isolated from mice that did not develop tumor 8–10 months post-injection of tumorspheres into the mammary fat pad were injected into the mammary fat pad of nude mice. BCa developed after two months and metastatic lesions were detected early, suggesting the acquisition of a more aggressive phenotype of DTC during metastatic latency within the BM microenvironment [33]. Furthermore, a new PCR-based method was developed to detect and isolate human PCa DTC from the murine BM niche, using human Alu sequences and by fluorescence-activated cell sorting and immunohistochemistry using anti-HLA antibody [34]. These strategies could be further engaged to explore the biology of PCa DTC.

Studying dormancy in animal models involves primary tumor growth, resecting the primary tumor, and then waiting for a period of time post-resection to ensure there is no tumor recurrence. It is time consuming (~1 year), expensive, and the results may fluctuate due to individual variation in each animal. In this regard, cell culture experiments that attempt to recapitulate PCa-microenvironment interactions, but take a shorter time (weeks) may be a fair alternative. However, a shorter timeframe to study tumor cell dormancy might be counterproductive since dormancy is per definition an event which occurs over a relatively long period of time. Having said that, some new models of dormancy *in vitro* include: a cell model with artificial BM proposed to study definitive BM components in cellular dormancy. BCa cells are mixed with a 3D-biomatrix and then placed in an inhibitory niche (a mixture of critical components of human BM using cell lines) or a supportive niche (primary human BM stromal cells) [17]. The results show that the direct interaction of cancer cells with the niche, in addition to secreted signals, is necessary for cancer dormancy maintenance; and BM fibroblasts and osteoblasts but not an endothelial cell alone, inhibit proliferation of BCa cells [17]. Vasculature-associated dormancy has also been modeled *in vitro* by forming 3D microvascular networks when cultured with fibroblasts, leading to sustained quiescence of BCa cells [27]. To date, in many model systems ‘dormant cells’ commonly refer to cells that are Ki67 negative [26;27]. However, not all Ki67 negative cells are dormant; the cells can be

senescent. To rule out senescent cells, senescence markers (e.g. beta-galactosidase) should be used [35].

Targeting dormant DTC

Owing to limited understanding of DTC biology, there are no targeted therapies to maintain DTC dormancy or to eradicate dormant DTC [28;36]. For a majority of PCa patients who are DTC positive after primary treatment and there is no other evidence of residual disease, no additional treatment is given until disease recurrence (e.g. PSA rise). Since around 20–57% of patients with no evidence of disease >5 years after surgery actually harbor DTC in their BM [2;5], could / should we target them to prevent the development of potential overt metastases?

Direct targeting

Aguirre-Ghiso and colleagues have recently put forward a hypothetical scheme for DTC monitoring to treat dormant residual disease [37]. At the time of primary tumor surgery, patients first have their BM analyzed for DTC content. Those patients negative for DTC are monitored for symptoms. However, those patients positive for DTC (who could have a chance of recurrence and metastasis, [5]) will have the DTC profiled for dormancy markers and divided into one of the three categories for treatment: (i) dormant and suitable for DTC eradication, (ii) dormant and suitable for DTC maintenance, or (iii) proliferative and recommended for anti-proliferation and dormancy-inducing therapy (if available) [37]. Any patient who is negative for DTC before surgery and then becomes positive during follow-ups will have the DTC profiled for therapy. In PCa, while the molecular markers for the three categories remain to be established, profiling individual DTC pre- and/or post-RP may give a more comprehensive picture of which populations of DTC are present, guiding a single or combinational DTC therapy. However, it is well recognized that it will be controversial to treat a patient with detectable dormant DTC but no other symptoms of disease; unless one identifies a subset of dormant DTC that is more prone to be reactivated.

Indirect targeting

In view of the extensive interaction of DTC and the bone, targeting the bone microenvironment to eradicate or maintain the dormancy status of DTC is rational. A recent BCa clinical trial demonstrated that zoledronic acid contributes to the eradication of DTC. They additionally argued that zoledronic acid may have a positive influence on survival in the adjuvant setting due to their effects on DTC [32]. Zoledronic acid, a highly potent inhibitor of osteoclast-mediated bone resorption, limits bone loss in men receiving androgen deprivation therapy [38]. Whether or not zoledronic acid impacts DTC in PCa patients and/or prevents the development of metastasis are definitely important questions to be answered. In addition, the possibility that the HSC niche induces PCa cell dormancy and therefore is a target for therapy has been thoroughly discussed in a recent review [24]

Limitations in studying dormancy in PCa

The biggest challenge of studying cancer dormancy in PCa is the lack of dormant cells isolated from patients. If one looks at DTC in the BM as one potential source of dormant

cancer cells, one can typically isolate only a few cells from a BM aspirate for further studies. Interrogation of this limited number of cells at a single cell level is another considerable challenge. For single cell analysis, individual cell information is obtained and cell heterogeneity can be determined, but gene expression bias may be introduced by the amplification process and some low abundance genes will not be detected. For DTC population analysis, cell heterogeneity is masked and the difference between subgroups of DTC in an individual may not be determined. Additionally, the gene expression profile of rare but important cells may be missed. To enable the study of clinically-relevant DTC, stimulating these cells to replicate to acquire a larger population by definition moves them out of dormancy and thereby perturbs the gene expression profile one acquires. Another limitation is that the various methods used to isolate DTC result in different sensitivity and specificity in DTC detection, thus findings from different laboratories are difficult to compare. To offset these clinical limitations, a few models of dormancy have been proposed. Yet, an inevitable question that will plague the field until we have characterized dormant DTC in patient samples is how well these models recapitulate what is seen in patients?

Conclusions and future perspectives

Dormant PCa DTC have to integrate autocrine and/or paracrine signals before they develop into a clinically-evident metastasis. Data obtained with newly developed *in vitro* and *in vivo* models in combination with the isolation and characterization of patient derived dormant cells will complement each other to provide answers as to what promotes and maintains cellular dormancy in PCa. The cancer cell dormancy field is still very young. However, with the knowledge gained from current models, ideas are already emerging to target dormant DTC or their microenvironment to maintain or eradicate these cells. To bridge laboratory and bedside observations, standardize methods needed to be adopted to detect and isolate DTC. Furthermore, characterization of DTC from PCa patients is necessary to (i) understand DTC biology in patients, (ii) identify molecular markers in dormant DTC, and (iii) correlate DTC molecular profiles with the risk of recurrence and the treatment response. Once dormant cells in patients have been characterized, then we can use pertinent models to discover possible treatment modalities to induce or maintain DTC dormancy in patients with disseminated disease.

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Abbreviations (most important)

BCa	Breast cancer
BM	Bone marrow

DTC	Disseminated tumor cells
PCa	Prostate cancer
PSA	Prostate-specific antigen
RP	Radical prostatectomy

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Figure 1.

A schematic diagram showing primary PCa and DTC in the bone. A single or a cluster of DTC (white arrow) was stained for pan-cytokeratin and PSA. PSA was detected in some DTC (green asterisk) but not the others (red asterisk). Blue is DAPI staining of the cell nucleus. Scale bar: 50 μ m. PCa, prostate cancer; DTC, disseminated tumor cells; PSA, prostate-specific antigen.

Figure 2.

A schematic illustration on some current concepts proposed in DTC dormancy in the bone. (a) Stress signaling: TGF- β 1 produced by bone marrow stromal cells upregulates the p38/ERK ratio in DTC which then remain dormant in a head and neck squamous cell carcinoma model. (b) Receptor ratio: PCa DTC express tyrosine kinase receptor Axl > Tyro3 when they bind to osteoblasts in the hematopoietic stem cell niche. Upon stimulation by GAS6 secreted by osteoblasts, DTC remain predominantly quiescent. (c) Perivascular niche: stable vasculature produces local endothelial- derived factors such as TSP-1 to sustain tumor cell dormancy, whereas sprouting vasculature secretes POSTN and TGF- β 1 to trigger DTC growth in a BCa model. DTC, disseminated tumor cells; TSP-1, thrombospondin-1; POSTN, periostin; TGF- β 1, tumor growth factor-beta 1; BCa, breast cancer; GAS6, growth arrest specific 6; TBK1, TANK binding kinase 1.