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Circulating tumor cells exhibit a biologically aggressive cancer phenotype accompanied by selective resistance to chemotherapy

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ABSTRACT

With prostate cancer (PCa), circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) portend a poor clinical prognosis. Their unknown biology precludes rational therapeutic design. We demonstrate that CTC and DTC cell lines, established from mice bearing human PCa orthotopic implants, exhibit increased cellular invasion *in vitro,* increased metastasis in mice, and express increased epithelial to mesenchymal transition biomarkers. Further, they are selectively resistant to growth inhibition by mitoxantrone-like agents. These findings demonstrate that CTC formation is accompanied by phenotypic progression without obligate reversion. Their increased metastatic potential, selective therapeutic resistance, and differential expression of potential therapeutic targets provide a rational basis to test further interventions.

Introduction

Prostate cancer (PCa) is a major public health concern. It is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the United States [35]. Localized PCa has a high survival rate and can be managed with localized therapy. However, once PCa has metastasized to distant sites, it is incurable and will inexorably lead to death. In order to move between distant sites throughout the body, cancer cells must move through the circulation. Circulating tumor cells (CTCs) in the blood are the subject of intense investigation because they denote a population at higher risk for poor outcome. However, currently therapy is not altered based upon CTC status. A lack of understanding of the biology of CTCs has served as a barrier to developing rational therapy tailored to these high risk patients.

The presence of CTCs has been linked to poor clinical outcome in several cancer types, including PCa. In patients with metastatic PCa, the presence of CTCs and/or of high numbers of CTCs denote a population with decreased overall survival as measured by either the CellSearch™ system [7,8,13,31,36] or by RT-PCR based methodology [6]. Further, in patients with metastatic PCa, a decline in CTCs after treatment with cytotoxic chemotherapy portends a more favorable prognosis than those who do not experience a decline [8]. In addition to overall survival, CTCs also predict responsiveness to hormonal therapy in patients with hormone-sensitive PCa [14], and a decline in CTCs is associated with responsiveness to therapy [34]. Finally, CTCs are increasingly recognized as having similar prognostic value in other cancer types, including colorectal [15,25], breast [25,42], lung [17], bladder [30], and pancreatic [16]. Despite our rapidly evolving ability to identify CTCs, and accumulating evidence that their presence predicts poor clinical outcome, these findings fail to provide guidance on differential therapeutic strategies for patients with CTCs.

Changes in the epithelial to mesenchymal transition (EMT) state of CTCs have been proposed as a mechanistic driver of CTC formation. Further, the loss of epithelial cell markers which accompany EMT act to offset the efficacy of technology which relies upon them for CTC detection. In multiple studies, breast cancer patients with high CTC counts have correlated with higher levels of EMT proteins, such as twist and phosphoinositide 3-kinase (Pi3K), as well as markers of stem cells [1,20]. However, the prognostic value of this correlation is still unclear. While some studies have been unable to correlate the presence of CTCs and EMT markers to bone marrow disseminated tumor cells (DTCs) in breast cancer patients [20], others have shown that EMT markers on CTCs change with metastatic progression. In one study, the percentage of twist and vimentin-positive CTCs increased in patients with metastatic

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versus early stage breast cancer [19], and an additional study demonstrated that patients with metastatic breast and PCa more frequently have CTCs with high levels of vimentin and n-cadherin, with low levels of E-cadherin [2]. This has also been confirmed in hepatocellular carcinomas, where expression of vimentin and twist are increased, while those of E-cadherin decreased, as compared to the primary tumor [24]. Multiple studies have shown the ratio of epithelial to mesenchymal markers on CTCs can be used to determine the likeliness of patient to respond to therapy [1,40].

In addition to EMT, CTCs constitute cells that are traversing the metastatic cascade. Therefore, it is important to additionally consider factors that drive transformation to a metastatic phenotype. Our group has identified several pathways that, through signaling pathway cross-talk, together serve to regulate cellular motility and transformation to a metastatic phenotype in human PCa (reviewed in [33]). We have demonstrated that heat shock protein 27 (HSP27) is a central downstream mediator of transformation to a metastatic phenotype [38]. HSP27 regulates the expression of matrix metalloproteinase 2 (MMP-2). MMP-2 degrades collagen IV, which is a major component of the prostate basement membrane. Both HSP27 and MMP-2 have been shown to be up regulated in aggressive PCa, and to predict the future development of metastasis in humans [9–12,28,29,37]. Our group has shown that it is possible to inhibit the pathway that activates HSP27 with a small molecule therapeutic [18], and that this will decrease human PCa metastasis in mice bearing orthotopic tumors. Further, we have also shown in a prospective phase II randomized controlled trial in men with localized PCa that therapeutically inhibiting HSP27 activation will decrease MMP-2 expression in human prostate tissue [39]. Therefore if CTCs differentially regulate HSP27 and/or MMP-2, they offer attractive targets to which therapeutic inroads are currently being made.

In this study, we used an orthotopic murine model of human PCa metastasis previously described by us [21,22,32] to generate CTC cell lines from blood and disseminated tumor cell (DTC) lines from the bone marrow. Importantly, this allowed us to compare the biological and functional characteristics of prospectively generated CTCs and DTCs, from two different compartments, to the parental cells from which they were derived. CTCs and DTCs exhibited increased invasion, as well as increased metastasis formation, as compared to parental cells. An examination of parental, CTC, and DTC cell lines, and resultant tumors in mice, revealed that CTCs and DTCs exhibited markers of an invasive EMT phenotype, as indicated by increases in Twist1, vimentin, HSP27, and MMP-2. We also evaluated CTC and DTC therapeutic responsiveness to a panel of cancer-relevant therapeutic agents. CTCs and DTCs exhibited selective resistance to growth inhibition by mitoxantrone-like agents. Together, these findings provide new insights into the biology of CTCs and DTCs. Their propensity for increased cell invasion and metastasis provide a mechanistic explanation for their clinical association with both metastatic disease as well as poor clinical outcome. Their selective resistance to specific types of therapeutic agents provides a rationale for implementing studies testing relative effectiveness based upon unique CTC and DTC sensitivity. Further, as HSP27 and MMP-2 can be targeted through several approaches now being developed in human trials, this similarly provides rationale to support CTC-specific interventions. Finally, the current study definitively demonstrates that EMT is a feature of CTC and DTC formation, and that it does not undergo obligate reversion once cells are removed from their circulatory environment.

Materials and methods

Mouse orthotopic implantation model

Orthotopic implantation of human PCa cells was performed as previously described by us [21,22,32]. Briefly, 2.5×10^5 cells were injected into the ventral lobe of the prostate of 4–6 week old inbred Balb/c athymic mice (Charles River Laboratories). Mice were fed ad labium with soy-free chow. All mice were treated, housed and managed under an IACUC approved protocol. Four to five weeks after implantation, blood was obtained via terminal cardiac puncture, bone marrow harvested from femurs, and primary tumor was weighed. In addition, lungs were formalin-fixed, paraffin embedded, serial sectioned, stained with H&E and for green fluorescent protein (GFP) by immunohistochemistry. The number of metastases to lungs was counted under light microscopy.

For the initial generation of CTC and DTC cell lines, mice were implanted with PC3-M cells that were stably transfected with green fluorescent protein (GFP) and constitutively active MAP2K4 as previously described by us [21,32,39].

Isolation and culture of CTCs

CTCs were isolated from animals as previously described by us [32]. Briefly, at necropsy blood was removed from the animal via terminal cardiac puncture and placed into sterile saline. Samples were centrifuged for 5 min at 3000 rpm, plasma was removed and ACK Lysis Buffer (154.95 mM Ammonium Chloride, 9.99 mM Potassium Bicarbonate, 0.0995 mM EDTA, Gibco) added for 5 min. Samples were centrifuged for 5 min at 3000 rpm, and the supernatant removed. The resultant pellet was resuspended in 1 ml cell culture media (RPMI media containing 5% FBS, 1% Penicillin–Streptomycin, 1% HEPES Buffer, and 1% L-glutathione, all purchased from Gibco) and added to a T75 flask containing 9 ml cell culture media. After 24 h, media was changed to cell culture media plus 500 μg/ml G418 (EMD Millipore). Once emergent colonies were outgrown (8–10 days), cells were sterile cell sorted for GFP-positive status using flow cytometry. Similar procedures were performed for the bone marrow samples. Bone marrow was ejected from the femurs using a syringe and sterile saline. Cells were cultured in cell culture media for 8–10 days, changed to media with G418 after the first 24 h, and then sterile cell sorted for GFP-positive status using flow cytometry. A schematic of the isolation procedure, with resultant cell lines names is shown in Fig. 1.

MTT assay

MTT assays were performed as previously described by us [26]. In brief, for the initial cell growth assay, cell concentrations of 1, 2.5, 5, 10, and 20×10^3 cells/well in 200 μl cell culture media were added to a 96 well plate and allowed to grow for 4 days. Four hours before the end of the experiment, 20 μl of 5 mg/ml MTT was added to each well. Cells were resuspended in 200 μl DMSO and analyzed on a microplate reader at OD_{550}. For the drug treatment assays, 1×10^4 cells/well in 200 μl cell culture media were plated in a 96 well plate, and drug was added 24 h after plating.

Cell invasion assay

Cell invasion assays were performed as previously described by us [4]. Briefly, 5×10^4 cells were plated on the top of a BD Biocoat Growth Factor Reduced Matrigel™ chambers with 8 μM pores (BD Biosciences) and allowed to invade for 24 h toward NIH 3T3 conditioned media in the lower chamber. Wells were plated in quadruplicate, with three wells measured for invasion and one as a loading control for cells in the upper chamber. After removing cells on the top wells for the three invasion wells, invading cells on the bottom membrane were fixed with 100% methanol for 5 min, and then stained with 0.5% crystal violet in 20% methanol for 10 min. Wells were washed with water and dried overnight. Cells were then imaged at 10× using light microscopy and the number of invading cells counted. Experiments were performed in triplicate and a p-value of 0.05 or less as measured by a two-sided t-test were determined to be significant.

Cell migration assay

Cell migration was performed as cellular invasion, but using a BD Falcon uncoated 8 μM pore membranes. Chambers were loaded with 2.5×10^4 cells per well into the upper chamber, and allowed to migrate for 8 h. Cells were stained, counted, and statistics performed as above.

Ten day colony formation assay

Twenty-four hours after plating 10^3 cells per well, in a six-well plate, 2 μM, 200 nM, 20 nM, 2 nM, 0.2 nM drug, or DMSO for controls, were added to each well. Colonies were allowed to grow for 10 days. Cells were then fixed with 1 ml of 100% methanol for 5 min, and then stained with 0.5% crystal violet in 20% methanol for 5 min. Plates were gently washed with dH_2O and left to dry overnight. Colonies in each well were counted and taken as a percentage of the control well. Experiments were performed in triplicate.

Western blot

Western blots were performed as previously described by us [4]. Antibodies used in this study were HSP27 (catalog #2402), vimentin (#5741), E-cadherin (#3195), all from Cell Signaling, and Desmoplakin (ab71690) from Abcam.