Microtubules Tip the Balance of Cytoskeletal Forces in Circulating Tumor Cells

Michael A. Matrone\textsuperscript{1}, Rebecca A. Whipple\textsuperscript{2}, Eric M. Balzer\textsuperscript{2}, and Stuart S. Martin\textsuperscript{2}

Abstract

Detection of circulating tumor cells (CTC) is advancing as an effective predictor of patient outcome and therapeutic response. Unfortunately, our knowledge of CTC biology remains limited, and the impact of drug treatments on CTC metastatic potential is currently unclear. Improved CTC imaging \textit{in vivo} and analysis of free-floating tumor cells now show that cytoskeletal regulation in CTCs contrasts starkly with tumor cells attached to extracellular matrix. In this review, we examine how persistent microtubule stabilization promotes the formation of microtentacles on the surface of detached breast tumor cells and enhances metastatic potential, \textit{Cancer Res; 70(20); 7737–41. ©2010 AACR.}

Introduction

To control morphology, cells perform a delicate balancing act of cytoskeletal forces. Termed the cellular tensegrity model, expansion of microtubules is counteracted by tension in the actin cytoskeleton to stabilize cell shape (1). Cytoskeletal aberrations contribute to many characteristics of aggressive tumors, such as increased cell motility, weakened adhesive contacts, and metastatic dissemination. For example, hypoxia-induced signaling can promote an epithelial-to-mesenchymal transition (EMT), which leads to numerous cytoskeletal alterations, including expression of chemotherapeutic-resistant tubulin isoforms and the intermediate filament (IF) vimentin (2, 3).

Recent studies show that altering the balance between microtubules and actin has serious implications for circulating tumor cell (CTC) dissemination (4, 5). \textit{In vitro} CTC modeling reveals that detached cells form microtentacles (McTN), dynamic, microtubule-enriched plasma membrane extensions that are antagonized by the actin cytoskeleton (McTN), showing that cellular mechanisms stabilize glu-microtubules, including capping of the plus end and crosslinking with IFs (8). In \textit{vivo}, cells forming McTNs are more efficiently retained in the microvasculature (8), reinforcing evidence that CTC attachment to the capillary endothelium is microtubule dependent (5). Here, we discuss the mechanisms of microtubule stabilization in CTCs and how these alterations influence metastatic efficiency (Fig. 1).

Mechanisms of Microtubule Stabilization in Cancer

Formation of detyrosinated tubulin

Microtubules are polarized and linear polymers composed of α- and β-tubulin heterodimers, which can be regulated through posttranslational modification, such as the cyclical removal and ligation of the COOH-terminal tyrosine on α-tubulin. An unidentified tubulin carboxypeptidase (TCP) removes this tyrosine from α-tubulin, exposing a glutamic acid residue to form detyrosinated microtubules (Glu-tubulin; ref. 9). This modification is reversed by tubulin tyrosine ligase (TTL), which replaces the tyrosine on free tubulin heterodimers to regenerate tyrosinated α-tubulin (Tyr-tubulin). In \textit{vivo}, glu-microtubules persist for hours, whereas tyr-microtubules undergoes turnover within 3 to 5 minutes (9). This stabilization effect is not observed with purified tubulin in \textit{vivo}, showing that cellular mechanisms stabilize glu-microtubules, including capping of the plus end and crosslinking with IFs (9).

Tubulin detyrosination is clinically associated with tumor aggressiveness and poor prognosis. In a study of 134 breast cancer patients, 65.4% of grade 3 primary breast tumors stained strongly for glu-microtubules, compared with 3.4% of grade 1 tumors (10). This glu-microtubule enrichment upon tumor progression may result from TTL inactivation, which is commonly observed in human carcinomas and sarcomas (10). Unlike TTL, the contribution of TCP to cancer progression is unknown. Despite more than 30 years of effort, TCP protein remains unidentified. What little is known about TCP stems from cell extract studies of chick brain embryos or cell lines, which have shown that the enzyme detyrosinates α-tubulin only in polymerized microtubules. In general, carboxypeptidases operate in a variety of mechanisms, using serine-, cysteine-, or metal ion-containing active
sites. Others possess affinities for terminal amino acids that have specific side chains. Phosphatase inhibitor studies indicate that TCP association with microtubules is regulated by serine-threonine phosphorylation, and not tyrosine phosphorylation, but it is not known if TCP, tubulin, or an intermediate is the phosphorylation-regulated component (11). In addition, unlike other carboxypeptidases, TCP remains unaffected by inhibitors of carboxypeptidase A, a pancreatic carboxypeptidase with catalytic affinity for COOH-terminal amino acids with aliphatic or aromatic side chains, such as tyrosine (12).

Tubulin detyrosination may also be associated with invasion and metastatic dissemination. Glu-microtubules orient in the direction of cell migration, implying their involvement in tumor invasiveness (13). In models of the hematogenous dissemination of CTCs, detachment of breast carcinoma cell lines increases glu-tubulin, which concentrates in McTNs (4, 6). Like the glu-microtubules from which they are formed, McTNs are persistent and are observed hours and days following cell detachment, especially in apoptosis-resistant cells (4). Furthermore, McTN formation and tumor cell reattachment are enhanced upon weakening of the actin cortex (4, 7), illustrating how cytoskeletal force imbalance can impact the metastatic potential of CTCs.

Crosslinking of microtubules to vimentin intermediate filaments

The cellular tensegrity model predicts that microtubules resist compressive buckling through a number of lateral interactions (1). Most notable is the association with IFs, nonpolarized ropelike structures that impart mechanical stability to animal cells through their association with other cytoskeletal structures and sites of cell-cell and cell–extracellular matrix (ECM) adhesion. For example, microinjection of nonpolymerizable and nontyrosinatable glu-tubulin causes collapse of IFs to the perinuclear region, showing how these two cytoskeletal systems coordinate to provide structural stability to the cell (9).

In malignancies, vimentin expression increases during the EMT associated with tumor invasion and metastasis, especially among breast cancers and melanomas. We have shown that vimentin expression enhances McTN formation (6). Metastatic breast carcinoma cell lines with elevated vimentin also exhibit increased McTNs. Conversely, tumor cell lines that express epithelial cytokeratins show fewer McTNs and decreased metastatic potential. Promoting vimentin phosphorylation and disassembly through protein phosphatase inhibition also reduces McTN formation (6). Furthermore, exogenous expression of dominant-negative vimentin decreases McTN frequency in metastatic cell lines that natively...
These data indicate that, even when the actin cortex is depolymerization significantly enhanced McTN formation, but combining tau expression and actin naked microtubules (16, 17). Similarly, actin depolymerization can withstand greater deforming forces compared with microtubule bundling, and individual tau-decorated microtubules provide structural strength to microtubules. Tau promotes metastasis during metastasis (8).

These data suggest that tau could provide a selective advantage during metastasis of breast tumors (from pathologic scores +1 to 0). In only 12% of patients did a loss of tau expression occur between primary and metastatic tumors. In only 12% of patients did a loss of tau expression occur between primary and metastatic tumors (8). These data suggest that tau could provide a selective advantage during metastasis (8).

Homo- and heterotypic cell aggregation

CTC survival in the circulation requires protection from mechanical damage due to shear and collision forces and from destruction by immune surveillance. Such protections are granted by the ability of metastatically efficient CTCs to homotypically and heterotypically aggregate. In vitro selection of tumor cell lines that efficiently cluster homotypically exhibited a greater ability to metastasize in vivo than nonaggregating parental cells (22). Similarly, our own studies show that McTNs enhance homotypic cell aggregation (4). Although formation of homotypic CTC aggregates increases the survival of disseminating cells by mechanical trapping, evidence suggests that CTC reattachment to the endothelium may also be activated through heterotypic aggregation of CTCs with platelets (22). Association of CTCs with platelets and other coagulation factors is implicated in venous thromboembolism, which correlates with CTC burden and poor prognosis in metastatic breast cancer patients (23). The CTC-platelet association involves activation of integrins and clotting factors to induce a fibrin coat that enhances CTC spreading following attachment to organ microvasculature. In addition, immune-mediated destruction of CTCs can be avoided through the direct association of CTCs with leukocytes. Given their ability to encircle adjacent cells during homotypic aggregation (Fig. 1B), McTNs may also facilitate heterotypic association of CTCs with platelets or with white blood cells.

Implications for Cancer Progression

Deformation of CTCs in transit

Optical deformability of cells is an emerging marker of cancer progression (18). A central determinant of metastatic efficiency is tumor cell survival during hematogenous dissemination. Metastasis of carcinoma CTCs is limited by the ability of the relatively large epithelial tumor cells to deform within the narrow size restrictions of the microvasculature. If CTC membranes are deformed beyond the limits of their surface area, the cells perish through shear-induced fragmentation. In contrast, metastatically efficient CTCs have been observed to undergo sphere-to-cylinder shape transformations within capillaries (19). These observations are supported by experimental deformations of suspended tumor cells with microfluidic optical stretchers, which indicate that tumor cells are more deformable than normal epithelial cells (18). The degree of actin crosslinking is directly implicated in CTC deformation. As malignancy progresses, the F-to-G actin ratio decreases, indicating a less polymerized actin cytoskeleton (20). Mutation in or altered expression of mediators of actin polymerization can also disrupt actin organization and crosslinking (21), which may weaken the cortical integrity. When microtubules are stabilized in concert with reduced actin integrity, CTCs produce increased McTNs (7, 8).
Increased intravascular retention

Currently, two models describe the successful reattachment of CTCs during blood-borne metastasis: a passive mechanism, whereby CTCs are mechanically trapped within the microvasculature; or an active mechanism, whereby specific adhesive interactions of CTCs permit microvasculature retention (24). In vivo studies by Korb and colleagues used intravitral microscopy to show that circulating HT-29 colon carcinoma cells adhere to liver sinusoidal capillaries in a microtubule-dependent manner (5). Specifically, pretreatment of these cells with the microtubule depolymerizer nocodazole significantly reduced CTC attachment. Surprisingly, inhibition of actin polymerization with cytochalasin-D significantly enhanced CTC reattachment (3). The cytoskeletal mechanisms supporting McTN formation (4, 6, 7) and McTN-dependent microvascular retention (8) match identically with these in vivo observations (5). As described above, tau stabilizes microtubules and increases McTNs in breast tumor cells, whereas whole-animal bioluminescence imaging shows that tau-expressing CTCs are more efficiently retained in the lung microvasculature compared with tau-deficient controls (8). These data (5, 8) and the ability of McTNs to penetrate endothelial layers (Fig. 1C) support an active role for microtubule stabilization during CTC reattachment within the microvasculature. Given that cells with McTNs attach more efficiently to ECM in vitro (4), further investigation is needed to determine whether McTNs are enriched in distinct adhesive proteins that may mediate CTC-endothelial attachment, such as integrins or selectins. Although selectin-mediated endothelial attachment permits tumor cell rolling, integrin-mediated ECM attachment is required for CTCs to arrest against the force of blood flow (25). These data support a model in which contact with the underlying ECM through transendothelial penetration is necessary to promote the earliest stages of CTC extravasation, and McTNs are capable of such transendothelial penetration (Fig. 1C).

Microtubule-Targeted Drugs and CTCs

Stabilizing drugs, like taxanes, prevent microtubule disassembly, stalling tumor cells in metaphase, and initiating cell death through spindle checkpoint activation or mitotic catastrophe. Although microtubule-targeted drugs are highly successful in primary tumor treatment, their success is limited for metastatic disease. The effects of microtubule-targeted chemotherapies on CTCs have not been thoroughly investigated. Evidence that taxanes enhance McTN formation and tumor cell reattachment (7) emphasizes that the effects of existing cancer drugs on CTCs should be examined to ensure that antimitotic therapies do not inadvertently increase metastatic potential. Emerging evidence that paclitaxel treatment actually increases lung colonization of CTCs (26), but a direct role for microtubule stabilization in CTC retention has not yet been excluded in this model.

Camara and colleagues showed that a potential consequence of taxane administration is the mobilization of CTCs. Reports indicate that newly diagnosed breast cancer patients receiving either combination or single-agent therapy, each with subsequent taxane administration, experienced as much as a 10,000 fold increase in CTCs during the taxane arm (27). These cell numbers remain elevated pre- and postsurgery, indicating persistent CTCs that resisted taxane treatment or new CTCs that were mobilized during surgery (27).

Growing evidence indicates that the ability of CTCs to successfully metastasize may increase because of the detrimental effects of chemotherapeutics. For example, cyclophosphamide has been observed to cause microvascular damage and to increase intravascular proliferation and extravasation of tumor cells. Likewise, anti-angiogenic therapies can also increase metastatic potential (28). Enhancement of metastasis could be a consequence of taxanes, which can alter the balance between microtubules and actin. Balzer and colleagues showed that jasplakinolide, an in vitro actin-stabilizing peptide that causes abnormal nucleation and aggregation of actin in vivo, increased McTN formation (7, 29). This effect was enhanced by paclitaxel-mediated microtubule stabilization, which additionally increased tumor cell reattachment and spreading on ECM (7). These data indicate that CTCs may be stimulated to adhere to blood vessel walls or to the underlying ECM after taxane-mediated microtubule stabilization or actin disruption. Although microtubule-targeted therapies are a common strategy to reduce the growth of attached tumor cells, proteins that alter actin polymerization are emerging as potential targets for chemotherapy (30).

Taxane resistance with tau expression may heighten the risk of successful metastasis because of an increased mobilization of McTN-forming CTCs. Tau contributes to taxane resistance by competing with paclitaxel for microtubule binding. Pulse-chase experiments measuring tritiated-paclitaxel incorporation into microtubules revealed that microtubules assembled in the presence of tau incorporate less paclitaxel compared with those assembled with tubulin alone. Consequently, the IC_{50} of paclitaxel required to kill tau-expressing breast tumor cells increases (15).

Further studies are required to evaluate the effects of microtubule-targeted chemotherapeutics on CTCs and metastatic risk. As new therapeutic targets are identified and new therapies developed, any possible detrimental effects of microtubule-based chemotherapies could be counteracted or avoided by defining a specific risk window during the course of treatment.

Future Considerations

Metastasis remains the leading cause of death among cancer patients. CTCs pose a particular challenge as our knowledge of the interaction between CTCs and the circulatory microenvironment is not well defined. It is now understood that tipping the cytoskeletal force balance toward microtubule stabilization and actin depolymerization causes CTCs to acquire characteristics that promote metastatic efficiency. Through stabilization by detachment-induced post-translational modifications and interactions with accessory proteins, microtubules can overcome the restrictive forces of cortical actin to protrude from the plasma membrane;
thus mediating McTN formation, cell-cell aggregation, and endothelial attachment of CTCs.

Considering the detrimental effects of chemotherapeutics on CTC mobilization and McTN induction, it raises the question of whether our anticancer effort is missing an important target. It remains critical to develop methods that distinguish whether drug-induced tumor shrinkage arises from tumor cell death or tumor cell dissemination, because these two effects have dramatically different consequences in a neoadjuvant setting. Although current drug development efforts and clinical endpoints focus on limiting the growth of either primary or metastatic tumors, it will be important to understand the effects of existing cancer drugs on CTCs in order to improve treatment strategies and develop novel therapies aimed at reducing the metastatic potential of CTCs.

Disclosure of Potential Conflicts of Interest

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