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Extracellular Matrix: A Gatekeeper in the Transition from Dormancy to Metastatic Growth

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Abstract

Metastases can develop after apparently successful treatment of a primary tumor, sometimes following a period of tumor dormancy that can last for years. However, factors that regulate metastatic tumor dormancy remain poorly understood. Here we review the potential contribution of interactions between tumor cells and the microenvironment in metastatic sites, in regulating tumor dormancy vs. metastatic growth. We focus particularly on the potential role of the extracellular matrix in regulating maintenance and release from dormancy. Tumor cells that fail to properly adhere to the extracellular matrix may enter a state of dormancy. The molecular and physical composition of the extracellular matrix can be affected by tumor cells themselves, as well as multiple stromal cell types. The roles of integrins, fibronectin, and collagen are discussed, as are factors that can change the extracellular matrix. A better understanding of the molecular details of the crosstalk between tumor cells and the extracellular matrix in secondary sites, and how these regulate the dormant state, may lead to improved therapeutic strategies to induce or maintain disseminated tumor cells in a dormant state, or alternatively to successfully eradicate dormant cells.

MeSH Keywords

Cell Adhesion; Collagen; Cytoskeleton; Extracellular Matrix; Fibronectins; Integrins; Metalloproteases; Neoplasm Metastasis; Neoplasms; Recurrence

Introduction

Metastasis – the dissemination of tumor cells from the primary tumor and growth at secondary sites – is the major cause of mortality in cancer patients and may occur years and even decades after successful removal of the primary tumor and adjuvant therapy.(1,2) This

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Conflict of Interest Statement

None declared.

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latency period is due to tumor dormancy, in which residual disease is present, but not clinically apparent. The mechanisms responsible for maintaining the survival and outgrowth of dormant tumor cells remain largely unknown. Two possible scenarios for tumor dormancy have been described, based on both experimental and clinical evidence. It has been proposed that dormant tumor cells may exist in a quiescent state for many years as solitary tumor cells.(3–5) These cells are resistant to conventional therapies that target actively dividing cells,(6–8) leading to possible disease recurrence following adjuvant therapy that targets actively dividing cells. Alternatively, tumor dormancy may exist as micrometastases where cellular proliferation is balanced by apoptosis.(9,10) Consequently, in this balanced state, there is no net increase in tumor mass over time. These micrometastases remain dormant because of lack of recruitment of the vasculature needed to nourish the tumor, known as the angiogenic switch and/or involvement of the adaptive immune system.(11–13)

Recent evidence indicates that dissemination of tumor cells may occur at an early stage of tumor progression.(7,14,15) These disseminated tumor cells can be found in the bone marrow, lymph nodes, and blood circulation of cancer patients and may be in a quiescent state.(1,7,16–18) If this is the case, recurrence after a period of tumor dormancy might depend on the rate at which genetic abnormalities progress in early disseminated tumor cells.(1,19) However, tumor dormancy can occur in tumor cells that have already acquired genetic alterations(20) and persist in a dormant state beyond the expected time for a genetic alteration to occur, suggesting that additional mechanisms may induce a tumor cell to enter or maintain a dormant phase, regardless of its genetic background. One mechanism that may regulate tumor dormancy is the interaction of the tumor cell with its microenvironment. Interactions of cells with their surroundings can have profound influences on gene expression and cellular behavior.(21–23) It has been postulated that a ‘non-permissive’ microenvironment encountered by a tumor cell may induce its dormancy, and that the failure of the tumor cell to engage with the surrounding microenvironment may trigger its quiescence.(22,24,25) The role of the microenvironment, as a modulator of survival and growth of the seeded tumor cells, was recognized more than a century ago by Stephen Paget. Paget proposed that metastasis will occur only when the tumor cell (the ‘seed’) and the microenvironment of a given organ (the ‘soil’) are compatible.(26,27) Consistent with this concept, Goodison *et al.* recently demonstrated in an experimental model that cancer cells may be seeded throughout the body, where they may remain dormant, only growing in specific, ‘favorable’ organs.(28) It is clear that many aspects of tumor biology, including tumor dormancy, can be regulated by interactions of tumor cells with their microenvironment.

In this review we will re-visit the ‘seed and soil’ theory, centering our discussion on the extracellular matrix (ECM), the microenvironment milieu that surrounds the dormant tumor cell. We will highlight recent studies demonstrating the role of the ECM in tumor dormancy. The ability of the dormant tumor cell to engage with the ECM through integrin signaling will be discussed. Furthermore, ECM remodeling by several stromal components leading to a potential establishment of a permissive microenvironment for the escape from tumor dormancy will be reviewed.

Engagement of the dormant tumor cell with the ECM

The microenvironment has been increasingly recognized as a critical regulator of cancer progression (reviewed in (1,5,21,22,25,29)). The ECM, a key component of the microenvironment, is in immediate contact with the tumor cells. The ECM functions as a critical source for growth, survival, motility, and angiogenic factors that significantly affect tumor biology and progression. Additionally, cell adhesion to the ECM triggers intracellular

signaling pathways that can regulate cell cycle progression, migration, and differentiation, through integrins and other cell surface receptors.(24,30,31) Thus, integrin-mediated interactions between tumor cells and the ECM are critical modulators of the metastatic potential of tumor cells.

Recently, we presented a study that clarifies potential mechanisms by which the microenvironment may regulate tumor dormancy.(32) In that study, solitary tumor dormancy and the transition to proliferation were recapitulated *in vitro* by utilizing a 3D *in vitro* culture system constituted from growth factor-reduced basement membrane, to mimic components of the ECM. Our results revealed that in the 3D culture system, cells with dormant behavior *in vivo* remained cell cycle arrested with elevated nuclear expression of p16 and p27. Our findings that the ECM can impose growth inhibitory signals on tumor cells were in concordance with previous reports.(33,34) Interestingly, the dormant tumor cells displayed distinct cytoskeletal organization with evidence of only transient adhesion to the ECM.(32) However, we demonstrated that the switch from quiescence to proliferative metastatic growth was strongly influenced by interactions with the ECM as a result of cytoskeletal reorganization and formation of actin stress fibers (Fig. 1). During the transition the tumor cells formed actin stress fibers via $\beta 1$ integrin signaling and downstream phosphorylation of myosin light chain by myosin light chain kinase. These findings are consistent with previous work implicating $\beta 1$ integrins in microenvironmental regulation of cell behavior(35), and were subsequently confirmed by others,(36) emphasizing the important role of the full engagement of the dormant tumor cell with the ECM as a mechanism to escape tumor dormancy(32). These observations are also consistent with previous studies in which downregulation of the urokinase receptor was shown to mediate signaling through the $\alpha 5 \beta 1$ integrin, forcing the cells into dormancy.(37,38) Furthermore, in transgenic mouse models for mammary or pancreatic beta cell cancer, knockdown of $\beta 1$ integrin resulted in inhibition of proliferation of the mammary tumor cells and senescence of the pancreatic beta tumor cells.(39,40) Thus, multiple lines of evidence indicate that lack of adhesion of the tumor cell to the ECM via integrins can lead a tumor cell to enter a dormant phase.

A solitary dormant tumor cell that fails to properly adhere to the ECM may initiate, under these stress conditions, mechanisms that lead to its long-term survival. For example, anchorage-independent survival of mammary tumors was shown to be mediated by secretion of laminin-5 by the detached mammary tumor cells. Laminin-5 as a component of the basement membrane induced tumor cell survival via $\alpha 5 \beta 1$ -mediated NF κ B activation. (41). Recently, it has been shown that detachment of epithelial cells from the ECM may lead to another survival mechanism called autophagy. Autophagy is a highly regulated self-digestion process that produces nutrients and energy for the cell through the breakdown of cytosolic components, and can lead to cell survival under stress conditions (reviewed in (42)). Evidence in the literature suggests that abrogated adhesion of epithelial cells to the ECM may induce autophagy through growth factor- and nutrient-sensing pathways, energy-sensing pathways, and integrated stress response.(42) Recently, Lu *et al.* reported that controlled induction of the tumor suppressor gene aplasia Ras homolog member I (ARHI) within human ovarian tumor cells induces autophagy and tumor dormancy. Interestingly, the tightly regulated autophagy signaling for survival of the cells was dependent on the presence of components from the tumor microenvironment such as ECM proteins. Absence of such factors led to excessive autophagy and programmed cell death.(43) Thus, failure of dormant tumor cells to properly engage with the ECM may trigger autophagy and promote long-term survival of the cells. In order to subsequently escape tumor dormancy tumor cells need to fully engage with the ECM components via integrin receptor(s), inducing downstream signaling and leading to cytoskeletal reorganization and proliferation.

Remodeling of ECM and the transition from dormancy to growth

Homeostasis of the ECM is maintained in the normal stroma by a tight balance between ECM synthesis, organization, cross-linking, and degradation. In the presence of tumor cells, ECM homeostasis is disrupted by the tumor cells themselves, by stromal components such as fibroblasts, macrophages, and leukocytes, (44,45) and by the interactions among these components and the tumor. In addition, bone marrow-derived hematopoietic progenitor cells may contribute to the microenvironment surrounding metastatic tumor cells.(46) Hence, induction of ECM remodeling by these multiple stromal components may lead to a permissive 'soil' that enables tumor cells to escape from dormancy.

ECM synthesis and organization: Priming the soil

Alterations in the expression of ECM-related genes have been identified in gene expression signatures related to poor prognosis and metastases in breast cancers.(47–51) Furthermore, a 'wound-healing' gene expression signature, which consists of genes related to extensive remodeling of the ECM, has been associated with poor outcome in breast, lung, and gastric carcinomas. (52,53) Indeed, changes in the ECM components such as production and organization of fibronectin have been implicated in eliciting the transition from dormancy to metastatic growth. Consistent with this idea, we found that a metastatic mammary cell line transitioned from a quiescent state to proliferation upon production of fibronectin and signaling through the $\beta 1$ integrin, whereas a related dormant mammary cancer cell line did not express fibronectin. (Fig. 2 and Fig. 6A in Barkan *et al.*(32)). However, addition of fibronectin was able to induce transient proliferation in the dormant cell line.(32) Similarly, in head and neck carcinoma cells, high levels of association of urokinase plasminogen activator receptor (uPAR) with the $\alpha 5 \beta 1$ integrin produced fibronectin fibrils, while disruption of the uPAR/integrin complex led to a drastic reduction in the number of fibronectin fibril-containing cells and forced the cells into a dormant state.(38) Interestingly, fibronectin has also been shown to contribute to the 'pre-metastatic niche'. The pre-metastatic niche has been described as the permissive microenvironment prepared at the future metastatic site by recruitment and clustering of bone marrow-derived cells (BMDC). These cells can be induced and recruited by the primary tumor to prime the 'soil' at the future metastatic site, prior to the colonization of the tumor cells.(46) Increased fibronectin expression in fibroblasts and fibroblast-like cells residing at the pre-metastatic site were reported to be vital for the adhesion of the BMDC that express $\alpha 4 \beta 1$ integrin.(54) Blocking the adhesion of BMDC to fibronectin reduced the formation of the BMDC clusters and inhibited metastasis.(54) Together, these studies suggest that fibronectin may be a fundamental component in establishing a permissive microenvironment for the transition of the dormant cell to metastatic growth.

Recently, lysyl oxidase (LOX) was shown to contribute to the establishment and maintenance of the premetastatic niche.(55) LOX released from hypoxic tumor cells at the primary site was able to induce cross-linking of collagen at the pre-metastatic niche at the distant site. This in turn induced increased matrix remodeling and matrix stiffening, leading to recruitment of BMDC to the site and establishment of a permissive microenvironment for metastatic growth.(55) These findings have important, potential clinical relevance, as high expression of LOX had been shown to be a prognostic marker for poor prognosis and for lymph node metastasis in several squamous carcinomas.(56)

Likewise, Type I collagen (Col-I) expressed by fibroblasts is another ECM component that has been identified as a prognostic marker for poor outcome, metastases, and tumor recurrence.(47,49,50) High levels of pro-collagen type I, a marker for Col-I synthesis, have been found in the serum of patients with recurrent breast cancer.(57) Increased breast density associated with increased stromal Col-I has been shown to promote tumor initiation,

progression, and increased risk of metastasis and local recurrence after mastectomy or radiotherapy.(58) Patients with invasive ductal carcinoma with fibrotic foci display an unusually dense collagenous stroma, which is associated with a higher risk of developing bone and lymph node metastasis, disease recurrence, and a worse prognosis.(59,60) Lymph node metastases in these patients are often associated with fibrotic foci. These clinical observations suggest that establishment of a fibrotic-like environment via induction of Col-I may provide a fertile 'soil' for the transition from dormancy to metastatic growth. Induction of fibrosis, with deposition of Col-I in the metastatic microenvironment, was shown to induce the transition of dormant mammary cancer cell line to metastatic growth through $\beta 1$ integrin signaling culminating in cytoskeleton reorganization (Barkan *et al.*, unpublished data). Hence, Col-I enrichment at the metastatic site may be a critical determinant of cytoskeletal reorganization in dormant tumor cells, leading to their transition from dormancy to metastatic growth.

Matrix stiffening is induced by increased Col-I deposition and cross-linking and has been shown previously to promote malignant transformation.(61) Increased matrix stiffening has been previously observed in fibrotic lungs, tissue exposed to high radiation, scar tissue, and in women with dense breasts (reviewed in (62)). Therefore, changes in the mechanical compliance of the matrix may also regulate the transition from cellular dormancy to growth. It is important to note that matrix stiffening occurs as part of the normal aging process in some organs. Aging in some tissues displays abrogated post-translational modifications of ECM proteins and cross-linking, thus yielding a stiffer matrix.(63) Furthermore, increased collagen production by fibroblasts has been found in aging tissue.(64) Thus, it is possible that recurrence of the disease after long latency may be a consequence of the ECM aging at the recurring site.

Processing the ECM: Unraveling the soil

The ECM can undergo degradation by matrix metalloproteases (MMP) that are prominently secreted by stromal cells (45) or by heparanase prominently expressed and secreted by tumor cells (65). MMPs, by 'unraveling' the ECM, may contribute to the establishment of a microenvironment that may support tumor dormancy or its switch to metastatic growth. Leukocytes can secrete MMPs, leading to the release of bioactive fragments of extracellular matrix that can inhibit angiogenesis, such as endostatin,(66) restin,(67) arrestin,(68) and all three chains of type IV collagen.(69) Similarly, macrophages, by expressing MMP12, can release angiostatic factors from the ECM, leading to inhibition of the angiogenic switch and metastatic growth.(70) Thus, release of angiostatic factors may be implicated in angiogenic dormancy. Conversely, stromal MMPs may release cytokines and angiogenic factors that are sequestered to ECM molecules such as basic fibroblast growth factor and VEGF,(44) and hence, initiate the angiogenic switch needed to transition from micrometastatic dormancy to metastatic growth. MMPs may also play a role in the formation of a permissive niche for the transition from solitary dormancy to metastatic growth. For example, leukocyte secretion of MMP2 and MMP9 were shown to activate latent TGF β residing in the ECM. Activation of TGF β may lead to Col-I synthesis and LOX expression, (71,72) thus establishing a permissive niche for metastatic growth. Remodeling of the ECM and release of angiogenic factors can be also initiated by the residing tumor cells. Heparanase preferentially expressed in human tumor cells, is an endoglycosidase that cleaves heparan sulfate (HS) chains of heparan sulfate proteoglycans, an essential and ubiquitous macromolecule associated with the cell surface and the extracellular matrix of a wide range of cells and tissues (65). Degradation and remodeling of the ECM by heparanase can release angiogenic factors (65,73) and, similar to MMPs, may initiate the angiogenic switch needed to transition from micrometastatic dormancy to metastatic growth.

In summary, and as diagrammed in Fig. 3, these studies suggest that the physical and biological composition of the ECM, which is regulated by multiple stromal cells and tumor cells, may help to determine the fate of the dormant tumor cell, and regulate entry or exiting the dormant state.

Future Directions

The crosstalk between the dormant tumor cells and the ECM as presented in this review suggests new avenues for regulation of tumor dormancy. The studies reviewed here raise several possibilities for designing future therapies aimed to either induce or maintain tumor dormancy, or conversely, to induce cell death in residual dormant cells. Finding ways to control ECM biosynthesis (71) and its physical organization may establish a non-permissive microenvironment that may lead to tumor dormancy or may induce excessive autophagy and cellular death of the dormant tumor cell. Inhibiting the expression of ECM components such as fibronectin, Col-I deposition and its cross-linking by LOX could serve as potential targets for establishing a non-permissive microenvironment that may prevent recurrence of the disease. Furthermore, hindering the interaction between dormant tumor cells and growth-promoting changes in the ECM, via integrin $\beta 1$ and its downstream signaling pathways, may be an important new avenue in preventing disease recurrence. Indeed, development of specific integrin inhibitors is currently under clinical investigation. Inhibition of fibronectin receptor $\alpha 5 \beta 1$ by humanized anti $\alpha 5 \beta 1$ antibody is currently in phase I trials for cancer, and blocking peptides for integrins $\alpha 5 \beta 1$ is in preclinical development (reviewed in (74)). In summary, the papers reviewed here put forward the notion that the ECM and its crosstalk with the dormant tumor cells may act as a gatekeeper in the transition from dormancy to metastatic growth.

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Figure 1. Cytoskeletal reorganization and formation of actin stress fibers during the switch from dormancy to metastatic growth

A) Confocal microscopy image (Magnification x63) of D2A1 cells cultured in the 3D basement membrane extract (BME) system stained with DAPI (blue), antibody against the phosphorylated form of myosin light chain (MLC-p) (red), and for F-actin (green) at day 1 (d1) and day 7 (d7). Quiescent D2A1 cells displayed cortical F-actin and MLC-p staining on day 1, whereas, during their transition from quiescence to growth (days 4–7) D2A1 cells formed actin stress fibers with elevated expression of MLC-p. **B)** Frozen sections of the lungs removed from mice injected with D2A1 cells stably expressing GFP (green) at the indicated time points and stained for F-actin (red) and DAPI (blue). One week (1 Wk) post-injection, D2A1-GFP cells reside as solitary dormant cells with cortical staining for F-actin while by week 3 (3 Wk) the metastatic lesion from the D2A1-GFP cell line formed actin stress fibers. Modified from Barkan *et al.*(32)

Figure 2. Fibronectin expressed by metastatic D2A1 cells signals through $\beta 1$ integrin leading to transition from dormancy to growth

A–C) Confocal images (magnification x63). **A)** D2A1 cells cultured on 3D BME express fibronectin during their transition from quiescence to growth (red staining) (day 7). **B)** Frozen section of lung lesions of D2A1-GFP cells (green) stained for fibronectin (red). **C)** Treatment of D2A1 cells with a neutralizing antibody against $\beta 1$ integrin, for 6 days, led to inhibition of MLC-p (staining red), loss of actin stress fiber formation (staining green) and its co-localization with MLC-p (staining yellow) and proliferation of D2A1 cells cultured either on 3D BME or on 3D BME supplemented with fibronectin (BME+Fibro). Treatment

with control IgG antibody had no effect. White bar = 20 μ m. Modified from Barkan *et al.*
(32)

Figure 3. ECM remodeling by stromal cells and escape from tumor dormancy

The crosstalk between the ECM at the secondary site with the disseminated tumor cell may decide the tumor cell's fate. Solitary tumor cells that fail to properly adhere to the ECM may enter a state of dormancy activating long-term survival programs such as regulated autophagy. In order to subsequently escape tumor dormancy tumor cells need to fully engage with the ECM components via integrin receptor(s), inducing downstream signaling leading to cytoskeletal reorganization and proliferation. Fibroblasts as well as the tumor cells may remodel the ECM to induce a permissive microenvironment for the tumor cell growth. Changes in the ECM components such as production and organization of Col-I and fibronectin may lead also to the establishment of the pre-metastatic niche by BMDC, leading to the transition from dormancy to growth. After exiting quiescence, the tumor cell mass can enter pre-angiogenic dormancy due to release of angiostatic fragments from the ECM by the MMPs of leukocytes. The angiogenic switch will occur once angiogenic factors are released from the ECM by stromal MMPs, resulting in metastatic growth.