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Matrix rigidity regulates the transition of tumor cells to a bone-destructive phenotype through integrin β3 and TGF-β receptor type II

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ABSTRACT

Cancer patients frequently develop skeletal metastases that significantly impact quality of life. Since bone metastases remain incurable, a clearer understanding of molecular mechanisms regulating skeletal metastases is required to develop new therapeutics that block establishment of tumors in bone. While many studies have suggested that the microenvironment contributes to bone metastases, the factors mediating tumors to progress from a quiescent to a bone-destructive state remain unclear. In this study, we hypothesized that the "soil" of the bone microenvironment, specifically the rigid mineralized extracellular matrix, stimulates the transition of the tumor cells to a bone-destructive phenotype. To test this hypothesis, we synthesized 2D polyurethane (PUR) films with elastic moduli ranging from the basement membrane (70 MPa) to cortical bone (3800 MPa) and measured expression of genes associated with mechanotransduction and bone metastases. We found that expression of Integrin β3 (*Iβ3*), as well as tumor-produced factors associated with bone destruction (*Gli2* and parathyroid hormone related protein (*PTHrP*)), significantly increased with matrix rigidity, and that blocking *Iβ3* reduced *Gli2* and *PTHrP* expression. To identify the mechanism by which Iβ3 regulates Gli2 and PTHrP (both are also known to be regulated by TGF-β), we performed Förster resonance energy transfer (FRET) and immunoprecipitation, which indicated that Iβ3 co-localized with TGF-β Receptor Type II (TGF-β RII) on rigid but not compliant films. Finally, transplantation of tumor cells expressing Iβ3 shRNA into the tibiae of athymic nude mice significantly reduced *PTHrP* and *Gli2* expression, as well as bone destruction, suggesting a crucial role for tumor-produced Iβ3 in disease progression. This study demonstrates that the rigid mineralized bone matrix can alter gene expression and bone destruction in an Iβ3/TGF-β-dependent manner, and suggests that Iβ3 inhibitors are a potential therapeutic approach for blocking tumor transition to a bone destructive phenotype.

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1. Introduction

Many of the most common tumors metastasize to bone, including breast, lung, and prostate cancer. It is estimated that 70% of breast cancer and 90% of prostate cancer patients with metastatic disease will develop bone metastases [1]. These metastases often develop years after the primary disease has been removed and are a major cause of morbidity and mortality [2,3]. The primary clinical treatments for bone metastases focus on inhibiting bone resorption. While resorption inhibitors, like bisphosphonates, extend time to the first skeletal-related event, they neither directly inhibit tumor burden nor significantly extend survival when given after

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the diagnosis of a bone metastasis [4]. Thus, newer approaches are needed to improve patient outcomes [4], which require a mechanistic understanding of why some tumors establish in bone.

Recent evidence indicates that metastasis to bone and other organs can be predicted from the genes expressed by the primary tumor [5–9], but the mechanisms by which gene expression patterns and metastatic traits specific to bone transpire in tumor cells remains an unanswered question. A seminal study has shown that the breast tumor stroma contributes to the selection of invasive triple-negative (TN) breast cancer cells that are conditioned for metastasis to bone [10]. While this "metastasis seed pre-selection" mechanism affirms the role of the breast stroma to bestow an advantage for tumor cells to establish in bone, the factors mediating progression of the tumor cells from the pre-osteolytic to the osteolytic phase are unknown [11]. When metastatic tumor cells establish in bone, they secrete parathyroid hormone-related protein (PTHrP), interleukin 6 and 8, and/or other factors that induce osteoblast expression of Receptor Activator of NF-κβ Ligand (RANKL) [12,13]. The consequent stimulation of osteoclast-mediated bone resorption results in release of TGF-β and other growth factors from the extracellular matrix that continues to drive the expression of PTHrP by the tumor cells [14,15], which is regulated by the transcription factor Gli2 [14]. However, it is still unclear how bone microenvironmental effects on TGF-β signaling regulate gene expression in tumor cells or why tumors recur in the bone many years after the primary tumor has been removed. More clearly understanding the events that induce progression from a pre-osteolytic to an osteolytic phenotype may allow clinicians to better predict which tumors may form bone metastases and may allow for improved treatments.

The mineralized extracellular matrix, which has an elastic modulus ranging from $10^3 - 10^6$ kPa [16] differentiates bone from other tissues. Expression of *Gli2* and *PTHrP* by tumor cells *in vitro* correlates with bone-like matrix rigidity, which has been attributed to cross-talk between TGF-β and Rho-associated kinase (ROCK) [16–18], a factor regulating cell contractility [19]. Integrin-mediated cell-matrix interactions generate an adhesion molecule-integrin-actomyosin complex that can be shifted between inactive and signaling states by activation of myosin II or matrix rigidity [20]. However, recent studies suggest that rigidity-mediated changes in gene expression are driven by uniform displacements (100–150 nm) of the matrix [21–23]. Considering that cells cannot generate displacements >100 nm on substrates more rigid than 10–100 kPa [21], 100 kPa has been proposed as the upper limit at which cells enter a state of isometric contraction and cannot respond to further changes in rigidity [24]. Thus, the previously reported correlations of tumor cell proliferation [25], invasiveness [25], and expression of bone metastatic genes [16] with rigidity over ranges comparable to mineralized bone (10^3–10^6 kPa) cannot be explained by uniform displacements of the matrix. These observations raise questions regarding the mechanisms by which matrix rigidity regulates tumor cell gene expression in the mineralized bone microenvironment.

We hypothesized that when tumor cells become established in bone, the "soil" of the bone microenvironment, which is > 10^3 more rigid than the primary site, stimulates their transition from the pre-osteolytic to the osteolytic phase. We further postulated that the transition to the osteolytic phenotype on substrates with bone-like rigidity is mediated by integrins, but not by uniform displacements of the matrix as reported previously [21–23] due to its high rigidity (>100 kPa). TGF-β Receptor type II (TGF-β RII) interacts physically with β3 integrin sub-unit (Iβ3) to enhance TGF-β-mediated stimulation of MAP-kinases (MAPKs) during epithelial-mesenchymal transition (EMT) of mammary epithelial cells (MECs) [26]. However, the role of matrix rigidity in promoting interactions between these receptors has not been explored. We used a 2D polyurethane (PUR) film monoculture system to design matrices with rigidities ranging from that of the basement membrane to cortical bone, which is far more rigid than previous studies have examined. *In vitro* studies demonstrated that *Iβ3* expression correlated with bone-like rigidity, which led to co-localization of Iβ3 with TGF-β RII and increased expression of *PTHrP*. Inhibition of tumor-produced Iβ3 by molecular and genetic interference decreased expression of *PTHrP* and *Gli2 in vitro* and reduced bone destruction *in vivo*. These observations provide evidence for a previously unexplored mechanism by which crosstalk between solid-state and soluble factor signaling switches on osteolytic signaling in tumor cells.

2. Materials and methods

2.1. Materials

Lysine diisocyanate (LDI) was purchased from Kyowa Hakko (New York, NY). Glycerol, stannous octoate, and ε-caprolactone were purchased from Sigma–Aldrich (St. Louis, MO). Glycolide and DL-lactide were supplied by Polysciences (Warrington, PA). COSCAT 83 bismuth catalyst was acquired from Vertullus (Hopewell, VA). Fibronectin (Fn) was purchased from Life Technologies (Grand Island, NY). The human breast cancer cell line, MDA-MB-231 was originally purchased from ATCC and a bone-tropic clone was generated by our laboratory [27]. The human squamous lung cancer cell line, RWGT2, was derived as previously described [28]. The human adenocarcinoma cell line, PC3 was purchased from ATCC. Dulbecco's Modification of Eagle's Medium (DMEM), Minimum Essential Medium α (α-MEM), and RMPI media were supplied by Mediatech (Manassas, VA). All media was supplemented with 10% Fetal Bovine Serum (Atlas, Fort Collins, CO) and 1% Penicillin and Streptomycin (Mediatech).

2.2. Synthesis of 2D substrates for cell culture experiments

2D films with varying rigidity were prepared for the cell culture experiments. The parameter typically used to quantify the rigidity of tissues and synthetic matrices is the elastic modulus, which is defined by the initial slope of the stress (σ) versus strain (ε) curve. Poly(ester urethane) (PUR) substrates were synthesized and characterized as described previously [29]. Briefly, an appropriate amount of poly(ε-caprolactone-co-glycolide) triol (Mn = 300, 600, 720 or 3000 g/mol) was mixed with an LDI-PEG prepolymer and COSCAT 83 catalyst (Vertellus) for 20s in a Hauschild SpeedMixerTM DAC 150 FVZ-K vortex mixer (FlackTek, Inc, Landrum, SC) (Fig. 1A). The targeted index (ratio of NCO to OH equivalents times 100) was 105. The resultant mixture was poured into the wells of a tissue culture plate and allowed to cure for 24 h at 60 °C.

2.3. Measurement of elastic modulus by nanoindentation

Nanoindentation measurements were performed as shown in Fig. 1B–E to determine the Young's modulus of the substrate (E_s) by the method of Oliver and Pharr [30]. The reduced modulus E_r was calculated from load-displacement data acquired in the nanoindentation measurements:

$$E_r = \frac{\sqrt{\pi}}{2}\frac{S}{\sqrt{A}} \quad (1)$$

where A is the indenter contact area and the stiffness S is calculated from the initial slope of the unloading curve. The Young's modulus of the substrate E_s is related to the reduced modulus, the Young's