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Research Paper

β2ARs stimulation in osteoblasts promotes breast cancer cell adhesion to bone marrow endothelial cells in an IL-1β and selectin-dependent manner

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ABSTRACT

Progression and recurrence of breast cancer, as well as reduced survival of patients with breast cancer, are associated with chronic stress, a condition known to impact the hypothalamic-pituitary axis and the autonomic nervous system. Preclinical and clinical evidence support the involvement of the sympathetic nervous system in the control of bone remodeling and in pathologies of the skeleton, including bone metastasis. In experimental mouse models of skeletal metastasis, administration of the βAR agonist isoproterenol (ISO), used as a surrogate of norepinephrine, the main neurotransmitter of sympathetic neurons, was shown to favor bone colonization of metastatic breast cancer cells via an increase bone marrow vascularity. However, successful extravasation of cancer cells into a distant organ is known to be favored by an activated endothelium, itself stimulated by inflammatory signals. Based on the known association between high sympathetic outflow, the expression of inflammatory cytokines and bone metastasis, we thus asked whether βAR stimulation in osteoblasts may alter the vascular endothelium to favor cancer cell engraftment within the skeleton. To address this question, we used conditioned medium (CM) from PBS or ISO-treated bone marrow stromal cells (BMSCs) in adhesion assays with bone marrow endothelial cells (BMECs) or the endothelial cell line C166. We found that ISO treatment in differentiated BMSCs led to a robust induction of the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). The CM from ISO-treated BMSCs increased the expression of E- and P-selectin in BMECs and the adhesion of human MDA-MB-231 breast cancer cells to these cells in short-term static and dynamic adhesion assays, and a blocking antibody against IL-1β, but not IL-6, reduced this effect. Direct IL-1β treatment of BMECs had a similar effect, whereas the impact of IL-6 treatment on the expression of adhesion molecules by BMECs and on the adhesion of cancer cells to BMECs was negligible. Collectively, these *in vitro* results suggest that in the context of the multicellular and dynamic bone marrow environment, sympathetic activation and subsequent βAR stimulation in osteoblasts may profoundly remodel the density but also the activation status of bone marrow vessels to favor the skeletal engraftment of circulating breast cancer cells.

1. Introduction

Breast cancer, the most common cancer diagnosed among women, frequently metastasizes to bone, causing osteolytic lesions, pain, fractures and poor life quality [1–3]. Current treatment options for breast cancer-associated skeletal-related events mainly target the action of osteoclasts with bisphosphonates [4,5], but the 5-year survival rate associated with the use of this class of drugs is improved in postmenopausal women only [6]. In addition, the use of adjuvant denosumab in an intense dosing schedule did not improve bone metastasis-free survival in patients with early-stage breast cancer who were receiving optimal locoregional and standard-of-care systemic adjuvant therapy [7]. Hence, despite the overall success of treating primary breast cancer, new treatments options are needed to reduce recurrence and increase the survival of women with advanced breast cancer.

Metastatic cancer cells circulate throughout the body at very early

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stages of the disease and can lodge into distant organs in a dormant stage for years until unknown mechanisms trigger their re-entry into the cell cycle and growth. These early disease stages represent an opportunity for treatment [8], as disrupting the cross-talk between disseminated cancer cells and their microenvironment might prevent progression to macrometastases and recurrence. Cancer patients, including those with triple-negative breast cancer (TNBC), have increased survival metrics when their treatment regimen includes a β-blocker [9–12]. β-blockers are a widely used drug for the treatment of high blood pressure, arrhythmia and anxiety, and work by blocking the communication between sympathetic nerves and target cells. Activation of the hypothalamic-pituitary axis (HPA) and of the sympathetic nervous system (SNS) are hallmarks of prolonged stress, and recent studies have shown that chronic stress exacerbates cancer progression in animal models of prostate [13], ovarian [14,15] and breast cancers [16–18], as well as in some cancer patient and survivor cohort studies [19–24]. Patients diagnosed with cancer experience significant psychological stress at the time of diagnosis and all along treatment [25–28], and the threat of relapse is likely to contribute to long-term stress and decline in health-related quality of life [29,30]. Prolonged stress is known to have deleterious effects on the physiology of multiple tissues, via the release of norepinephrine (NE) from nerve terminals, which then binds post-synaptic adrenergic receptors (βAR) on target cells, including bone-forming cells [31]. During normal bone remodeling in mice, osteoblasts respond to the synthetic βAR agonist isoproterenol (ISO) by an increase in RANKL and VEGF production, leading to increased osteoclastogenesis and vascular bone density *in vivo*, respectively [18,32]. We have shown in the context of preclinical bone metastasis models that these SNS-induced changes in the bone marrow environment promote breast cancer bone colonization following intracardiac injection of MDA-MB-231 breast cancer cells, by favoring breast cancer cell migration (in a RANKL-dependent fashion) and by increasing the density of bone marrow blood vessel (in a VEGF-dependent fashion) [18,32]. Independent studies have shown that chronic SNS activation promotes breast primary tumor growth and metastasis via changes in stromal homeostasis as well, which involved an influx of pro-inflammatory macrophages and an increase in primary tumor vasculature density [16,33].

The vasculature is a crucial component for primary tumor growth, but also for the metastatic process in distant organs. Interactions with endothelial cells must occur in order for tumor cells to extravasate from the circulatory system and to establish at a secondary site [34,35]. The bone is a highly vascularized tissue, and osteoblasts are known regulators of angiogenesis during skeletal development and bone regeneration [36,37]. Our previous findings suggest SNS activation effectively increases bone marrow vessel density in adult mice [32]. The extravasation process of cancer cells is very similar to lymphocytes diapedesis after injury or infection [38,39]. During the resulting inflammation, endothelial cells are activated by inflammatory cytokines to express adhesion molecules and synthesize chemokines that are presented on their luminal surface [40]. Similar to the leukocyte extravasation process, tumor cells are recruited to secondary sites by chemotactic gradients of cytokines and growth factors [41]. Inflammatory mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) for instance have been shown to favor cancer cell interactions with endothelial cells by altering the levels of adhesion proteins present on the endothelium [42]. The activated endothelium expresses inducible adhesion molecules such as E-selectin, P-selectin, ICAM1, VCAM1 and multiple β-integrins, all of which can facilitate arrest, docking, and extravasation of metastatic breast and prostate tumor cells into bone [43–45]. Selectins are members of the carbohydrate-binding proteins family. These molecules are involved in adhesive interactions between endothelium and leukocytes or platelets within the blood circulatory system. There are three members of the selectin family: P-, E-, and L-selectin. The expression of these adhesion molecules on the endothelium is temporally coordinated to ensure efficient inflammatory response [46]: whereas L-selectin mediates fast rolling of leukocytes on the endothelium, P- and E-selectins support rolling at lower velocities [40]. Selectins binds to various classes of molecules (mucins, sulfated glycolipids, glycosaminoglycans) and most of these molecules were shown to be functional selectin ligands *in vivo* [47]. Prostate [48], colorectal [49], pancreatic [50] and breast cancer cells [51] expressing high levels of ligands for selectins have been shown to exhibit more aggressive oncogenic properties *in vitro, in vivo,* and in patient samples.

In this study, we investigated the putative impact of sympathetic nerve activation on the adhesive properties of the activated bone endothelium for metastatic breast cancer cells, via *in vitro* assays designed to probe the communication and interaction between osteoblasts, endothelial cells and breast cancer cells.

2. Materials and methods

2.1. Cell lines

Human GFP^+ MDA-MB-231 and murine GFP^+ 4T1 mammary tumor cells were cultured with 10% FBS DMEM High Glucose (ThermoFisher, #1965118), BMSCs with 10% FBS α-MEM (Fisher scientific, #SH3026501), mouse C166 endothelial cells and BMECs with complete ECM (ScienCell, #1001) at 37 °C and 8% CO_2.

2.2. Primary mouse bone marrow stromal cells

Hindlimbs from WT C57BL/6 mice were used to prepare primary mouse bone marrow stromal cells (BMSCs). Femur and tibia were stripped of skin and muscles, distal and proximal epiphyses were cut off, and each bone was inserted into a punctured 0.5 mL tube placed into a 1.5 mL tube. Tubes were centrifuged for 4 min at 4000 g. Resulting pellets were resuspended in complete α-MEM (Fisher Scientific, #SH3026501), and cells were plated at 1×10^6 cells/mL. Cultures were grown in 10% FBS α-MEM for 7 days and then switched to an osteogenic medium (10% α-MEM containing 50 μg/mL ascorbic acid [Sigma, #A5950] and 10 mM β-glycerophosphate [Sigma, #G9891-25 G]) for 7 more days.

2.3. Primary mouse bone marrow endothelial cells

Primary mouse bone marrow endothelial cells (BMECs) were harvested as described for BMSCs. Flushed cells were resuspended in complete ECM (ScienCell, #1001). Tissue culture dishes were coated for 20 min at 37 °C with 0.8 μg/cm^2 fibronectin (Gibco, #33016015) then cells were plated at 3×10^6 cells/mL. Cultures were then grown in complete ECM for 7 days.

2.4. Gene expression assay

For all gene expression assays, total RNA was extracted from cells using TRIzol (Invitrogen, #15596-026). Following DNAse I treatment (ThermoFisher, #18068015), cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813). Real-time PCR was performed using SYBR Green Supermix (Biorad, #1708884) gene expression assays on a Biorad CFX96 Real-Time System with appropriated primers (see Supplementary Table 1). Amplification specificity was verified by the presence of a single peak on the melting curve of the amplicon. Gene expression was analyzed by the ΔΔCt method.

2.5. Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, then blocked in 1% bovine serum albumin for 1 h at room temperature. Immunodetection of CD62E, CD31, and endomucin was