Biochimica et Biophysica Acta 1843 (2014) 815–826

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Microenvironmental stimuli affect Endothelin-1 signaling responsible for invasiveness and osteomimicry of bone metastasis from breast cancer

Paola Bendinelli [a], Paola Maroni [b], Emanuela Matteucci [a], Alessandro Luzzati [b], Giuseppe Perrucchini [b], Maria Alfonsina Desiderio [a,*]

[a] Dipartimento di Scienze Biomediche per la Salute, Molecular Pathology Laboratory, Università degli Studi di Milano, Italy
[b] Istituto Ortopedico Galeazzi, IRCCS, Milano, Italy

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 4 December 2013
Accepted 20 December 2013
Available online 27 December 2013

Keywords:
Bone metastasis
Endothelin-1 axis
Growth factors
Microenvironment
Osteomimicry

ABSTRACT

The present study was undertaken to clarify the function(s) of Endothelin-1 and its receptors ET_AR and ET_BR in osteolytic-bone metastasis from breast cancer, and their regulation by hepatocyte and transforming growth factors (HGF, TGF-β) and hypoxia. The aim was to evaluate the adaptability of bone metastasis to microenvironmental stimuli through Endothelin-1-mediated epithelial-mesenchymal transition (EMT), or the reverse process MET, and through osteomimicry possible key features for bone colonization. We compared low (MCF-7) and high (MDA-MB231) invasive-breast carcinoma cells, and 1833-bone metastatic clone, with human pair-matched primary breast-carcinomas and bone metastases. Parental MDA-MB231 and the derived 1833-clone responded oppositely to the stimuli. In 1833 cells, TGF-β and hypoxia increased Endothelin-1 release, altogether reducing invasiveness important for engraftment, while Endothelin-1 enhanced MDA-MB231 cell invasiveness. The Endothelin-1-autocrine loop contributed to the cooperation of intracellular-signaling pathways and extracellular stimuli triggering MET in 1833 cells, and EMT in MDA-MB231 cells. Only in 1833 cells, HGF negatively influenced transactivation and release of Endothelin-1, suggesting a temporal sequence of these stimuli with an initial role of HGF-triggered Wnt/β-catenin pathway in metastatization. Then, Endothelin-1/ET_AR conferred MET and osteomimetic phenotypes, with Runt-related transcription factor 2 activation and metalloproteinase 9 expression, contributing to colonization and osteolysis. Findings with human pair-matched primary ductal carcinomas and bone metastases gave a translational significance to the molecular study. Endothelin-1, ET_AR and ET_BR correlated with the acquisition of malignant potential, because of high expression already in the *in situ* carcinoma. These molecular markers might be used as predictive index of aggressive behavior and invasive/metastatic phenotype.

1. Introduction

Advanced breast carcinomas are prone to give metastasis at the skeleton, viscera and lymph nodes. Bone metastases can develop also ten years after surgical removal of the primary tumor, because osteotropic malignant cells may have a long period of quiescence before secondary outgrowth [1]. Insights into the molecular mechanisms of organotropism should be provided to devise new therapies aimed at the metastasis, or at the metastasis-microenvironment cross-talk [1,2]. Osteomimetic properties are suggested for breast carcinomas with bone tropism, but the underlying regulatory mechanisms and the relevance for colonization are still elusive [3–6].

Literature does not indicate whether skeletal metastasis from breast cancer coopt signaling pathways normally used by host bone, as evidenced for metastases colonizing other tissues [7]. In view of the plasticity of the metastatic phenotype [8,9], it is difficult to consider the gene signature of primary carcinoma the only factor responsible for bone tropism. For example Src, a candidate molecular target for therapy of bone metastasis, is active in human-primary breast carcinoma and in MDA-MB231 breast carcinoma cells [10,11]. By *in vivo* selection of the latter cell line, Massagué and colleagues isolated the 1833 subpopulation with enhanced ability to give bone metastasis [12]. In the xenograft model, prepared with the 1833 clone, Src phosphorylation is regulated by hepatocyte growth factor (HGF) [13]. Osteolytic bone metastasis is largely prevented by the concomitant blockade of HGF signaling pathway and the pleiotropic Src activity [14]. Transforming growth factor β (TGFβ) is one of the most potent regulators of Endothelin-1 (ET-1) levels in endothelial cells, through functional cooperation of Smad and AP-1 transcription factors and the co-activator CREB-binding protein

Abbreviations: HGF, hepatocyte growth factor; TGFβ, transforming growth factor β; EMT, epithelial-mesenchymal transition; ET-1, Endothelin-1; MMP, metalloproteinase; Runx2, Runt-related transcription factor 2; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor-2; 3′-UTR, 3′-untranslated region

* Corresponding author at: Università degli Studi di Milano, Dipartimento di Scienze Biomediche per la Salute, Via Luigi Mangiagalli, 31, 20133 Milano, Italy. Tel.: +39 02 50315334; fax: +39 02 50315338.
E-mail address: a.desiderio@unimi.it (M.A. Desiderio).

0167-4889/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.12.015

[15]. In normal mammary cells, TGFβ increases the expression of metalloproteinases (MMP) 2 and 9, implicated in migration and invasion [16]. TGFβ stimulates MMP 13 production in chondrosarcoma cells [17], and activates IL-11 and connective tissue-derived growth factor in 1833-bone metastatic cells [12].

The establishment of bone metastasis involves bidirectional interactions between cancer cells and the bone microenvironment [18]. Though bone is a hard calcified tissue, bone marrow is a hospitable environment *per se* due to the abundant sinusoids and growth factor/cytokine networks. After engraftment, the metastatic cells seem to perturb the microenvironment and exploit the surrounding cells to their own ends [13,19].

Of note, many of the permissive metastasis–stroma interactions depend on growth factor binding to specific receptors, as reported for TGFβ and HGF [7]. The impact of growth factor signaling on metastatic colonization is not limited to a simple proliferation/cell survival advantage at secondary sites, but cell signaling may also lead to malignant traits such as epithelial-mesenchymal transition (EMT) or the reverse process MET [7,9,20,21]. Hypoxia is a physical stimulus of tumor microenvironment that induces EMT in tumors, through distinct mechanisms including the upregulation of HIF-1α [22,23]. The production of the regulatory peptide ET-1 is affected by cytokines/growth factors and HIF-1α contributing, therefore, to EMT [24,25], but the involvement of ET-1 in the formation of bone metastasis is poorly understood. For the choice of ET-1 in the present study, we relied on the fact that the activation of ET-1 axis, formed by the ligand ET-1 and its receptors ET_AR and ET_BR, is now recognized as a common mechanism underlying the progression of various solid tumors, including breast cancer [25]. In fact, the ET-1 axis in tumor microenvironment regulates cell–cell and cell–matrix interactions, and immunomodulation [25], and is also involved in new blood vessel formation [26]. Moreover, chromatin methylation state affects ET-1 and ET_AR expression in bone metastatic cells [27].

Our aim was to clarify the role played by ET-1 in the network of biological and physical stimuli of bone metastasis microenvironment, considering its influence on osteomimetic, invasive and EMT/MET phenotypes in conjunction with the critical growth factors HGF and TGFβ as well as with hypoxia in 1833-bone metastatic clone, compared with parental MDA-MB231 cells. We examined the regulatory function of ET-1 axis on Runt-related transcription factor 2 (Runx2), typical of osteoblast phenotype [6], and on the Wnt/β-catenin transcription pathway, critical for bone-metastatic process [13]. In molecular medicine the evaluation of ET-1 axis components could be important for grading, and to this end we analyzed human specimens of transformed mammary tissue at different disease stages till the occurrence of bone metastasis. Since invasive-ductal carcinomas estrogen receptor (ERα)/progesterone receptor (PR) positive and epidermal growth factor receptor-2 (HER2) negative were examined, we evaluated some molecular events in a comparable MCF-7 cell line.

The elevated expression of ET_AR in primary breast carcinoma associates with reduced disease-free survival time in a subgroup of patients with a putative favorable prognosis according to classic prognostic factors, and correlates with poor histological differentiation and incidence of distant metastasis [28]. ET-1, ET_AR and ET_BR show variable expression in primary breast carcinoma, which is positively correlated with ER status, lymphovascular invasion and inflammatory component [28]. Metastatic prostate cancer cells release ET-1, which stimulates bone formation and inhibits bone resorption giving osteosclerotic lesions [29].

Here, we add new data demonstrating the central role of ET-1 axis in the network of molecular players for breast-cancer metastatic process. In 1833-bone metastatic cells, the various microenvironmental stimuli differently influenced ET-1 production, and the autocrine/paracrine control of invasiveness. ET-1 autocrine production also regulated ET_AR expression. In particular, our results indicate that ET-1 activated signaling cascades for osteomimicry, by functioning oppositely with respect to HGF. Finally, dysplastic lesions remarkably expressed ET-1/ET_AR/ET_BR, potential markers for the incidence of invasive-ductal carcinomas – ERα/PR positive and HER2 negative – and predictive index of progression towards bone metastasis.

2. Materials and methods

2.1. Materials

Recombinant-human HGF, recombinant-human TGFβ1, and human Endothelin-1 immunoassay (ELISA) were from R&D System (Abingdon, UK). Human Endothelin-1 Calbiochem® was from Merck Chemicals Ltd (Nottingham, UK). BQ-123 was from Enzo Life Sciences (Lausen, Switzerland). Anti-ET_AR, anti-ET_BR and anti-Snail antibodies were from Abcam (Cambridge, UK). Anti-E-cadherin (clone 36) was from Transduction Laboratories (Bedford, MA, USA). Anti-Endothelin-1/2/3 (H-38), anti-N-cadherin (H-4), and anti-Vimentin (V9) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP 2 and anti-MMP 9 were from Cell Signaling Technology (Boston, MA, USA). Alexa Fluor488 and 568 antibodies were from Molecular Probes (Eugene, OR, USA).

2.2. Cell lines

Invasive parental MDA-MB231 breast carcinoma cells and the derived 1833-bone metastatic clone were kindly given by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York), and cultured as reported before [13]. The comparative study of transcriptomic profile of the two cell lines identifies a gene set whose expression pattern is associated with, and promotes the formation of, metastasis to bone [12]. Non-invasive MCF-7 breast carcinoma cells were from European Cell Cultures Collection, Salisbury, UK [30].

2.3. ELISA assay

ET-1 was measured in the conditioned medium of cells deprived of serum for 48 h, and exposed for 24 h to TGFβ1 (5 ng/ml), HGF (100 ng/ml) or hypoxia, following manufacturer's protocol. For hypoxic stimulation, cells were placed in an incubator chamber that was thoroughly flushed with a gas mixture containing 5% CO_2, 1% O_2 and nitrogen-balanced.

2.4. Western blot analysis

Total extracts (100 μg of protein), prepared from cells treated with 100 ng/ml HGF, 5 ng/ml TGFβ1 or hypoxia, were examined by Western blot and immunoblotted with the indicated antibodies [20]. Densitometric analysis was performed after reaction with ECL plus chemiluminescence kit from Thermo Fisher Scientific (RockFord, IL, USA).

2.5. Immunofluorescence

MDA-MB231 and 1833 cells (4×10^4) on coverslips were exposed to TGFβ, hypoxia or ET-1. The antibodies used were: anti-Endothelin-1 (1:50), anti-ET_AR (1:500), anti-Vimentin (1:50), anti-Snail (1:50) and anti-E-cadherin (1:5000). Secondary reactions with fluorescent antibodies were performed. The images were collected under Eclipse 80i, Nikon Fluorescence microscope [20].

2.6. Matrigel-invasion assay and transfection of siRNAET_AR

The cells were exposed to TGFβ1 (5 ng/ml) or hypoxia, in the presence or absence of 1 μM BQ123 [31]: 4-hour inhibitor pretreatment and 20-hour treatments were performed. Some cells were treated with HGF (200 ng/ml) or ET-1 (10 or 50 ng/ml) for 24 h. Matrigel invasion chambers from BD-Biocoat Cellware (Becton-Dickinson Labware, Bedford, MA, USA) were used to