EXPERIMENTAL CELL RESEARCH 312 (2006) 3822–3834

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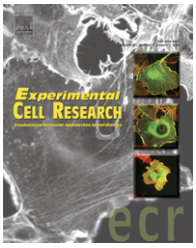

Research Article

PTH-related protein upregulates integrin α6β4 expression and activates Akt in breast cancer cells

*Xiaoli Shen, Miriam Falzon**

Department of Pharmacology and Toxicology, and Sealy Center for Molecular Science, University of Texas Medical Branch, 10th and Market Streets, Galveston, TX 77555, USA

ARTICLE INFORMATION

Article Chronology:
Received 6 July 2006
Revised version received
14 August 2006
Accepted 14 August 2006
Available online 17 August 2006

Keywords:
Parathyroid hormone-related protein
Integrin α6β4
Breast cancer
Akt
GSK-3

ABSTRACT

Breast cancer is the most common carcinoma that metastasizes to bone. Tumor-produced parathyroid hormone-related protein (PTHrP), a known stimulator of osteoclastic bone resorption, is a major mediator of the osteolytic process in breast cancer. We have previously shown that PTHrP increases breast cancer cell proliferation, survival, migration, and pro-invasive integrin α6β4 expression. To determine the role of integrin α6β4 in these PTHrP-mediated effects, we utilized two strategies to modulate expression of the α6 and β4 subunits in parental and PTHrP-overexpressing MDA-MB-231 and MCF-7 cells: overexpression of α6β4 by transfection with constructs encoding the α6 and β4 subunits, and suppression of endogenous α6β4 expression by transfection with siRNAs targeting these subunits. We now show that the effects of PTHrP are mediated via upregulation of integrin α6β4 expression. We also show that integrin α6β4 expression is modulated at the mRNA level, indicating a transcriptional and/or post-transcriptional mechanism of action for PTHrP. PTHrP expression also increased the levels of phosphorylated Akt, with a consequent increase in the levels of phosphorylated (inactive) glycogen synthase kinase-3 (GSK-3). The role of PTHrP in breast cancer growth and metastasis may thus be mediated via upregulation of integrin α6β4 expression and Akt activation, with consequent inactivation of GSK-3.

Introduction

The process of tumor formation, invasion, and metastasis involves cellular attachment to the extracellular matrix, local proteolysis of the basement membrane, and migration through the stroma to gain access to the circulation for the establishment of metastasis. Cancer cells also adopt survival mechanisms, thereby avoiding apoptosis [1]. These properties are dependent on inherent tumor cell characteristics [2,3], and on the presence in the metastatic microenvironment of several growth factors, matrix molecules, and cytokines [2,4]. One of these factors is parathyroid hormone-related protein (PTHrP), which plays an important role in the local bone breakdown that is characteristic of tumor-induced osteolysis [2,5,6].

PTHrP was initially identified as the factor responsible for humoral hypercalcemia of malignancy (reviewed in [7]). The protein is also expressed by normal fetal and adult tissues [8]. PTHrP undergoes extensive post-translational processing to generate mature secretory forms representing N-terminal, mid-region, and C-terminal portions. Each of these secretory forms appears to act via cell surface receptors [9,10]. To date, only the parathyroid hormone/PTHrP (PTH1) receptor that binds parathyroid hormone (PTH), PTHrP, and N-terminal

* *Corresponding author*. Fax: +1 409 772 9642.
E-mail address: mfalzon@utmb.edu (M. Falzon).

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doi:10.1016/j.yexcr.2006.08.011

analogs has been cloned [11]. PTHrP also functions via an intracrine pathway after translocation to the nucleus or nucleolus, and contains multibasic clusters in the 88–106 region, similar to the nuclear or nucleolar signals found in viral and mammalian transcription factors [12–15].

PTHrP is necessary for mammary gland development. In fact, mice devoid of PTHrP expression in all tissues except the skeleton completely lack mammary epithelial structures [16]. PTHrP is not readily detectable in the peripheral circulation of normal subjects except during lactation [17]. In contrast, 65 to 90% of breast cancer patients have detectable plasma PTHrP levels [18,19]. The invasive component of a majority of breast tumors is positive for PTHrP, while the intraductal component is negative or very weakly positive [20–23].

The mechanisms through which PTHrP exerts its effects in breast cancer are not fully understood. We have previously shown that PTHrP increases the proliferation, survival and migration of the breast cancer cell line MCF-7 [14,15]. We have also shown that PTHrP increases the cell surface expression of the integrin α6 and β4 subunits in a number of cell lines [15,24,25]. Integrin α6β4 is an adhesion receptor for most of the known laminins [26]. Multiple studies have shown that integrin α6β4 contributes to the functions of carcinoma cells [27–33]. The β4 extracellular domain associates exclusively with the α6 subunit to form α6β4 complexes [26,34], whereas the α6 subunit also associates with the β1 subunit to form α6β1 heterodimers [26]. Integrin β4 was initially identified as a tumor-related antigen associated with metastasis [35]. The β4 subunit was also recently implicated in ligand-independent signaling, such that integrin α6β4 enhances migration and invasion on non-laminin substrates such as collagen. These α6β4 effects cannot be blocked with antibodies that block the adhesion of α6β4 to laminin [29]. Since PTHrP regulates integrin α6β4 expression, and this integrin also regulates cell survival and migration, here we asked whether upregulation of integrin α6β4 expression plays a role in the observed effects of PTHrP on cell survival and migration. As model systems, we used the MDA-MB-231 and MCF-7 cell lines. Both cell lines express PTHrP; expression is higher in MDA-MB-231 than in MCF-7 cells [14,15]. These two cell lines were selected based on their different metastatic capability; only MDA-MB-231 cells metastasize to bone when injected into nude mice [36], reflective of the in vivo situation in breast cancer patients. In contrast, only PTHrP-overexpressing, but not parental, MCF-7 cells metastasize to bone [36,37]. Manipulation of PTHrP expression in these cell lines thus presents an appropriate model with which to correlate PTHrP expression with cell survival and migration, important determinants of tumor cell aggressiveness. Integrin α6β4 also activates phosphoinositol 3-kinase (PI3-K) [28]. We therefore also asked whether PTHrP activates Akt, a downstream effector of PI3-K.

Materials and methods

Materials

Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA). Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD). The R-PE-conjugated anti-α1 (CD49a; clone SR84), anti-α2 (CD49b; clone 12F1-H6), anti-α3 (CD49c; clone C3 II.1), anti-α5 (CD-49e; clone IIA), anti-α6 (CD49f; clone GoH3), anti-β1 (CD29; clone MAR4), and anti-β4 (clone 439-9B) antibodies, as well as their isotype controls [mouse IgG1, 6-PE (clone MOPC-1), mouse IgG2a, 6-PE (clone G155-178) and rat IgG2a, 6-PE (clone R35-95)] were obtained from BD PharMingen (San Diego, CA). The anti-p-Akt antibodies (p-Thr 308 and p-Ser 472/473), anti-total Akt antibody, and their isotype controls were also obtained from BD Pharmingen. The anti-p-GSK-3α/β (Ser21/Ser9) antibody was obtained from Cell Signaling Technologies (Danvers, MA), and the anti-total GSK-3α/β antibody was purchased from Affinity Bioreagents (Golden, CO). The FluoroBlok inserts for analysis of migration were purchased from BD PharMingen. Calcein-AM was obtained from Molecular Probes (Eugene, OR). The small interfering RNAs (siRNAs) targeting PTHrP and the integrin α6 and β4 subunits, and the corresponding nonspecific siRNA sequences were purchased from Dharmacon (Lafayette, CO).

Plasmid constructs

A cDNA encoding human PTHrP (obtained from Genentech, Inc., South San Francisco, CA) was digested with *Eco*RI and *Hind*III and subcloned in the sense orientation into the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). The (+) refers to the orientation of the multiple cloning site within the vector, relative to the direction of transcription from the T7 promoter. This construct was transfected into cells using FuGENE™ 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). pcDNA 3.1(+) was used as the empty vector control. The cDNAs encoding the integrin α6 and β4 subunits, cloned into the vector pRC CMV, were obtained from Dr. L. Shaw [28].

Cell culture and transfection

MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown at 37°C in a humidified 95% O_2–5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum (FBS) and L-glutamine.

The cells were stably transfected with the PTHrP-pcDNA3.1(+) construct or pcDNA3.1(+) as the empty vector, using FuGENE™ 6 Transfection Reagent. Two days after transfection, 600 μg/ml G418 (Geneticin; Life Technologies Inc.) was added, and resistant clones were selected. Single clones of stably transfected cells, isolated by limiting dilution in 96-well plates, were transferred to individual flasks and cultured in medium containing 150 μg/ml of G418. Individual clones overexpressing PTHrP were tested for PTHrP secretion using an immunoradiometric "sandwich" assay (IRMA; Nichols Institute, San Juan Capistrano, CA), and transfected mRNA levels were measured by real-time Polymerase Chain Reaction (PCR) analysis. The characterization of the PTHrP-overexpressing MCF-7 cell clones has been described [14,15].

The siRNA sequences targeting PTHrP or the integrin α6 and/or β4 subunits were transfected into MDA-MB-231 and MCF-7 cells by electroporation. Cells were plated in medium containing 10% FBS. At 75% confluence, the cells were trypsinized, washed with medium containing 10% FBS, and