Molecular and Cellular Endocrinology 446 (2017) 59–69

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Growth hormone-releasing hormone (GHRH) promotes metastatic phenotypes through EGFR/HER2 transactivation in prostate cancer cells

Laura Muñoz-Moreno, Ana M. Bajo, Juan C. Prieto*, María J. Carmena

Department of Systems Biology, Unit of Biochemistry and Molecular Biology, University of Alcalá, Alcalá de Henares 28871, Spain

ARTICLE INFO

Article history:
Received 22 September 2016
Received in revised form
6 February 2017
Accepted 8 February 2017
Available online 11 February 2017

Keywords:
GHRH
Cell adhesion
Cell migration
VEGF secretion
Prostate cancer

ABSTRACT

The involvement of growth hormone-releasing hormone (GHRH) in several relevant processes that contribute to prostate cancer progression was analyzed. Firstly, we evaluated GHRH effects on cell proliferation and adhesion in human cancer prostate cell lines, LNCaP and PC3, by using specific assays (BrdU incorporation and collagen adhesion). The expression levels of the main marker molecules of these processes were measured by RT-PCR, Western blotting and zymography assays. GHRH increased both cell proliferation and proliferating cell nuclear antigen (PCNA) levels in LNCaP cells and in PC3 cells; however, such a rise was faster in the PC3 cells that represent the most aggressive stage of prostate cancer. Furthermore, GHRH significantly reduced cell adhesion and E-cadherin levels in LNCaP and PC3 cells and up-regulated the total and nuclear expression of β-catenin in PC3 cells. In addition, we assessed cell cycle, cell migration and VEGF secretion in PC3 cells. GHRH augmented the number of cells in G2/M-phase but diminished that corresponding to G1-phase. Cell-cycle specific markers were evaluated since GHRH effects may be related to their differential expression; we observed a decrease of p53, p21, and Bax/Bcl2 ratio. Furthermore, GHRH increased the expression of CD44, c-myc and cyclin D1, MMP-2 and MMP-9 activity, and VEGF secretion. We also observed that EGFR and/or HER2 transactivation is involved in cell adhesion, cell migration and VEGF secretion produced by GHRH. Consequently, present results define GHRH as a proliferative, anti-apoptotic and migratory agent in prostate cancer.

1. Introduction

Prostate cancer (PC) is the second most common cause of cancer death in men worldwide (Siegel et al., 2016). Progression of prostate cancer results in metastases in bone and other tissues, causing bone pain, skeletal complications, and patient mortality. Thus, the knowledge of molecular events leading to prostate cancer progression is essential for the development of improved therapies for patients with advanced prostate cancer.

Growth hormone-releasing hormone (GHRH) is a neuropeptide hormone, secreted by the hypothalamus, which binds to its receptor (GHRH-R) in pituitary somatotrophs and activates synthesis and secretion of growth hormone (GH) (Barabutis and Schally, 2010; Kiaris et al., 2011). Both, GHRH and its receptors (pituitary type and truncated splice variants), are expressed by various extra-hypothalamic sites as observed in surgical specimens and tumor cell lines of a diversity of human cancers (Busto et al., 2002; Garcia-Fernandez et al., 2003; Halmos et al., 2002; Havt et al., 2005; Kahán et al., 1999). In fact, the presence of GHRH and SV1 isoform of GHRH receptors in LNCaP and PC3 cells have been previously described (Chopin and Herington, 2001). Moreover, there are various reports that support that GHRH and its tumoral SV receptors may form an autocrine mitogenic loop in prostate cancer LNCaP cell line (Barabutis and Schally, 2008a, 2008b; Plonowski et al., 2002). Furthermore, several *in vitro* and *in vivo* studies show the effect of antagonistic analogs of GHRH on cell proliferation and apoptosis in prostate cancer (Barabutis and Schally, 2008a, 2008b; Barabutis et al., 2010; Stangelberger et al., 2012; Muñoz-Moreno et al., 2013; Fahrenholtz et al., 2014). However, the effects of GHRH on processes that characterize a more aggressive molecular phenotype of prostate cancer have not been extensively studied.

On the other hand, it is known that G-protein coupled receptors (GPCRs) are able to activate tyrosine kinase receptors (RTKs) (Delcourt et al., 2007; Pyne and Pyne, 2011). Interestingly, GHRH is involved in the transactivation of the signaling of epidermal growth

* Corresponding author. Department of Systems Biology, Unit of Biochemistry and Molecular Biology, University of Alcalá, Alcalá de Henares 28871, Spain.
E-mail address: juancarlos.prieto@uah.es (J.C. Prieto).

http://dx.doi.org/10.1016/j.mce.2017.02.011
0303-7207/

factor receptor (EGFR/HER1/ErbB1) and human epidermal growth factor receptor-2 (HER2) in prostate cancer (Munoz-Moreno et al., 2014). This fact is extremely important since the formation of EGFR/HER2 heterodimers is related to mitogenic signaling pathways implicated in prostate cancer progression towards androgen independence (Di Lorenzo et al., 2002; Berger et al., 2006).

The beginning of the cancer process is related to both an uncontrolled growth and loss of tumor cell adhesion. In regard to the former, numerous molecules regulate the cell cycle including the proliferating cell nuclear antigen (PCNA) that acts as a processivity factor for DNA polymerase δ during DNA replication (Maga and Hubscher, 2003; Wang, 2014). In addition, p21 protein, a cyclin-dependent kinase (CDK) inhibitor, is capable to bind to both cyclin-CDK and PCNA. Through its binding to PCNA, p21 inhibits replication by blocking activity of PCNA to stimulate polymerases (Moldovan et al., 2007; Waga and Stillman, 1998). Furthermore, p21 is able to inhibit the binding to cyclin-CDK complex leading to cell growth arrest in cell cycle (Abbas and Dutta, 2009). The antiproliferative actions of p21 may occur by a p53-dependent mechanism (Pérez-Sayáns et al., 2013). In addition, p53 induces apoptosis through the regulation of apoptotic genes. Thus, p53 activates and represses the transcription of Bax (pro-apoptotic) and Bcl2 (anti−apoptotic), respectively, resulting in the activation of the programmed cell-death process (Mirzayans et al., 2012). Instead, the disruption of both cell-cell and cell-extracellular matrix interactions are mediated by cell adhesion molecules. Loss of one of them, E-cadherin, a member of the cadherin family, has been associated with the epithelial-mesenchymal transition (EMT), the mesenchymal-epithelial transition (MET) and a higher motility, invasiveness and resistance to apoptosis allowing aggressive phenotype in various human cancers (Mărgineanu et al., 2008; Beuran et al., 2015; Le Bras et al., 2012; Onder et al., 2008). Furthermore, the cytoplasmic domain of E-cadherin recruits several molecules including β-catenin, providing anchorage to the cytoskeleton (Schmalhofer et al., 2009). The β-catenin also acts as a transcriptional co-activator in the Wnt signaling pathway used during development to control cell fate decisions and implicated in many cancers (Schmalhofer et al., 2009; Howard et al., 2011; Huber and Weis, 2001). Loss of E-cadherin causes β-catenin release from the membrane, which is associated with the transcription factor Lef/TCF (Lymphoid enhancer factor/T cell factor) in the cytoplasm. This complex translocates to the nucleus where β-catenin-Lef/TCF activates transcription of genes involved in tumor progression such as cyclin D1, CD44, c−myc, metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) (Thakur and Mishra, 2013). Furthermore, metastatic process that starts with the disruption of cell-cell local interaction and alteration of basement membrane carry on with invasion and infiltration of surrounding tissue, and penetration into blood or lymphatic vessels with consequent transportation of neoplastic cells through the blood circulation (Hanahan and Weinberg, 2011).

The aim of this study was to determine the role of GHRH in the progression of prostate cancer by analyzing the participation of this hormone in important cellular processes such as cell proliferation, cell adhesion, cell migration and angiogenesis, typically associated with metastatic phenotypes in prostate cancer. In addition, we observed the involvement of EGFR and/or HER2 in the effects of GHRH on such processes.

2. Materials and methods

2.1. Peptides

GHRH (1−29)NH_2 was purchased from PolyPeptide (Strasbourg, France), EGFR tyrosine kinase inhibitor (AG-1478) from Calbiochem (Darmstadt, Germany), and HER-2 tyrosine kinase inhibitor (AG-825) from Tocris Bioscience (Bristol, United Kingdom).

2.2. Cell cultures

Two human prostate cancer cell lines that exhibit different features of prostate cancer progression were used. LNCaP is an androgen-responsive cancer cell line which may represent early stage of the disease. PC3 is an androgen-unresponsive cell line that may represent recurrent prostate cancers that have achieved androgen independence. Cell lines were obtained from the American Type Culture Collection. LNCaP and PC3 cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). All culture media were also supplemented with 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B from Life Technologies, Carlsbad, CA, USA). The culture was performed in a humidified 5% CO_2 environment at 37 °C. After the cells reached 70−80% confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin/0.2% EDTA, and seeded at 30,000−40,000 cells/cm^2. The culture medium was changed every 3 days.

2.3. Cell proliferation assays

LNCaP and PC-3 cells (2×10^5 cells) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B) for 16 h. Then, cells were subjected for 24 h to different treatments. In the last 30 min of incubation, cells were labeled with 10 μM bromodeoxyuridine (BrdU). Thereafter, cells were washed with PBS, fixed with ice-cold absolute ethanol, and stored at −20 °C for 30 min. Fixative was removed by centrifugation and pellets were washed with PBS. DNA was partially denatured by incubation with 1 N HCl for 30 min at room temperature and then cells were washed three times with PBS containing 0.05% Tween-20 (pH 7.4) and 0.1% BSA. Cells were incubated with 20 μl of anti-BrdU monoclonal antibody with FITC (BD Bioscience, San Agustin de Guadalix, Spain) for a 30 min-period in darkness. For flow cytometry analysis, cells were stained with propidium iodide (PI) staining solution (50 μg/ml PI and 10 μg/ml RNase). The number of BrdU-positive cells was counted using a FACSCalibur cytometer (BD Bioscience). Results obtained were analyzed with the Cyflogic v 1.2.1 program.

2.4. Cell adhesion assays

Concentrated type-I collagen solution was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 h at 37 °C. Plates were washed twice with PBS (pH 7.4). Cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were resuspended in RPMI medium/0.1% (w/v) BSA (pH 7.4) and treated with 0.1 μM GHRH for 30 min. Then, LNCaP and PC3 cells were plated at 2.5×10^4 cells per 100 μl. The assay was terminated at the indicated time intervals by aspiration of the wells. Cell adhesion was quantified by adding 1 mg/ml of the substrate 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) followed by 1 h incubation. Isopropanol (50 μl) was added to each well to dissolve the dark blue formazan precipitates and absorbance was read at 570 nm in a plate reader (ELX 800, Bio-Tek Instruments, Winooski, VT, USA). Results were expressed as the relative percentage of absorbance compared with the corresponding control cells.