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Original Articles

Autocrine human growth hormone stimulates the tumor initiating capacity and metastasis of estrogen receptor-negative mammary carcinoma cells

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ABSTRACT

The oncogenic effects of autocrine human growth hormone (hGH) have been intensively investigated in estrogen receptor-positive mammary carcinoma (ER + MC) cells. We demonstrated herein that autocrine hGH promoted cancer stem cell (CSC)-like properties of estrogen receptor-negative mammary carcinoma (ER-MC) cells *in vitro*. In xenograft studies, autocrine hGH increased the tumor initiating capacity of ER-MC cells. We also observed that autocrine hGH promoted migration and invasion of ER-MC cells *in vitro*, and metastasis *in vivo*. Thus, inhibition of hGH is a potential therapeutic strategy to prevent tumor initiation and metastasis of ER-MC.

Introduction

Mammary carcinoma exhibits heterogeneous pathology at the molecular, cellular and clinical levels [1,2]. Differences in molecular characteristics and gene profiling predict distinct outcomes and response to treatment in mammary carcinoma patients [2]. Despite progress in the diagnosis and treatment of mammary carcinoma, the estrogen receptor-negative subtype of mammary carcinoma (ER-MC) is still associated with poorer prognosis compared with other molecular subtypes [3]. Thus, further study is warranted to effectively target this clinically challenging subgroup of mammary carcinoma.

Recent reports have identified the presence of CSCs in solid tumors [4], and implicated them in tumor initiation, growth, epithelial-to-mesenchymal transition (EMT) [5], chemotherapeutic resistance and tumor recurrence [4], thereby compromising complete therapeutic eradication of the tumor. CSCs have also been postulated to possess a crucial role in tumor metastasis [6]. Hence, understanding the basic molecular mechanisms involved in CSC function is pertinent for the development of targeted therapeutic approaches to ablate the CSC population, and to maximize clinical response of subgroups of mammary carcinoma that are resistant to conventional therapies.

Human growth hormone (hGH) secreted from the anterior pituitary gland promotes somatic growth through stimulation of hepatic insulin-like growth factor-I (IGF-I) synthesis and secretion, and also through IGF-I independent mechanisms [7]. A functional association between endocrine or circulating GH and various cancers has been described in both humans and animal models. Acromegalic patients with increased circulating GH exhibit a higher incidence of colon, thyroid, and possibly mammary carcinoma [8,9]. In addition, mammary carcinoma patients have significantly higher levels of serum GH and IGF-I in comparison to individuals without cancer [10,11]. Conversely, two recently published studies, albeit with limitations, observed that the incidence rate of cancer among individuals with a GH receptor (GHR) mutation which disrupts GH-mediated signaling was much lower than their normal relatives [12,13]. The oncogenic potential of endocrine GH in animal models has also been widely reported [14–16].

In addition to its endocrine functions, GH also possesses important autocrine and paracrine effects, and is secreted locally in the mammary gland [17–19]. GH is required for appropriate pubertal mammary gland development and is essential for ductal elongation and differentiation of ductal epithelia into terminal end buds

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[20,21]. Increased *hGH* mRNA and protein expression has been observed in mammary carcinoma [18,19]. Histopathological analyses have demonstrated that hGH expression in mammary carcinoma is significantly and positively correlated with lymph node metastasis, higher clinical stage and *HER2*-positive tumors [19]; In addition, tumor expression of hGH predicts a poor survival outcome in patients with mammary carcinoma or endometrial carcinoma [19]. We have previously reported that autocrine hGH promotes the oncogenicity of human mammary- and endometrial-carcinoma cells by enhanced cell proliferation, survival, and invasion [22,23]. The oncogenic effect of autocrine hGH in endometrial carcinoma cells is meditated through activated STAT3 [24]. Remarkably, forced expression of hGH is capable of oncogenically transforming the human mammary epithelial cell with consequent tumor formation [25]. In addition to enhanced oncogenicity, autocrine hGH has been observed to promote epithelial-to-mesenchymal transition (EMT) [26], angiogenesis [27] and therapy resistance [28–30] in mammary carcinoma cells. However, the functional effect of autocrine hGH on the tumor initiating potential and metastasis of ER-MCs has not previously been established.

We report herein that forced expression of hGH in ER-MC cells promotes a CSC-like phenotype and increases migration and invasion *in vitro*. In addition, forced expression of hGH enhances MDA-MB-453 cells' tumor initiating and metastatic potential *in vivo*.

Materials and methods

Cell lines and stable cell construction

Human ER-negative mammary carcinoma cell lines MDA-MB-453 and SKBR3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to recommended conditions. Stable transfection was performed on both MDA-MB-453 and SKBR3 cells with a pcDNA3 expression vector harboring the entire genomic region of *hGH* gene (cell lines designated MDA-MB-453-hGH and SKBR3-hGH) or the pcDNA3 empty vector (MDA-MB-453-Vec and SKBR3-Vec) using FUGENE 6 (Roche, IN). Selection of the transfected cells was achieved with G418 at 800 μg/ml (MDA-MB-453) or 1000 μg/ml (SKBR3) G418 for 4 weeks, and stable expression of hGH was verified through RT-PCR and western blot as described below.

RT-PCR and western blot analysis

RT-PCR was carried out as described previously [27]. Primers used were:

hGH:
F-CCGACACCCTCCAACAGGGA
R-CCTTGTCCATGTCCTTCCTG

β-actin:
F-ATGATATCGCCGCGCTCG
R-CGCTCGGTGAGGATCTTCA

Western blot analysis was performed as described previously [31]. The rabbit anti-hGH polyclonal antibody was obtained from Dr. Parlow (National Hormone and Peptide Program, Torrance, CA). The mouse anti-β-ACTIN antibody was from Sigma Aldrich.

Total cell counts and three-dimensional Matrigel growth assay

1×10^4 MDA-MB-453 stable cells and 2×10^4 SKBR3 stable cells were plated in 6-well plates in 10% FBS. Counts were conducted every 2 days for 10 days (SKBR3) or 12 days (MDA-MB-453). For three-dimensional Matrigel growth assay, 1000 stable transfected cells were seeded with 4% Matrigel (BD Biosciences, San Diego, CA) in Matrigel pre-coated 96-well plates. After 7 days in culture, pictures were taken using a light microscope at 20× magnification, and cell growth was determined by MTT assay.

Mammosphere formation assay

Monolayer cells were collected in trypsin–EDTA and carefully resuspended in Dulbecco's modified Eagle's medium F12 (Invitrogen) supplemented with 20 ng/ml recombinant human EGF (Sigma Aldrich), 20 ng/ml recombinant human basic FGF (BD Biosciences), 2% B27 (Invitrogen), penicillin–streptomycin (Invitrogen), and 5 μg/ml bovine insulin (Sigma Aldrich).

A single cell suspension was generated by passing cells through a 40 μm cell strainer (BD Biosciences) and assessed under a light microscope. Single cells were then plated in ultra-low attachment 96-well plates (Corning, MA) at 1000 cells/100 μl. Subsequent passages were grown with the same density, and mammosphere number was counted as described previously [32]. Suspension cultured MDA-MB-453 and SKBR3 parental cells were placed under B2036 treatment at different concentrations, and the control groups were treated with BSA.

In vitro *cell migration and invasion assays*

Migration and invasion assays were performed as described previously [26]. Briefly, Corning Transwell chambers with porous membranes (8 μm pore size) were used for the study of cell migration, while membranes pre-coated with 10% Matrigel were used to determine cell invasion according to the manufacturer's instructions. The upper chamber contained cell suspension of 1×10^5 cells/ml in 0.2 ml serum-free media. The lower chambers contained 0.5 ml medium supplemented with 10% FBS, which acts as a chemoattractant. Following 24 hours incubation, migrated cells on the lower side of the membranes were fixed using cold methanol for 5 min and stained with 5 μg/ml Hoechst 33342 (Invitrogen) for 15 min at room temperature. Quantitative data of migrated or invaded cells were presented as the average number of cells per microscopic field over 3 random fields per membrane and performed in triplicate.

Aldefluor assay

The Aldefluor assay was conducted as per the manufacturer's instruction. In brief, stably transfected cells were harvested in trypsin–EDTA, passed through 40 μm cell strainers, and collected by gentle centrifugation. Cells were then resuspended in Aldefluor assay buffer containing aldehyde dehydrogenase (ALDH) substrate BODIPY™ aminoacetaldehyde (BAAA) at 3 μM/10^6 cells/ml, and incubated for 30 min at 37 °C. An aliquot of each cell sample was treated with a specific ALDH inhibitor diethylaminobenzaldehyde (DEAB), as a negative control. Subsequent flow cytometry was carried out using a FACS LSR II (BD Biosciences).

Side population analysis

The protocol for side population analysis was slightly modified from Goodell et al. [33]. Harvested surviving cell populations were incubated in media containing 2% FBS and Hoechst 33342 (5 μg/ml) for 90 min at 37 °C ± the ABC transporter inhibitor verapamil (50 μM, Sigma Aldrich). Analysis of the samples was then carried out by flow cytometry (FACS LSR II, BD Biosciences).

Xenograft analyses

All animal studies were performed in conformance with an approved protocol by the Animal Care and Ethics Committee of the University of Science and Technology of China. Xenografts were generated as previously described [23,25]. For the tumor initiating assay, MDA-MB-453-Vec and MDA-MB-453-hGH cells were suspended in 100 μl PBS with serial dilutions and injected into the fourth mammary fat pad of 3- to 4-week-old BALB/c Nude mice (Slac Laboratory Co, Shanghai, China). Primary tumor sizes were measured with a caliper twice every week. After one month of inoculation, the nude mice were sacrificed to determine tumorigenicity, which was determined by tumor incidence (i.e., the number of tumors/number of injections). For tumor metastasis, 5×10^6 MDA-MB-453-Vec and MDA-MB-453-hGH cells were injected into the fourth mammary fat pad of 3- to 4-week-old BALB/c Nude mice. Tumor sizes were measured twice every week. In this assay, the latency of tumor formation is approximately one week, and tumors were collected one month after inoculation. At necropsy, primary tumors, lungs and livers were fixed in 4% paraformaldehyde and counterstained with hematoxylin and eosin (H&E) to assess the morphology of the primary tumor and the presence of micrometastasis in the lung and liver.

RNA analysis

Analysis of *hGH* and *ALDH1* gene expression was performed in 24 ER-BC patients with invasive mammary ductal carcinoma who underwent surgery at The First Affiliated Hospital of Anhui Medical University (Hefei, China) between 2001 and 2002, as described in previous studies [34]. The relative amount of gene transcripts was normalized to *GAPDH*. The Pearson correlation coefficient was used to analyze the correlation between the expression levels of *hGH* and *ALDH1*.

Statistics

All data were presented as means ± SEM of triplicate determinants. Data were analyzed using an unpaired two-tailed *t* test or χ^2 test.

Results

Forced expression of hGH enhances cell proliferation in ER-MC cells

We have previously published the functional effects of hGH in ER + MC cells [35,36]. However, histopathology studies also