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DHEA increases epithelial markers and decreases mesenchymal proteins in breast cancer cells and reduces xenograft growth

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ABSTRACT

Breast cancer is one of the most common neoplasias and the leading cause of cancer death in women worldwide. Its high mortality rate is linked to a great metastatic capacity associated with the epithelial-mesenchymal transition (EMT). During this process, a decrease in epithelial proteins expression and an increase of mesenchymal proteins are observed. On the other hand, it has been shown that dehydroepiandrosterone (DHEA), the most abundant steroid in human plasma, inhibits migration of breast cancer cells; however, the underlying mechanisms have not been elucidated.

In this study, the *in vitro* effect of DHEA on the expression pattern of some EMT-related proteins, such as E-cadherin (epithelial), N-cadherin, vimentin and Snail (mesenchymal) was measured by Western blot and immunofluorescence in MDA-MB-231 breast cancer cells with invasive, metastatic and mesenchymal phenotype. Also, the *in vivo* effect of DHEA on xenograft tumor growth in nude mice (nu^-/nu^-) and on expression of the same epithelial and mesenchymal proteins in generated tumors was evaluated.

We found that DHEA increased expression of E-cadherin and decreased N-cadherin, vimentin and Snail expression both in MD-MB-231 cells and in the formed tumors, possibly by DHEA-induced reversion of mesenchymal phenotype. These results were correlated with a tumor size reduction in mouse xenografts following DHEA administration either a week earlier or concurrent with breast cancer cells inoculation.

In conclusion, DHEA could be useful in the treatment of breast cancer with mesenchymal phenotype.

1. Introduction

Dehydroepiandrosterone (DHEA) is a steroid produced from cholesterol in the adrenal gland under the regulation of adrenocorticotropic hormone (ACTH) (Prough et al., 2016). DHEA is also produced in organs such as brain, placenta, ovaries and testes (Olech and Merril, 2005) and is stored in its sulfated form DHEA-S (Racchi et al., 2003). DHEA and DHEA-S are the most abundant circulating steroids in the human plasma (Teng et al., 2015; Nestler et al., 1988). DHEA and DHEA-S levels peak between the ages of 20 and 30 years and declines at a rate of 2% per year (Teng et al., 2015; Orentreich et al., 1984).

Several studies showed that DHEA decreased levels in plasma are related to changes in cardiac tissues causing the development of cardiovascular disease, cholesterol reduced in blood, activation of the immune response, changes in female fertility and neuronal functions, modifications of metabolism, Alzheimer's disease and cancer, among others (Olech and Merril, 2005; Nestler et al., 1988; Khorram et al., 1997; Tchernof et al., 1996; Ebeling and Koivisto, 1994; Regelson et al., 1990).

DHEA has protective properties against cancer given its potent antiproliferative effect (Schwartz et al., 1986). Recently, our research group showed that DHEA inhibits proliferation and migration of breast cancer cells such as MCF-7, MDA-MB-231 and Hs578T (López-Marure et al., 2011). We also showed that DHEA inhibits invasion, colonies formation and growth of spheroids in MCF-7, MDA-MB-231 and ZR-75-30 cells (López-Marure et al., 2016). The anti-tumor effects of DHEA have also been demonstrated *in vivo* (McCormick et al., 1996). DHEA and DHEA-S inhibited pancreatic cancer in xenografts of nude male mice (Muscarella et al., 1998; Melvin et al., 1997); besides, DHEA repressed carcinogenesis in the mammary gland and metastasis in

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Sprague-Dawley rats (Ratko et al., 1991). Despite the beneficial properties of DHEA on breast cancer, the mechanisms by which DHEA inhibits migration and invasion in these cells have not been elucidated.

Breast cancer is one of the most common cancers in women over 20 years old around the world, its incidence and mortality has gradually increased in the last 10 years. Since 2005, breast cancer is the leading cause of cancer death for women in Mexico (www.inegi.gob.mx, 2014; Knaul et al., 2008). Its high mortality is due mainly to the great metastatic capacity, which is associated with epithelial-mesenchymal transition (EMT).

EMT is present during embryogenesis, carcinogenesis, metastasis, and tumor recurrence. During EMT, cells lose their epithelial features and acquire mesenchymal properties such as loss of polarity and cell adhesion, increased mobility and invasiveness, resistance to apoptosis, and morphological changes (Diepenbruck & Christofori, 2016). The contrary process is the mesenchymal-epithelial transition (MET), where autonomous mesenchymal cells gradually become tightly linked and polarized epithelial cells (Kim et al., 2016).

The onset of EMT requires activation of transcription factors (Snail/Slug, Twist and ZEB1/2, Twist, FoxC2, TCF3, Goosecoid homeobox and LBX1) (Voutsadakis, 2016), enzyme production and changes in the levels of miRNAs, and the expression of specific epithelial and mesenchymal markers, including E-cadherin, β-catenin, N-cadherin, vimentin and others (Kalluri & Weinberg, 2009). EMT involves reduced cell-cell adhesion through transcriptional repression of cadherins in adherens junctions, the tight junction components occludin and claudin and a desmosome element, desmoplakin (De Wever et al., 2008). The expression of intermediate filaments also changes during EMT, replacing cytokeratin by vimentin (Omary et al., 2009).

A marked feature of EMT is downregulation of E-cadherin, the major transmembrane component of the adherens junctions, essential for induction and maintenance of epithelial cell polarity (Thiery, 2002). E-cadherin acts as a tumor suppressor gene since it maintains normal cell-cell adhesion. Altered E-cadherin expression is enough for the initiation of EMT (Onder et al., 2008).

Several pathways initiated in cancer have the ability to trigger EMT transcription regulators. For example, the Snail family of transcription factors, triggers the EMT by directly repressing E-cadherin and other adhesion molecules expression and to activate EMT associated with epithelial tumor progression (Cano et al., 2000); therefore, Snail is expressed in primary human tumors (Cheng et al., 2001).

Since EMT stimulates tumor progression and metastasis resulting in high mortality among breast cancer patients and in view of the protective effect of DHEA in this malignancy, we hypothesized that changes in levels of EMT-related proteins may underlie the inhibitory effects of DHEA on migration and invasion of breast cancer cells reduced tumor growth *in vivo*. To test this, the expression of three mesenchymal proteins (N-cadherin, vimentin and Snail) and one epithelial (E-cadherin) was measured in MDA-MB-231 breast cancer cells with an invasive and mesenchymal phenotype. The effect of DHEA on tumors xenografts in nude mice was also evaluated.

2. Methods

2.1. Materials

Monoclonal antibodies (mAbs) against vimentin (V9) and N-cadherin (CD325) were purchased from eBioscience (San Diego, CA, USA), the anti-beta-actin (AC-15) mAb was acquired from Abcam (Cambridge, MA, USA), the anti-E-cadherin and Snail polyclonal antibodies (H-108 and T-18, respectively) and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA). Fluoroshield™ with DAPI histology mounting medium and all other reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA). The SuperSignal® system was provided by GE Healthcare (Buckinghamshire, UK) and Universal Blocking Reagent was obtained from BioGenex (Fremont, CA, USA). RPMI-1640 medium and other cell culture reagents were purchased from GIBCO/BRL (Grand Island, NY, USA).

2.2. Cell culture conditions

MDA-MB-231 cells were cultured in RPMI containing 10% newborn bovine serum (NCBS) and 1% anti-mycotic and incubated at 37 °C, 5% CO_2 and 95% relative humidity. Cells were exposed to different pharmacologically relevant concentrations of DHEA (1, 10 and 100 μM) for 24 and 48 h.

2.3. Protein extraction and quantification

For each experiment, 1×10^6 MDA-MB-231 cells were seeded in 60 mm Petri dishes. Cells at 70% confluence were treated with DHEA for 24 and 48 h. Then culture medium was removed and cells were washed with Hepes buffer (150 mM NaCl, 4.4 mM KCl, 10.9 mM Hepes, 12.2 mM glucose, pH 7.4) and incubated at 4 °C for 20 min with 300 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40, 100 mM NaF, 0.2 mM $NaVO_3$, 1 μg/mL aprotinin, 1 mM PMSF, 1 μg/mL leupeptin). After incubation, cells were detached with a cell scraper and homogenates were centrifuged at 14,000 rpm for 3 min to remove cell debris, supernatants were collected and kept at − 80 °C.

To obtain protein derived from xenograft tumor, tumors were frozen with liquid nitrogen and macerated in a mortar to obtain very small pieces of tissue, subsequently 300 μl of lysis buffer were added to tissue and after was homogenized with a POLYTRON® PT 6100 homogenizer. Homogenates were centrifuged at 14,000 rpm for 10 min to remove cell debris, supernatants containing protein were collected and kept at − 80 °C.

Protein concentration was determined using the Bradford reagent, measuring absorbance at 595 nm in a GENESYS™ 10S UV–Vis spectrophotometer (Thermo Fisher Scientific). Protein concentration was calculated with reference to a standard curve based on bovine serum albumin (BSA).

2.4. Western blot

Proteins were separated on 12% polyacrylamide gels. Gel preparation: 2.5 mL of solution 1 (0.75 M Tris-HCl, 0.2% SDS, pH 8.8), 2 mL of solution 2 (30% acrylamide-bisacrylamide in 37.5:1 ratio), 1 mL of water, 7 μL of TEMED, 25 μL of 13% ammonium persulfate. Samples were denatured at 95 °C for 5 min. Then, 30 μg of protein from total lysate were loaded per lane along with 5 μl of 3 × sample buffer (0.3 M Na_2HPO_4, 30% glycerol, 260 mM SDS, 0.74 mM bromophenol blue, 1% glycerol and 9.7 mM DTT). The total sample was loaded on the polyacrylamide gel and run at 120 V for 90 min. After electrophoresis, proteins were transferred to a PVDF membrane at 250 mA for 120 min at 4 °C, with 800 mL of transfer solution containing: 25 mM Tris-base, 192 mM glycine and 20% methanol, pH 8.3. Following transfer, membrane was blocked with a solution of 8% fat-free milk in TBS-T (20 mM Tris, 137 mM NaCl, 3 mM KCl and 0.1% Tween-20) for 1 h with constant stirring. Subsequently, three washes were performed with TBS-T solution for 5 min each. Membrane was incubated overnight with primary antibodies. Then, blots were washed three times for 5 min in TBS-T, and incubated with 8% fat-free milk in TBS-T for 15 min. Secondary antibody was added for 1 h before washing the blots three times with TBS-T for 5 min. The coupled peroxidase was detected by chemiluminescence using the SuperSignal® system and the ChemiDoc™ MP Imaging System (Bio-Rad). Densitometric analysis of proteins was performed with the Image Lab™ V 4.0 Software (Bio-Rad).

2.5. Immunofluorescence

For each experiment, 3×10^5 MDA-MB-231 cells were cultured on