EXPERIMENTAL CELL RESEARCH 319 (2013) 2282–2295

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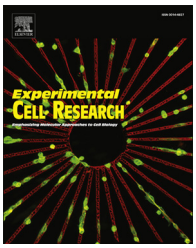

Research Article

Insulin-like growth factor binding protein-3 (IGFBP-3) plays a role in the anti-tumorigenic effects of 5-Aza-2′-deoxycytidine (AZA) in breast cancer cells

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ARTICLE INFORMATION

Article Chronology:
Received 27 July 2012
Received in revised form
28 May 2013
Accepted 18 June 2013
Available online 26 June 2013

Keywords:
IGFBP-3
AZA
Breast cancer
Cell survival

ABSTRACT

Breast cancer progression is associated with loss of estrogen receptor (ER-α), often due to epigenetic silencing. IGFBP genes have consistently been identified among the most common to be aberrantly methylated in tumours. To understand the impact of losing IGFBP-3 tumour expression via DNA methylation, we treated four breast cancer cell lines (MCF-7, T47D, Hs578T and MDA-MB-231) with a DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (AZA) to determine IGFBP-3's role in the effects of AZA on total cell number and survival relative to changes in the ER. AZA induced cell growth inhibition, death and a reduction in the formation of colonies, despite increasing ER-α expression in ER-negative cells but reducing ER-α in ER-positive cells. Regardless of the differential effects on the ER-α, AZA consistently increased the abundance of IGFBP-3 and negating this increase in IGFBP-3 with siRNA reduced the AZA-induced growth inhibition and induction of cell death and virtually negated the AZA-induced inhibition of colony formation. With ER-α positive cells AZA increased the abundance of the tumour suppressor gene, p53 and induced demethylation of the IGFBP-3 promoter, whereas with ER negative cells, AZA epigenetically increased the transcription factor AP2-α, which when silenced prevented the increase in IGFBP-3. IGFBP-3 plays an important role in the anti-tumorigenic effects of AZA on breast cancer cells.

Introduction

Worldwide, more than a million women are diagnosed with breast cancer every year. In developed countries, breast cancer has become the most common malignancy in women [1]. Estrogen receptors (ERs) and their ligand estrogen play a crucial role in the initiation and promotion of mammary carcinogenesis [2]. Blocking estrogen actions is the mainstay of treatment for breast

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http://dx.doi.org/10.1016/j.yexcr.2013.06.011

cancer. However, more than one-third of breast cancers are ER-α negative and resistant to anti-estrogen therapy. In addition, many cancers that respond to hormone therapy ultimately lose expression of ER-α and develop into an aggressive hormone-resistant cancer [3].

Recent studies have shown that epigenetic events may play a key role in tumour progression [4]. Hypermethylation of the promoter sites on the genes usually causes transcriptional repression or silencing of the gene [5]. The ER-α gene is one of the most important genes aberrantly methylated in breast cancer [6–8]. About half of primary breast cancers and over 70% of ER-negative breast tumors have detected methylation in the 5′ region of the ER-α promoter [9]. Unlike gene mutations, DNA methylation is reversible and can be inhibited by DNA methytransferase (DNMTs) inhibitors, such as 5-aza-2′-deoxycytidine (5-AZA-dCyd) [10]. 5-AZA-dCyd can also cause direct cytotoxic effects through the formation of DNA adducts [10,11].

Considerable cross-talk exists between estrogen and the insulin-like growth factor (IGF) axis and both are linked to breast cancer progression [12]. The IGF axis is composed of two ligands (IGF-I and IGF-II), cell surface receptors (IGF-IR, IGF-IIR, and hybrid IGFR-insulin receptors), six high affinity IGF binding proteins (IGFBP-1 to 6), and IGFBP proteases [13]. Many prospective epidemiology studies, in vivo tumour models and in vitro experiments suggest that members of IGF axis are critical regulators of mammary epithelial cell and breast cancer growth and contribute to cancer risk and progression [13]. Furthermore, when screening tumours for aberrant DNA methylation IGFBPs, particularly IGFBP-3 have consistently been among the most commonly identified genes [14,15]. IGFBP-3 is the main IGFBP found in human serum and in addition to modulating the actions of IGFs, IGFBP-3 can also act intrinsically in either a positive or negative manner depending on the context [16]. In keeping with these in vitro data, epidemiology studies have also reported both positive and inverse association of IGFBP-3 with pre-menopausal breast cancer [17]. In breast cancer cells, the expression of IGFBPs has distinct patterns correlated with their ER-α status. The ER-α negative cell lines secrete predominantly IGFBP-3 [18]. Furthermore IGFBP-3 is known to mediate the effects of a number of different inhibitors of cell growth and inducers of apoptosis, such as TGF-beta, p53, vitamin D and tamoxifen [19–23]. In this study we treated four different breast cancer cell lines with the DNA methytransferase inhibitor, AZA to determine the role played by IGFBP-3 in the effects of AZA on total cell number, cell survival and colony formation.

Materials and methods

Cell culture

The human breast cancer cell lines MCF-7, T47D, MDA-MB-231 and Hs578T were purchased from ATCC and were maintained as described previously [24,25].

Detection of protein abundance radiommunoassay (RIA)

Levels of IGFBP-3, IGF-I and -II in conditioned media were measured by radioimmunoassays as described previously [26,27].

Cell counting: trypan blue dye exclusion

All cells were collected following trypsinisation and the resulting cell suspension was loaded onto a haemocytometer (1:1) with the dye trypan blue, which is excluded by viable cells. Cells were counted from which total cell number and the percentage of dead cells relative to control were calculated.

Tritiated thymidine incorporation

Proliferation was also measured using [^{3}H]-thymidine incorporation as described previously [25].

Western immunoblotting analyses

Preparation of cell lysates were performed as described previously [28]. The protein content of each sample was determined using a Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Cheshire, UK) to ensure equivalent amounts of protein were loaded. Protein samples from cell lysates or cell conditioned media were resolved by electrophoresis on 8 or 12% polyacrylamide-sodiumdodecyl sulfite (SDS-PAGE) and transferred to nitrocellulose membranes. Non-specific binding sites on the membranes were blocked for a minimum of 2 h with 5% (w/v) milk in Tris-buffered saline (TBS)/2% Tween before probing with anti-GAPDH (1:5000, Chemicon, Nottingham, UK, MAB 374), anti- ER-α (1:750, Santa Cruz, Heidelberg, Germany, sc-73479), anti-IGFBP-3 (1:10,000, in-house), anti- activator protein-2alpha (AP-2α) (1:1000, Santa Cruz, Heidelberg, Germany, sc-184). After removal of excess unbound primary antibody with three TBST washes, appropriate secondary antibodies conjugated to peroxidase were added for 1 h. Peroxidase binding was visualized by enhanced chemiluminescence and detected as described previously [29].

Transfection with siRNA

Cells were seeded in growth media (GM) in 24-well plates (0.08×106 cells per well) in the presence or absence of target siRNA or non-silencing (NS) with or without AZA (1 μM). HiPerFect reagent from Qiagen (Cat #. 301705) was used for the transfection reaction. Cells were then switched to serum-free media (SFM) for a further 48 h before being assessed for changes in cell number and cell death or DNA synthesis using cell counting or a tritiated thymidine incorporation assay respectively. The target sequences of the primers are as follows: IGFBP-3 siRNA: TTCAAAGATAATCATCATCAA (Qiagen, West Sussex, UK Cat #. SI00012516), AP2-α siRNA: CCGGGTATTAACATCCCAGAT (Qiagen, West Sussex, UK, Cat #. 1027415), ER-α siRNA GAGACTTGAATTAATAAGTGA (Qiagen, West Sussex, UK Cat #. SI02781401) and NS siRNA: AATTCTCCGAACGTGTCACGT (Qiagen, Cat #. 1027310).

Colony formation assay

For colony formation assay, IGFBP-3 was silenced in MCF7 or MDA-MB-231 cells for 24 h in universal tubes (same concentrations of reagents as above). Cells were then counted. The same number of live cells for each sample (10,000 cells and 50,000 cells for MCF7 and MDA-MB-231 respectively) was seeded in soft agarose gel (Lonza, 50101) in petri dishes. AZA was added to the