Annexin-1 Regulates Growth Arrest Induced by High Levels of Estrogen in MCF-7 Breast Cancer Cells

Emily Zhao-Feng Ang,1 Hung Thanh Nguyen,1 Hui-Ling Sim,1 Thomas C. Putti,2 and Lina H.K. Lim1

1Inflammation and Cancer Laboratory, Department of Physiology and NUS Immunology Program, and 2Department of Pathology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Abstract

Estrogen, a naturally occurring female steroid growth hormone, has been implicated as a major risk factor for the development of breast cancer. Recent research into this disease has also correlated Annexin-1 (ANXA1), a glucocorticoid-inducible protein, with the development of breast tumorigenesis. ANXA1 is lost in many cancers, including breast cancer, and this may result in a functional promotion of tumor growth. In this study, we investigated the expression of ANXA1 in MCF-7 cells treated with estrogen and the regulation of estrogen functions by ANXA1. Exposure of MCF-7 breast cancer cells to high physiologic levels (up to 100 nmol/L) of estrogen leads to an up-regulation of ANXA1 expression partially through the activation of cyclic AMP-responsive element binding protein and dependency on activation of the estrogen receptor. In addition, treatment of MCF-7 cells with physiologic levels of estrogen (1 nmol/L) induced proliferation, whereas high pregnancy levels of estrogen (100 nmol/L) induced a growth arrest of MCF-7 cells, associated with constitutive activation of extracellular signal-regulated kinase 1/2 and up-regulation of cell cycle arrest proteins such as p21watcris. Silencing of ANXA1 with specific small interfering RNA reverses the estrogen-dependent proliferation as well as growth arrest and concomitantly modulates extracellular signal-regulated kinase 1/2 phosphorylation. We confirm that ANXA1 is lost in clinical breast cancer, indicating that the antiproliferative protective function of ANXA1 against high levels of estrogen may be lost. Finally, we show that ANXA1-deficient mice exhibit faster carcinogen-induced tumor growth. Our data suggest that ANXA1 may act as a tumor suppressor gene and modulate the proliferative functions of estrogens.

(Mol Cancer Res 2009;7(2):266–74)

Introduction

Mammary cancer is one of the most common cancers among females and the second leading cause of cancer mortality for all women today. There is substantial evidence suggesting that high estrogen levels in postmenopausal women may be associated with an increased risk to breast cancer (1). In addition, studies have also shown that mammary tumors may be initiated by carcinogenic metabolites of estrogen, which may react with DNA to induce oncogenic mutations (2, 3). Estrogens, acting via its receptors, regulate the growth, proliferation, and metastatic potential of breast cancer cells as well as the growth and differentiation of cells in the female and male reproductive systems.

Annexin-1 (ANXA1; also known as lipocortin-1) is the first member of the Annexin superfamily of proteins, which consists of a relatively large family of calcium and phospholipid-binding proteins (4). First discovered as a 37-kDa protein factor induced by glucocorticoids to suppress the inflammatory mediators of the eicosanoid family (5), its endogenous physiologic function(s) have remained largely unclear (6). Nevertheless, since its discovery as a major cellular substrate for tyrosine phosphorylation by the epithelial growth factor receptor (7), ANXA1 has been implicated in various pathways known to be involved in cancer (ref. 4; for a review, see ref. 6) through the modulation of mitogen-activated protein kinase signaling, calcium ion mobilization, apoptosis (5), cell proliferation (8, 9), and inhibition of cell growth (10, 11).

ANXA1 has been shown to be strongly down-regulated in prostate cancer (12), head and neck cancer (13), and esophageal cancer (14); it was observed to be up-regulated in hepatocarcinoma (15) and pancreatic cancer (16). In addition, although earlier studies have shown ANXA1 to be up-regulated in mammary adenocarcinoma (17, 18), a recent report has shown that decreased ANXA1 expression was correlated with development and progression of the disease as determined by a tissue microarray analysis (19). This differential expression of ANXA1 in normal and malignant mammary cells highlights the possibility that ANXA1 may be regulated differently in normal and tumorigenic tissues possibly by hormonal influence.

The diverse functions of ANXA1 point to its importance in cellular homeostasis. Its endogenous function is still under debate, but most have focused on its anti-inflammatory actions, shown to mimic those of glucocorticoids (20). ANXA1 is glucocorticoid-inducible (21-22) through transcriptional activation of the glucocorticoid response element as well as through rapid de novo synthesis (23). Glucocorticoids were not effective in ANXA1-deficient mice in two models of acute inflammation, suggesting that ANXA1 could be the...
endogenous protein mediating the anti-inflammatory actions of glucocorticoids (24).

In this study, we investigated if disparate expression of ANXA1 in breast cancer in clinical studies could be linked to its regulation by estrogen or 17β-estradiol (17β-E), the major estrogen synthesized by the ovaries of the female human. In addition, we show that ANXA1 regulates the proliferative functions of estrogens in MCF-7 breast cancer cells. Research into this area can enhance our understanding of the possible role of ANXA1 in tumorigenesis, which is at present fairly limited, as evidence in support of any contributory role(s) for ANXA1 in the development of the disease is still mainly circumstantial (4). At the same time, such investigations can also perhaps pave the way for novel therapeutics for the disease via targeting of the protein and/or lead to the possible identification of ANXA1 as a potential biomarker in breast cancer development and progression.

Results

We first examined the expression of ANXA1 in clinical breast cancer samples, as the known literature is conflicting (17-19). Ten samples of different breast carcinomas (ductal, medullary, and colloid) were obtained and processed by the Department of Pathology at the National University of Singapore. Normal breast tissue and ducts, which were tumor-adjacent stained positive to ANXA1 (Fig. 1A and B). However, ANXA1 expression was not visible in low-grade ductal carcinoma (Fig. 1A and B). All areas of tumor in ductal carcinoma in situ (Fig. 1C) and invasive ductal carcinoma (Fig. 1D) were ANXA1 negative. However, myoepithelial cells and epithelial cells stained positive for ANXA1.

Effects of 17β-E on ANXA1 Expression

As breast cancer is heavily influenced by estrogen, the effects of estrogen or its active metabolite 17β-E on ANXA1 expression were investigated in estrogen receptor (ER)-positive MCF-7 breast cancer cells. Cells were treated with 0.01 nmol/L to 1 μmol/L 17β-E for 72 h in charcoal-stripped medium. Treatment with 17β-E induced an up-regulation of ANXA1 mRNA expression in a time- and dose-dependent manner, evident after 48 h with 1 nmol/L 17β-E (Fig. 2A). The highest level of ANXA1 was observed with 100 nmol/L 17β-E at 72 h. Similarly, ANXA1 protein expression was induced in a dose-dependent manner (Fig. 2B), evident at a concentration of 1 nmol/L 17β-E, with the highest expression of ANXA1 at 72 h with 100 nmol/L 17β-E (1.9-fold increase from vehicle control). Cytosolic and total ANXA1 levels in MCF-7 cells after 72 h 17β-E treatment were similar (Fig. 2C), indicating that the increase in total ANXA1 observed was not likely due to translocation but an induction of ANXA1 expression or transcription by 17β-E. This, coupled with the considerable time lag between drug administration and the observable increase in ANXA1 levels, suggests that the induction of ANXA1 expression by 17β-E is a result of gene transcription. In addition, as ANXA1 phosphorylation on Tyr21 has been proposed to be a prerequisite for its proliferative effects in hepatic cancer cells in vitro and in vivo (15), ANXA1 phosphorylation was also examined in MCF-7 cells using immunoprecipitation of ANXA1 and probing with phosphotyrosine, threonine, and serine antibodies. However, no changes in ANXA1 phosphorylation after 17β-E treatment were observed (data not shown).

Role of ER-α in ANXA1 Induction

To assess if ER-α was involved in 17β-E-induced ANXA1 expression, the MDA-MB-231 cell line (negative for ER-α) was used. After treatment with the same concentrations of 17β-E, no increase in ANXA1 expression was observed at all time points (data not shown). This indicates that ER-α may be involved in the induction of ANXA1 by 17β-E. To further verify that the ER is involved in the up-regulation of ANXA1, ICI 182780, a selective inhibitor against ER-α, was added 1 h before the addition of 17β-E. Pretreatment of cells with ICI 182780 resulted in a complete inhibition of 17β-E-induced ANXA1 expression after 72 h (Fig. 3). These data suggest that 17β-E acts via the ER-α to regulate ANXA1 expression in MCF-7 cells.

Regulation of ANXA1 Expression by Cyclic AMP-Responsive Element

17β-E has been shown to activate cyclic AMP (cAMP)-responsive element (CRE) binding (CREB) proteins to induce transcriptional activity. As the time course of ANXA1 induction was suggestive of gene transcription, the promoter region of ANXA1 was examined and found to contain a similar, near-identical 8-nucleotide sequence (TGATGTCA) to the CRE consensus sequence (TGACGTCA). Thus, to investigate the mechanisms through which 17β-E could induce ANXA1 in MCF-7 breast cancer cells, CRE transcriptional activity was examined using a CRE promoter luciferase reporter assay. Treatment of MCF-7 cells with 17β-E (0.1-10 nmol/L) resulted in significant activation of CRE-luciferase reporter activity in a concentration-dependent manner (Fig. 4A). To investigate if CRE is involved in 17β-E-induced ANXA1 expression, MCF-7 cells were pretreated with 100 nmol/L SQ22536, a cell-permeable adenylate cyclase inhibitor 1 h before 17β-E incubation. To prove the efficiency of the inhibitor, CRE-luciferase reporter activity was measured after SQ22536 pretreatment. SQ22536 almost completely repressed the transcriptional activity of CRE after 17β-E treatment (Fig. 4B). To examine if 17β-E-induced ANXA1 expression is regulated by CRE, SQ22536 was added before 17β-E treatment. SQ22536 pretreatment abrogated ANXA1 induction at 24 and 48 h with all concentrations of 17β-E stimulation (Fig. 4C) but only partially inhibited ANXA1 expression at 72 h (10 nmol/L: 1.8-fold increase with SQ22536 versus 2.3-fold increase without SQ22536). Furthermore, induction of ANXA1 mRNA expression after 17β-E treatment was also significantly inhibited with SQ22536 pretreatment at all time points (data not shown). The data suggest that 17β-E-induced ANXA1 expression in MCF-7 breast cancer cells can be modulated partially through CREB activation.

Effect of 17β-E on MCF-7 Viability and Proliferation

An overexpression of ANXA1 was shown to reduce cell proliferation in RAW mouse macrophages (11); thus, we next
investigated the effect of 17β-E on viability and proliferation. Cells were treated with 0.01, 1, or 100 nmol/L 17β-E for 24, 48, and 72 h. Cell viability and proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Cell Titer 96 AQueous One Solution Cell Proliferation Assays. 17β-E treatment induced a time-dependent increase in absorbance (metabolism of MTT) compared with the vehicle control, maximal at 0.01 nmol/L 17β-E (Fig. 5A). However, on a higher concentration of 17β-E at 100 nmol/L, cell viability was similar to that of the vehicle control, significantly lower than that of 0.01 and 1 nmol/L 17β-E. This was confirmed with the use of the Cell Titer proliferation assay (Fig. 5B), although more proliferation was observed with 100 nmol/L 17β-E compared with MTT assay. Drug-treated cells exhibited significant cell proliferation when compared with the vehicle control. Once again, cells treated with 100 nmol/L 17β-E exhibited the lowest proliferation relative to the control (33.7% increase in proliferation) when compared with lower concentrations of 17β-E (1 nmol/L; 56.3% increase in proliferation) at 72 h. This was not due to apoptosis, as Annexin V-FITC/PI staining showed no apoptosis induced by estrogen (data not shown). These data indicate that normal physiologic concentrations of 17β-E induce proliferation, whereas higher concentrations of estrogen induce a growth arrest in MCF-7 breast cancer cells.

To investigate if 17β-E-induced growth arrest and ANXA1 levels were associated with extracellular signal-regulated kinase 1/2 (ERK1/2) activation and p21waf/cip expression in MCF-7 breast cancer cells, ANXA1, p21waf/cip, and phosphorylated ERK1/2 were examined in MCF-7 cells after 72 h of drug treatment. As shown in Fig. 5C, 100 nmol/L 17β-E markedly induced the phosphorylation of ERK1/2, whereas ERK1/2 phosphorylation was not observed with lower concentrations of ERK1/2. The expression of p21waf/cip was slightly up-regulated when cells were treated with 100 nmol/L 17β-E, correlating with low proliferation rates observed with this concentration of 17β-E, suggesting that the sustained activation of ERK1/2 and induction of p21 and ANXA1 may reduce proliferation at high concentrations of 17β-E.

Mediation of 17β-E-Induced Proliferation and Growth Arrest by ANXA1

Next, to investigate if 17β-E-induced proliferation and growth arrest is mediated by ANXA1, MCF-7 cells were treated with either a scramble small interfering RNA (siRNA) or ANXA1 siRNA. Proliferation analysis of scramble siRNA-transfected or ANXA1 siRNA-transfected cells treated with 0.01, 1, or 100 nmol/L 17β-E for 72 h showed that silencing ANXA1 reverses the proliferation arrest at higher concentrations of 17β-E (Fig. 6A). Treatment of scramble siRNA-transfected cells with 100 nmol/L 17β-E induced an increase in proliferation at low nanomolar concentrations and a decline in proliferation at high concentrations (1.2-fold increase in proliferation at 100 nmol/L versus 1.7-fold at 0.01 nmol/L) similar to parental MCF-7 cells. These changes in proliferation were reversed in ANXA1 siRNA-transfected MCF-7 cells where a concentration-dependent increase in proliferation was now observed at 100 nmol/L (1.5-fold increase in proliferation at 100 nmol/L versus 1.25-fold at 0.01 nmol/L). These data suggest that ANXA1 may play a role in the proproliferative and antiproliferative effects brought about by 17β-E. The effect of ANXA1 silencing was also determined on ERK1/2 phosphorylation. In scramble siRNA-transfected cells, 1 and 100 nmol/L
17β-E induce ANXA1 expression and ERK1/2 activation, whereas ERK1/2 phosphorylation was almost completely blocked in ANXA1 siRNA-transfected cells (Fig. 6B). This indicates that ANXA1 is critical for proliferation arrest induced by high levels of 17β-E mediated by ERK1/2 phosphorylation. Furthermore, treatment of MCF-7 cells with UO126 does not affect the up-regulation of ANXA1 expression by 17β-E (data not shown), indicating that ANXA1 is upstream and not downstream of ERK1/2 activation. These data suggest that the induction of ANXA1, an antiproliferative protein by high levels of 17β-E, may negatively regulate proliferation and may be involved in the growth arrest observed with a high concentration of 17β-E.

7,12-Dimethylbenz[a]anthracene-Induced Carcinogenesis in ANXA1-Deficient Mice

Finally, we wished to determine if the antiproliferative effect of ANXA1 could be shown in a carcinogen-induced tumor model in vivo and if ANXA1 was important in in vivo breast tumor development and growth. We used the ANXA1-deficient (knockout) BALB/c mouse and compared them with normal BALB/c mice (wild-type) orally administered with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) in sesame oil. There was no difference between wild-type and knockout mice in tumor incidence from weeks 0 to 30 (Fig. 7A). A significant difference in tumor incidence was only observed at week 38 (P = 0.034). Furthermore, DMBA carcinogen-induced tumors were significantly different in both wild-type and knockout mice after 28 days of first palpation (P < 0.01; Fig. 7B). ANXA1 knockout mice exhibited significantly faster growth, with a significantly larger tumor size after 24 days of first palpation (P < 0.05) compared with wild-type mice. These data indicate that ANXA1 can act as an important regulator of breast cancer growth in vivo.

Discussion

Estrogen, the main female hormone, is thought to play a role in breast cancer development and growth. In this study, we have correlated the loss in ANXA1 in clinical breast cancer to a functional regulation of cellular proliferative ability induced by estrogen. Submicromolar (100 nmol/L) estrogen (or its active metabolite 17β-E) induces growth arrest in minimally invasive MCF-7 breast cancer cells, whereas lower nanomolar concentrations promote proliferation of breast cancer cells (25). These lower concentrations are in line with the physiologic concentrations of estrogen found in women. Physiologic levels of 17β-E in premenopausal women ranges from 30 to 300 pg/mL midcycle (0.1-1 nmol/L), whereas higher levels of estrogen (100 nmol/L) may be found in pregnant women. Nulliparous women are more susceptible to breast cancer, and it was suggested that short-term exposure to high pregnancy levels of estrogen may in fact reduce mammary carcinogenesis (26), whereas longer-term estrogen exposure to lower concentrations may result in carcinogenesis. Micromolar concentrations of estrogen have been shown to inhibit proliferation of rhesus ovarian surface epithelial cells (27), via the blocking of serum growth factors, activation of the G1-phase cell cycle checkpoint, and induction of p21, possibly through the up-regulation of p53.

Evidence associating ANXA1 expression and function with glucocorticoid treatment is indisputable (21-23, 28). This induction of ANXA1 expression can be blocked by the glucocorticoid receptor antagonist RU486 (29). However, evidence on the induction of ANXA1 expression by the chemically similar sex hormones is lacking and controversial. Some groups show that sex hormones do not induce ANXA1 expression (5), whereas others show that ANXA1 can be induced by 17β-E, albeit in CEM-CCRF lymphoma cells, and in a de novo mechanism (30), mediated through cAMP and CREB (31). High levels of steroids are found in cell culture serum and may already have induced ANXA1 up-regulation, which would mask subsequent induction of ANXA1. Thus, the use of charcoal-stripped medium in our study reduced the variability in responses and increased the sensitivity of cells to...
exogenous steroid treatment. In our study, ANXA1 mRNA and protein expression is only significantly induced after 48 h with the increasing concentrations of estrogen. Due to the considerable time lag between drug administration and the evident increase in ANXA1 expression, the induction of ANXA1 is likely to be a result of gene transcription activated either directly or indirectly by the binding of 17β-E to its receptor, similar to the binding of glucocorticoids to the glucocorticoid receptor (23). In addition, it may be possible that the up-regulation of ANXA1 is due to the action of 17β-E on ER-α and not ER-β, because the ER-α antagonist ICI 182780 effectively inhibited ANXA1 up-regulation, and treatment of MDA-MB-231 cells, which are ER-negative or express only ER-β and not ER-α (32) with 17β-E, had no effects on ANXA1 expression. Although the roles of ER-β remain elusive (33), studies have suggested that although actions of 17β-E via ER-α are typically proliferative, the effects of 17β-E on ER-β are, in contrast, anti proliferative and tumor suppressive (34, 35).

It was recently shown that the promoter region of the mouse ANX-A1 gene contains a CRE and that a CREB is required for cAMP-induced ANXA1 synthesis (36). 17β-E has also been shown to influence CRE-mediated gene transcription through rapid phosphorylation of CREB (37). 17β-E can rapidly elicit cAMP production within minutes to initiate downstream signaling cascades, one of which is the cAMP-protein kinase A-CREB signaling pathway (38). We show in our present study that the early up-regulation of ANXA1 is transcriptionally up-regulated by CRE in MCF-7 breast cancer cells.

A low ANXA1 expression was shown to induce a prolonged stimulation of the mitogen-activated protein kinase pathway and subsequent increased cell proliferation in RAW macrophages (39); an overexpression of ANXA1 led to a constitutively sustained stimulation of the same pathway via activation of ERK1/2 and was accompanied by a reduction in cell proliferation due to an up-regulation of cell cycle arrest proteins such as p21waf/cip (11). In our study, Western blot analyses showed that treatment of the cells with high concentrations of 17β-E induced ERK1/2 activation that was sustained even after 72 h, unlike treatment with lower concentrations of 17β-E, which resulted in an ERK1/2 activation that was not sustained. 17β-E has been shown to activate the mitogen-activated protein kinase pathway in other studies (37) and a constitutive activation of the mitogen-activated protein kinase pathway leads to antiproliferative signals generated to restrict cell proliferation, whereas transient activation may induce proliferation (40, 41). Our data suggest that exposure to high levels of 17β-E induces a constitutive activation of ERK1/2, resulting in growth arrest, and that ANXA1 is upstream of ERK1/2 and in turn regulates ERK1/2 activity.

Our transfection studies have shown that ANXA1 is critical, at least in part, for the regulation of proliferation observed for cells treated with 17β-E, as ANXA1 siRNA-transfected cells treated with 100 μmol/L 17β-E exhibited the highest proliferation rates compared with scramble-siRNA transfected cells, whereas proliferation was inhibited during treatment with lower physiologic concentrations of 17β-E. This suggests that ANXA1 is inherently an antiproliferative protein up-regulated to counter the overactivation of proliferative signals induced by 17β-E. This is similar to previous studies where ANXA1 was shown to regulate the proliferation of RAW macrophages (11) and mediated steroid-induced growth arrest in lung cancer cells (21). We have shown in this study that ANXA1 siRNA-transfected cells are unable to sustain prolonged ERK1/2 activation after 72 h when treated with high 17β-E doses, suggesting that ANXA1 is required for constitutive ERK1/2 activation observed in cells treated with high 17β-E concentrations over a prolonged period. Removal of constitutive ERK1/2 activation by the suppression of ANXA1 expression may hence eliminate growth arrest. The finding that low concentrations of 17β-E induce proliferation, whereas ANXA1 is induced does not necessarily negate its role as a tumor suppressor, as the mechanisms of carcinogenesis versus growth or differentiation may be different. Interestingly, we also observe that siRNA to ANXA1 inhibits proliferation induced by low concentrations of 17β-E. This suggests that ANXA1 does play an active role in the proliferation induced by 17β-E perhaps through the inhibition of ERK1/2 activation.

This study links clinical data with the in vitro evidence that ANXA1 influences breast proliferative ability. The results with the DMBA-induced tumors in ANXA1-deficient mice are consistent with the clinical data that have arisen in recent years.
Previous clinical studies reported that ANXA1 was lost in several cancers such as esophageal (42) and prostate (12) and, recently, breast (ref. 19; for a review, see ref. 6). We have also shown in our study that ANXA1 expression is lost in breast cancer samples. With particular reference to breast cancer, an overexpression of ANXA1 was reported in tumor ducts compared with normal ducts (17). However, a recent report on tissue microarray (containing 1,158 informative breast tissue cores of different histologies, including normal, hyperplasia, in situ and invasive tumors, and lymph node metastases) showed that, when compared with normal or hyperplasia tissues, a significant reduction in ANXA1 expression in ductal carcinoma in situ and invasive ductal carcinoma was observed (19). The loss or deregulation of ANXA1 expression in breast cancer is still under investigation. It may be possible that the regulation of ANXA1 through specific microRNAs may be involved in the changes in expression of ANXA1 in breast cancer. Mutation studies on esophageal cancers showed that allelic loss of ANXA1 frequently occurs, whereas somatic mutations were rare. It may be possible that, in normal breast tissue expressing ANXA1, high concentrations of estrogen may induce a growth arrest. However, in breast tumors with low or no ANXA1 expression, high concentrations of estrogen may induce uncontrolled proliferation.

In summary, the experimental results generated within this study directly link ANXA1 to a role in tumorigenesis in vitro and in vivo. We have shown that prolonged exposure of MCF-7 cells with 17β-E (after at least 48 h) induces a dose-dependent up-regulation of ANXA1 and that a higher expression of ANXA1 induced by high levels of 17β-E correlated with growth arrest despite the fact that 17β-E is a well-established growth-stimulatory hormone in mammary cells. In addition, we have also shown that ANXA1 has direct antiproliferative effects on cells possibly via a constitutive overactivation of ERK1/2 and that the antiproliferative role of ANXA1 is, in turn, directly responsible, at least in part, for the growth arrest observed when cells were exposed to high 17β-E concentrations for a prolonged period. As such, we believe that ANXA1 may act as a tumor suppressor in cells and that its expression may be increased in times of need or stress to put a brake on cellular proliferation. Any mutations that occur in pathways that up-regulate ANXA1 may hence render the cells incapable of inhibiting any uncontrolled proliferation and may thus, in turn, explain why a decreased ANXA1 expression has been implicated in breast cancer development and progression (19).

Materials and Methods

Reagents

17β-E, DMBA, HEPES, okadaic acid, and sodium vanadate were purchased from Sigma. Protease inhibitor and ANXA1 antibodies were purchased from BD Biosciences. cAMP kit was purchased from Cayman Chemical. CRE-luciferase reporter assay was purchased from Stratagene.

Cell Culture and Treatment

ER-α-positive human breast adenocarcinoma cell line, MCF-7, was obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. These monolayer cells were maintained in a 37°C incubator with 5% CO₂. Cells were checked regularly under the light microscope and subcultured when they reached 70% to 80% confluence. Twenty-four hours before drug treatment, cells were incubated with incomplete medium.
containing 10% charcoal-stripped fetal bovine serum. Fetal bovine serum was incubated with activated charcoal to remove steroids and other growth factors. MCF-7 cells were seeded in 25 cm² flasks and incubated in charcoal-stripped medium 1 day before treatment with vehicle (DMSO) or 17β-E (0.001 nmol/L-1 μmol/L) for 24, 48, and 72 h. The same was done with MDA-MB-231 breast cancer cells.

**Western Blot**

Following drug treatment, cells were washed with ice-cold 1× PBS and immediately placed at −20°C for 5 min. Cells were scraped in the presence of a lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 10% NP40, 1× protease inhibitor, and phosphatase inhibitors: 50 μmol/L okadaic acid and 200 mmol/L sodium vanadate]. The supernatant was collected and stored at −20°C until evaluation by SDS-PAGE analysis. Equal amounts of protein from each sample were subjected to SDS-PAGE at a constant voltage of 125 V. The proteins were then transferred onto nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer apparatus (Bio-Rad Laboratories). Protein expression and phosphorylation were determined by Western blotting with specific antibodies, and expression signals were obtained by enhanced chemiluminescence. Protein expression was normalized to α-tubulin levels and ERK1/2 phosphorylation was normalized to total ERK1/2 expression. Where applicable, Western blots were scanned at 600 dpi for densitometric quantification using the ImageJ software. Densitometric values were normalized with that of a control band (α-tubulin or ERK1/2) and further normalized to control samples.

**Reverse Transcription-PCR**

Total RNA was extracted from cells using Trizol reagent. Procedures were carried out in accordance to the manufacturer’s instructions. All RNA samples were stored at −70°C and analyzed within a week by subsequent reverse transcription-PCR and DNA gel electrophoresis analysis. Reverse transcription-PCR was done on total RNA extracted from MCF-7 cells using the Qiagen One-Step Reverse Transcription-PCR Kit. Procedures were carried out in accordance to the manufacturer’s instructions using 1 μg template RNA per reaction. The following primers were used: ANXA1 forward primer, 5′-CTGGAAAGCTTGTGTCAGAATTTCACAGCAG-3′; ANXA1 reverse primer, 5′-CTCCTCATGATGTCCATCCCAACCAAGAGCC-3′; GAPDH forward primer, 5′-AACACAGTCCATGCCATCAC-3′; and GAPDH reverse primer: 5′-TCCACACACCGTGTTGCTGTA-3′. PCR was done and PCR products were separated by 1.5% agarose gel electrophoresis in 1× TAE electrophoresis buffer and visualized by ethidium bromide staining. The resulting fluorescent bands were then video-digitized by a GelDoc 1000UV-Gel camera and PCR products were identified as a single band corresponding to expected molecular sizes.
**Transfection Studies**

MCF-7 cells were transfected with Dharmacon SMARTpool ANXA1 siRNA (Dharmacon). Transfection procedures were carried out in accordance to the manufacturer’s instructions. Two sets of control experiments were also done, whereby the cells were either transfected with scrambled ANX-A1 siRNA or Oligolectamine transfection reagent (Invitrogen) alone [scrambled siRNA sequence: sense strand r(GGGGA-CAUAGUAAACGUUG)dTdT and antisense strand: r(CAC-GUUUACGUAUGUCCCC)dTdT].

**Proliferation Assays**

Viability was determined by quantitative measurement of the conversion of MTT (Sigma) to a water-insoluble formazan by the mitochondrial dehydrogenase enzyme in metabolically active cells. Cell growth rate was determined at 24 and 48 h after treatment using MTT assay. Proliferation was measured using Cell Titer 96 AQeuous One Solution Cell Proliferation Assay (Promega). Cells (1 × 10^5) were plated in each well of a 96-well plate and left to adhere for a minimum of 4 h, after which the cell culture medium was aspirated and replaced with 100 μL charcoal-stripped medium. After 24 h, this was replaced with fresh charcoal-stripped medium and drugs were added in the appropriate concentrations. After drug treatment, 20 μL Cell Titer 96 AQeuous One Solution Reagent was added to each well and the plates were incubated in a 37°C incubator with 5% CO₂ for 3 h. The absorbance intensities were then measured at 490 nm and the cell proliferation relative to the control sample was calculated.

**Luciferase Assay**

The commercially available promoter-reporter assay was used to measure CRE transcriptional activities and done as described by the manufacturer. Briefly, the firefly luciferase reporter constructs, under the control of the appropriate enhancer elements and transactivator constructs, were provided in the PathDetect in vivo signal transduction pathway cis-reporting system. MCF-7 cells were grown to 80% confluence in charcoal-stripped RPMI 1640 and seeded at 2 × 10^5 into each well of 6-well tissue culture dishes, after which they were serum starved for 16 h. The cells were either transfected with scrambled ANX-A1 siRNA alone or Oligolectamine transfection reagent (Invitrogen) followed by the firefly luciferase reporter constructs, under the control of the appropriate enhancer elements and transactivator constructs, and incubated for 3 h. The absorbance intensities were then measured at 490 nm and the cell proliferation relative to the control sample was calculated.

**DMBA-Induced Carcinogenesis**

All animal work was approved by the Institutional Animal Care and Use Committee and followed the National Advisory Committee for Laboratory Animals Research. BALB/c mice (6-8 weeks) were obtained from Laboratory Animal Centre, Singapore. Annexin-1-deficient mice on BALB/c background were bred in pathogen-free conditions in our animal facility. Mice were kept on a 12 h light/dark cycle with food and water provided ad libitum. DMBA was dissolved in sesame oil and 8- to 12-week-old female mice were gavaged p.o. with 1 mg (0.1 mL) DMBA once a week for 4 weeks. Mice were monitored for 42 weeks and tumors were palpated twice weekly and tumor volume was calculated as follows: length × width × width / 2. Mice were killed by cervical dislocation either at the end of the study or earlier if they displayed significant weight loss, signs of distress, or palpable tumors >2 cm in diameter.

**Statistical Data Analysis**

Data were tested for normality and paired sets of data (treated versus control) were compared using Student’s t test (two-tailed). Comparisons between multiple treatment groups from the same experiment were made using one-way ANOVA. When significant differences were found between groups, Tukey’s post hoc test was used to test significance. The Fisher’s exact test was used to analyze tumor incidence in mice, whereas a two-way repeated-measures ANOVA was done for tumor size, and the Bonferroni post hoc test was used to test significance between groups. In all cases, P < 0.05 was accepted as statistically significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Florence Ng for expert technical assistance and Prof. Roderick Flower (William Harvey Research Institute) for generously providing us the ANXA1-deficient animals.

**References**


Annexin-1 Regulates Growth Arrest Induced by High Levels of Estrogen in MCF-7 Breast Cancer Cells

Emily Zhao-Feng Ang, Hung Thanh Nguyen, Hui-Ling Sim, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-08-0147

Cited articles  This article cites 41 articles, 13 of which you can access for free at: http://mcr.aacrjournals.org/content/7/2/266.full.html#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at: /content/7/2/266.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.