Expression Pattern of the Pro-apoptotic Gene *PAR-4* During the Morphogenesis of MCF-10A Human Mammary Epithelial Cells

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Abstract The histological organization of the mammary gland involves a spatial interaction of epithelial and myoepithelial cells with the specialized basement membrane (BM), composed of extra-cellular matrix (ECM) proteins, which is disrupted during the tumorigenic process. The interactions between mammary epithelial cells and ECM components play a major role in mammary gland branching morphogenesis. Critical signals for mammary epithelial cell proliferation, differentiation, and survival are provided by the ECM proteins. Three-dimensional (3D) cell culture was developed to establish a system that simulates several features of the breast epithelium in vivo; 3D cell culture of the spontaneously immortalized cell line, MCF10A, is a well-established model system to study breast epithelial cell biology and morphogenesis. Mammary epithelial cells grown in 3D form spheroids, acquire apicobasal polarization, and form lumens that resemble acini structures, processes that involve cell death. Using this system, we evaluated the expression of the pro-apoptotic gene PAWR (PKC apoptosis WT1 regulator; also named PAR-4, prostate apoptosis response-4) by immunofluorescence and quantitative real time PCR (qPCR). A time-dependent increase in PAR-4 mRNA expression was found during the process of MCF10A acinar morphogenesis. Confocal microscopy analysis also showed that PAR-4 protein was highly expressed in the MCF10A cells inside the acini structure. During the morphogenesis of MCF10A cells in 3D cell culture, the cells within the lumen showed caspase-3 activation, indicating apoptotic activity. PAR-4

was only partially co-expressed with activated caspase-3 on these cells. Our results provide evidence, for the first time, that PAR-4 is differentially expressed during the process of MCF10A acinar morphogenesis.

Keywords Apoptosis · Breast cancer · MCF10A · PAR-4 · Three dimensional (3D) cell culture · Gene expression

Abbreviations

BM	basement membrane
ECM	extra-cellular matrix
PAR-4	prostate apoptosis response-4
qPCR	quantitative real time PCR

Introduction

The mammary gland has a very well organized and functional architecture composed of epithelial and stromal cells surrounded by the components of the extracellular matrix (ECM) [1]. The development and maintenance of the mammary gland depends upon a complex communication system. This includes cell-cell contacts and cell-ECM interactions, which lead to the formation of ducts and acini that are functional milk secretory units characterized by a hollow lumen surrounded by polarized epithelial cells [1, 2].

The three-dimensional (3D) cell culture system uses a commercially available matrix, such as Matrigel™ that is derived from the Engelbreth-Holm-Swan (EHS) murine tumor; it is a well-established model to study breast epithelial cell biology and morphogenesis in the context of a tissue organized structure [3]. Pioneer studies conducted by Bissell's group demonstrated that normal and transformed mammary cells can be distinguished by their

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behavior in 3D cell culture [4, 5]. In 3D cell culture, normal mammary cells are able to follow the acinar morphogenic process that includes proliferation, differentiation, and cell death. This results in a well-organized acinar structure in which cells in contact with ECM components acquire apicobasal polarity, leading to reduction in cell proliferation and death of the cells inside the acini. This morphogenic process is complete in one to two weeks. However, cancer cells derived from adenocarcinomas of the breast maintain the proliferative capability, forming solid spheroids without a hollow lumen or display disorganized masses of cells [5].

Evasion of apoptosis, or programmed cell death, is a hallmark of cancer and is an active, energy dependent process involving biochemical and molecular events regulated by a series of distinct genes [6]. Apoptosis is involved in mammary gland development, lactation, and involution [7]. Changes in a cell's microenvironment that reduces cell survival factors such as low access to nutrients, hypoxia, and loss of contact with the basement membrane contribute to the death of cells inside the acini and cavitation, leading to lumen formation [8]. Selective apoptosis and autophagy are the major processes involved in the selective removal of cells centrally located in the spheroid, leading to acinar lumen formation [9, 10]. Disruption of this structure results in re-population of the luminal space and is observed in the tumorigenic process of the breast [11].

In the present study, 3D cell culture, confocal microscopy, and quantitative real time PCR (qPCR) were used to evaluate the expression pattern of the pro-apoptotic gene, PAR-4, during the morphogenesis of MCF10A cells. Experimental evidence indicates that PAWR (PKC apoptosis WT1 regulator; also named PAR-4, prostate apoptosis response-4) is one of the central players in cancer cell survival and could be a target for cancer-selective targeted therapeutics [12]. The PAR-4 protein is ubiquitously expressed and localized in the cytoplasm of diverse normal tissues and cell lines, in both the cytoplasm and nucleus of many tumors and cancer cells, and very recently has been shown to be secreted [13–15]. Expression of PAR-4 may increase most cancer cell's sensitivity to apoptosis, especially in hormone-independent cancer cell lines, including breast cancer cells [14]. PAR-4 protein expression is induced by many conditions, such as growth factor withdrawal, *TNF*, ionizing radiation, and high levels of calcium [13, 16]. In cultured embryonic rat hippocampal neurons, withdrawal of trophic factors increases the level of PAR-4 mRNA expression [17]. The PAR-4 mechanism of apoptosis induction involves Fas/FasL translocation to the plasma membrane, phosphorylation of the SAC domain and nuclear translocation, and inhibition of bcl-2 and the pro-survival factor NF- κ B [12]. Alterations in PAR-4 have been associated with the development of different types of tumors [18]. Recently, we demonstrated that reduced nuclear expression of PAR-4 is associated with

a poor prognosis in breast cancer [19], however, to date, little is known of the role of PAR-4 in the mammary gland morphogenesis and breast cancer.

Material and Methods

Cell Culture MCF10A, MCF-7, and MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC). The MCF10A cells were cultured at 37°C in an atmosphere of 5% CO₂ and 95% air in F12/DMEM supplemented with 5% horse serum, 50 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1 µg/ml cholera toxin. MCF-7 and MDA-MB-231 cells were maintained in RPMI 1640 medium (Sigma Chemical Corporation) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10% fetal bovine serum (FBS) (Invitrogen Life Technologies) in a humidified atmosphere of 5% CO₂. For the morphogenesis assay and immunofluorescence, MCF10A cells were grown in monolayers until they reach 100% confluence and were then trypsinized to obtain a suspension containing single cells. MCF10A cells (2×10^3) in media containing 3% of growth factor-reduced Matrigel (BD Biosciences) were plated in 8-well chamber slides on a bed (30 µl) of growth factor-reduced Matrigel, as described by Debnath et al. [3]. The expression of PAR-4 and activated caspase 3 in the acini structures was analyzed by immunofluorescence on days 3, 5, 7, and 10. The medium containing 3% Matrigel was replaced every 2 days. Immunofluorescence was performed as described by Debnath et al. [3] with a few modifications, including permeabilization with 0.5% Triton-X100 for 45 min and overnight incubation with primary antibodies at room temperature. The acini structures were stained with mouse anti-PAR-4 monoclonal antibody (Santa Cruz, Inc, catalog sc-1666; 1:50), anti-laminin V mouse monoclonal antibody conjugated with Alexa Fluor 488 (Chemicon; catalog MAB19562X; 1:100), and/or a rabbit anti-activated-caspase 3 polyclonal antibody (Cell Signaling Technology, Inc, catalog 9661; 1:50). The conjugated secondary anti-mouse-Alexa Fluor 546 and anti-rabbit-Alexa Fluor 488 were purchased from Invitrogen and diluted 1:300. Nuclear staining was performed with Hoechst 33342 (Invitrogen; 1:5000) for 15 min. A Zeiss LSM Meta 510 scanning confocal microscope was used for immunofluorescence analysis and image capture.

Real Time PCR (qPCR) For RNA extraction, MCF10A cells were isolated from the Matrigel with PBS containing 2.5 mM EDTA. Total RNA was extracted from MCF10A cells using the RNA/DNA/Protein purification kit (Norgen Biotek Corporation, Ontario, Canada) and cDNA synthesis

was carried out using the high capacity archive kit (Applied Biosystems, California, USA) according to the manufacturer's instructions. mRNA levels of PAR-4 were measured by qPCR with GAPDH as the internal standard; qPCR was performed using the SYBR Green PCR Core reagent (Applied Biosystems) and processed in the GeneAmp

7500 Sequence Detection System (Applied Biosystems). The oligonucleotides used were: GAPDH, forward 5'-CCTCCAAAATCAAGTGGGGCG-3', reverse 5'-GGGGCAGAGATGATGACCCTT-3'; PAWR, forward 5'-CCAGAGAAGGGCAAGAGCTCGG-3', reverse 5'-ATTGCATCTTCTCGTTCCGC-3'. The relative gene

Fig. 1 Expression pattern of PAR-4 in MCF10A cells grown in monolayers and three-dimensional (3D) cell culture. **a** Immunofluorescence localization of PAR-4. MCF10A cells grown in monolayers were fixed using 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then incubated with primary monoclonal antibody anti-PAR-4 (red). **b** and **c**, Representative confocal microscopic images of MCF10A cells cultured in growth factor reduced Matrigel for 5 days and examined PAR-4 (red) and laminin V (green) by immunofluorescence. Nuclei were contrasted with Hoestch (blue). Equatorial cross sections of the acini were obtained using a Zeiss LSM Meta 510 laser scanning confocal microscope system (40 $\times$)

Fig. 2 PAR-4 mRNA expression pattern during MCF10A morphogenesis. mRNA was extracted from MCF10A cells cultured in growth factor reduced Matrigel for 3, 5, 7, and 10 days and the relative expression of PAR-4 mRNA was determined by quantitative real time PCR using GAPDH as the reference gene. Bars represent the relative gene expression after treatment, compared to control cells (NS). Data are expressed as the mean $\pm$ SD of three experiments

expression was calculated by $2^{-\Delta\Delta CT}$, where CT = fluorescence threshold value; ΔCT = CT of the target gene - CT of the reference gene (GAPDH); and $\Delta\Delta CT$ = ΔCT of the target sample - ΔCT of the reference sample (cells growing in 3D for 3 days). The results were expressed in *n*-fold differences in mRNA expression relative to the expression of GAPDH and the reference sample.

Western Blot For blotting, whole-cell lysates were prepared from MCF10A, MCF-7, and MDA-MB-231 cells cultured in monolayers and maintained in media supplemented with 10% serum. Thirty μ g of lysates were analyzed on 10% SDS-polyacrylamide gels followed by transfer at 100 V for 2 h to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL, USA). Protein concentration was measured using the Coomassie Plus Protein Assay Reagent™ (Pierce Biotechnology, Rockford, IL, USA). Blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1%

of Tween 20 (TBS-T) for 1 h at room temperature and then incubated with anti-PAR-4 monoclonal antibody (Santa Cruz, Inc, catalog sc-1666; 1:500) for 1.5 h at room temperature. Membranes were then washed and incubated with a peroxidase-conjugated secondary antibody, anti-mouse IgG, for 1.5 h. The incubations were performed in 3% albumin in TBS-T. For detection of β -actin, the membranes were striped with an acid solution and blocked again with 5% skim milk for 1 h at room temperature. Then, the membranes were incubated with anti- β -actin antibody (diluted 1:1000) followed by a peroxidase-conjugated secondary antibody, anti-mouse IgG, both for 1 h at room temperature. After washing the membranes with TBS-T, the signals of reactive proteins were developed with an enhanced chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ).

Results and Discussion

In MCF10A cells grown in monolayers, PAR-4 protein expression was predominantly localized in the cytoplasm and only displayed by a few cells (Fig. 1a). Using three-dimensional (3D) cell culture, we evaluated the expression pattern of PAR-4 during the morphogenesis of MCF10A cells. As shown in Fig. 1b and c, high levels of PAR-4 expression were displayed by the cells within the mammosphere, especially from day 5 through the process of cavitation that leads to lumen formation. qPCR analysis revealed that PAR-4 mRNA expression was up-regulated in a time-dependent fashion during the process of MCF10A morphogenesis. As shown in Fig. 2, PAR-4 was up-regulated at the transcriptional level during the acinar morphogenesis and reached the highest level on day 7. We also evaluated PAR-4 protein expression in the mammary epithelial cells MCF10A, MCF-7, and MDA-

Fig. 3 PAR-4 protein expression in different mammary epithelial cell lines. **a** PAR-4 protein expression was examined by Western blot analysis of protein extracts from the mammary luminal cells MCF10A and from the breast cancer cell lines MCF7 and MDA-MB-231

cultured in media supplemented with 10% serum. Protein expression was analyzed with the mouse monoclonal antibody anti-PHLDA1 (Santa Cruz) and the β -actin antibody (Chemicon) for normalization. **b** Graphic representation of the quantified results from the experiment

MB-231 (Fig. 3a and b). The breast cancer cells, MCF-7 and MDA-MB-231, showed appreciable levels of PAR-4 protein expression compared with the normal epithelial cell line MCF10A. The level of PAR-4 protein expression was significantly higher in MCF-7 cells compared with MDA-MB-231 cells. PAR-4 has been classified as a tumor suppressor gene with pro-apoptotic activity that has a function in both ligand-mediated extrinsic and mitochon-

drial mediated intrinsic apoptosis [15, 20]. In normal or immortalized cells, PAR-4 expression is not sufficient to induce apoptosis, but it increases cellular sensitivity to several apoptotic stimuli [21]. Loss of interaction between the cells and components of the extra-cellular matrix, leading to anoikis, is required for lumen formation [10]. Loss of cell adhesion and reduced availability of steroids and growth factors could be the factors that induce PAR-4

Fig. 4 Expression of PAR-4 and activated caspase 3 in MCF10A cells grown in three-dimensional (3D) cell culture. A to D, Representative confocal microscopic images of MCF10A cells cultured in growth factor reduced Matrigel for 3, 5, 7 and 10 days and examined for PAR-

4 (red) and activated caspase 3 (green) by immunofluorescence. Nuclei were contrastained with Hoestch (blue). Equatorial cross sections of the acini were obtained using a Zeiss LSM Meta 510 confocal microscope system (scale bar: 10 μ m)

expression, which subsequently marks and sensitizes cells for apoptosis. We can also speculate that hypoxia could lead to up-regulation of PAR-4 expression, which in consequence may increase the sensitivity of the cells inside the acini structure to cell death.

During the morphogenesis of MCF10A cells in 3D cell culture, the cells within the mammosphere showed apoptotic activity. Caspase 3 staining detected active luminal apoptosis during mammary epithelial cell morphogenesis. We found PAR-4 was only partially co-expressed with activated caspase-3 in these cells (Fig. 4). Interestingly, the morphogenesis of MCF10A can be altered by oncogene activation, such as ERBB2 over-expression, or by tumor suppressor gene inactivation such as BRCA1, that leads to increased proliferation and disruption of lumen formation [11]. Here we provide, for the first time, preliminary evidence that the tumor suppressor gene PAR-4 is differentially expressed during acinar morphogenesis of MCF10A cells in three-dimensional cell culture. Further in vitro and in vivo studies to investigate the role of PAR-4 in mammary gland morphogenesis and breast cancer development are warranted.

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