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Expression of Protein Kinase C α and the MZF-1 and Elk-1 Transcription Factors in Human Breast Cancer Cells

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Abstract

Recently, our research into hepatocellular carcinoma (HCC) has shown that the transcription factors Myeloid Zinc Finger-1 (MZF-1) and Ets-like-protein 1 (Elk-1) are related to protein kinase C alpha (PKCα) expression. The purpose of this study was to determine the correlation of the expression of PKCα with the expressions of Elk-1 and MZF-1 in various differentiated breast cancer cell lines: MDA-MB-231, Hs57BT, SKBR3, MDA-MB-468 and MCF-7. The malignant potential in the five lines of breast cancer cells was examined by using a cell proliferation/migration/invasion assay and the protein and mRNA levels of PKCα, Elk-1 and MZF-1 were examined by Western blot and RT-PCR analysis, respectively. The results showed that there were obvious signs of migration and invasion of cells in MDA-MB-231 and Hs57BT cells, little signs of cell migration and invasion in MDA-MB-468 cells, and no sign in SKBR3 and MCF-7 cells. Moreover, the highest expression levels of PKCα, Elk-1 and MZF-1 were also observed in MDA-MB-231 and Hs57BT cells when compared to the other breast cancer cell lines. These findings confirm that elevated expression of PKCα in breast cancer cells may be correlated with the potential of cell migration and invasion, and suggest an association between the expression of PKCα and the expression of the transcription factors Elk-1 and MZF-1.

Key Words: PKCα, Elk-1, MZF-1, human breast cancer cells

Introduction

Protein kinase C (PKC) is an important family of signaling molecules that regulate proliferation, differentiation, transformation and apoptosis in cells (26). The ten PKC isoforms are divided into conventional (cPKCs: α, β, βII, and γ), novel (nPKCs: δ, ε, η, and θ) and atypical (aPKCs: ξ and ι/λ) subclasses depending on their requirement for Ca^{2+}, phosphatidylserine and diacylglycerol (29). The α-isoform of

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protein kinase C (PKCα) is widely expressed in tissues regulating apoptosis, proliferation, differentiation, migration and adhesion (25). However, this isoform has been suggested to play an important role in tumorigenesis, invasion and metastasis (1, 12, 14, 16, 19, 22, 34, 35). In fact, overexpression of PKCα has been detected in tissue samples of prostate, breast, high grade urinary bladder and liver cancers by immunohistochemistry (7, 15, 17, 32, 33). Activation of PKCα has also been observed in breast cancer cells as well as in breast tumor samples (3, 27), while other researchers have found down-regulation of the PKCα protein in breast cancer (2, 13). Overexpression of PKCα is associated with decreased levels of the estrogen receptors in breast cancer cells (35, 38), and causes human breast cancer cells to show a more aggressive and metastatic phenotype, *i.e*. anchorage-independent growth in soft-agar and tumorgenicity in nude mice (31). Moreover, breast cancer patients with the $PKC\delta^+/PKC\alpha^-$ phenotype respond to anti-estrogen therapy six-times longer than patients with the $PKC\delta^+/PKC\alpha^+$ phenotype (4). PKCα, therefore, represents an interesting and challenging research target for the development of new therapeutic agents.

Recently, our data from research on cells of other organs had shown the expression of PKCα to be higher in the poorly-differentiated hepatocellular carcinoma (HCC) cell lines HA22T/VGH and SK-Hep-1 as compared with the well-differentiated ones, and the expression of PKCα was associated with the expressions of Ets-like-protein 1 (Elk-1) and Myeloid Zinc Finger-1 (MZF-1) transcription factors (9-11, 36, 37). The focus of this research is to investigate whether the correlation of PKCα with Elk-1 and MZF-1 is also found in breast cancer cells. Expression of PKCα in breast cancer cells with the potential of cell proliferation, migration and invasion associated with the expression of the transcription factors Elk-1 and MZF-1 is discussed in this study.

Materials and Methods

Materials

Anti-Elk-1, anti-MZF-1 and anti-β-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-labeled anti-mouse secondary antibody was purchased from Promega (Madison, WI, USA). The polyclonal PKCα antibody was obtained from the rabbits day 42 after immunization as described in previous study (32).

Cell Culture

Five breast cancer cell lines, MDA-MB-231, Hs57BT, SKBR3, MDA-MB-468 and MCF-7, were purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). These cell lines were cultured with their respective media (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 μg/ml streptomycin (Sigma Chemicals Co., St. Louis, MO, USA) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell Proliferation Assay

Cell proliferation was determined by the yellow tetrazolium MTT assay. The cells were seeded in 24-well plates at 1×10^4 cells/well and cultured in DMEM containing 10% FBS at 37°C overnight. The cells were treated with or without various plasmids and incubated for 24 h or 48 h. After incubation, the medium was replaced with fresh medium and the cells were incubated with 5 mg/ml MTT for 4 h before dissolving in 1 ml isopropanol for 10 min. The optical density at 570 nm was then measured using a spectrophotometer.

Migration Assay

Migration assay was performed using the 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) plated with 8-μm pore size polycarbonate membrane filters (Neuro Probe, Gaithersburg, MD, USA). The lower compartment was filled with DMEM containing 20% FBS. Cells at 2×10^5 cells/well in serum-free DMEM were placed in the upper part of the Boyden chamber and incubated for 12 h. After the incubation, the cells were fixed with methanol and stained with 0.05% Giemsa for 1 h. The cells on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed in distilled water until no additional stain leached. The cells were then air-dried for 20 min. The migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at 200× magnification.

Invasion Assay

Invasion assay was performed using a 48-well Boyden chamber with polycarbonate filters. The upper side was pre-coated with 10 μg/ml Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Cells at 2×10^5 cells/well in serum-free DMEM were placed in the upper part of the Boyden chamber and incubated at 37°C for 24 h. The rest of the experimental procedures were the same as that in the migration assay. Invasive phenotypes were

determined by counting the cells that invaded to the lower side of the filter with microscopy at 200× magnification.

Western Blot

The cultured cells were washed twice with PBS and then lysed with a lysing buffer containing 50 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40 and 0.3% sodium deoxycholate. The cell lysates were centrifuged at 12000 ×g and 4°C for 15 min. The supernatant was collected and the protein concentration was determined by the Bradford method. Equal amounts of protein extracts (50 µg) were subjected to 12.5% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA, USA). After blocking, the membrane was incubated with the specific anti-PKCα antibody (1:500), anti-Elk-1 (1:500), anti-MZF-1 (1:500) or β-actin antibody (1:2000). The blots were then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:3000) at room temperature for 2 h. Proteins were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from cell specimens by the guanidinium thiocyanate-phenol method. The RNA integrity was assessed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The total amount of RNA was determined spectrophotometrically. RT-PCR assay was performed according to De Petro *et al*. (6) with slight modifications. An aliquot of total RNA (1 µg) was reverse transcribed. The RT product (2 µl) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris-HCl, and 2 mM $MgCl_2$) to a final volume of 50 µl, containing 0.5 µM dNTPs (final concentration, 0.8 mM) and 0.5 unit of Super-Therm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers used in RT-PCR were as described previously (11). The PCR products were analyzed by 1.5% agarose gel electrophoresis and direct visualization after SYBR Green I (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging System (Eastman Kodak Company, New Haven, CT, USA).

Statistical Analysis

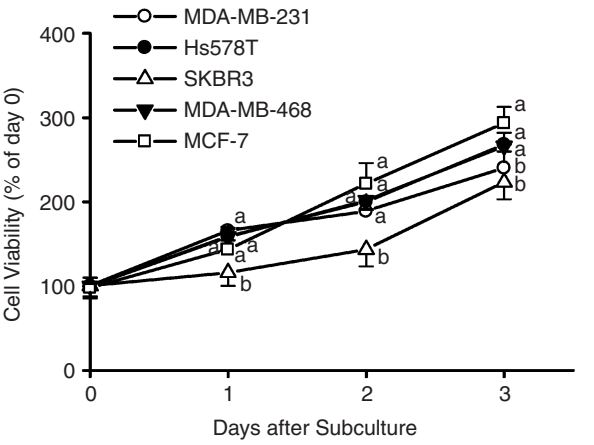

Fig. 1. Cell growth in five breast cancer cell lines. Cell growth was determined 1-4 days after subculture using the MTT assay as described in Materials and Methods. Absorbance values obtained from untreated cells on day 0 after subculture were taken as 100%. The data are presented as means ± SE of three replicates from three independent experiments. Means at the same day after subculture not sharing the same superscripts differ significantly ($P < 0.05$).

Data were expressed as means ± SEM and analyzed using analysis of variance (ANOVA). Student's *t*-test was used in two-group comparisons. The association between the various factors was determined using the Pearson correlation. $P < 0.05$ was considered to be statistically significant.

Results

Cell Proliferation, Migration and Invasion

The malignant potential in the five breast cancer cell lines was examined by using a cell proliferation/migration/invasion assay. As shown in Fig. 1, the highest proliferation rate was exhibited by the MCF-7 cell line at a doubling time of 38.5 h, and the slowest proliferation rate was exhibited by the SKBR3 cell line at a doubling time of 65.1 h.

The results also show that the potentials of cell migration and invasion in MDA-MB-231 and Hs57BT cells were highest amongst the cell lines tested (Figs. 2 and 3). Furthermore, MDA-MB-468 cells showed little sign of migration and invasion, while SKBR3 and MCF-7 showed even less migration and invasion.

Expressions of PKCα, Elk-1 and MZF-1

The protein levels of PKCα, Elk-1 and MZF-1 were examined by Western blot analysis. In the results, the PKCα expressions were significantly higher in MDA-MB-231 and Hs57BT cells than in the other

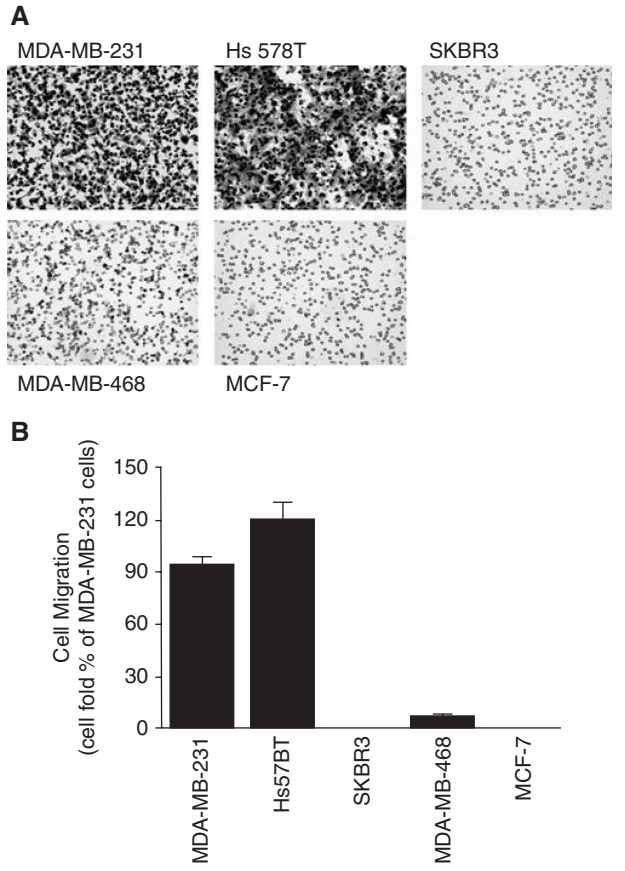

Fig. 2. Cell migration (A) and statistical analysis (B) of cell migration in five breast cancer cell lines. Migration assays were performed on cell cultures as described in Materials and Methods. The data are presented as means ± SE of three replicates from three independent experiments.

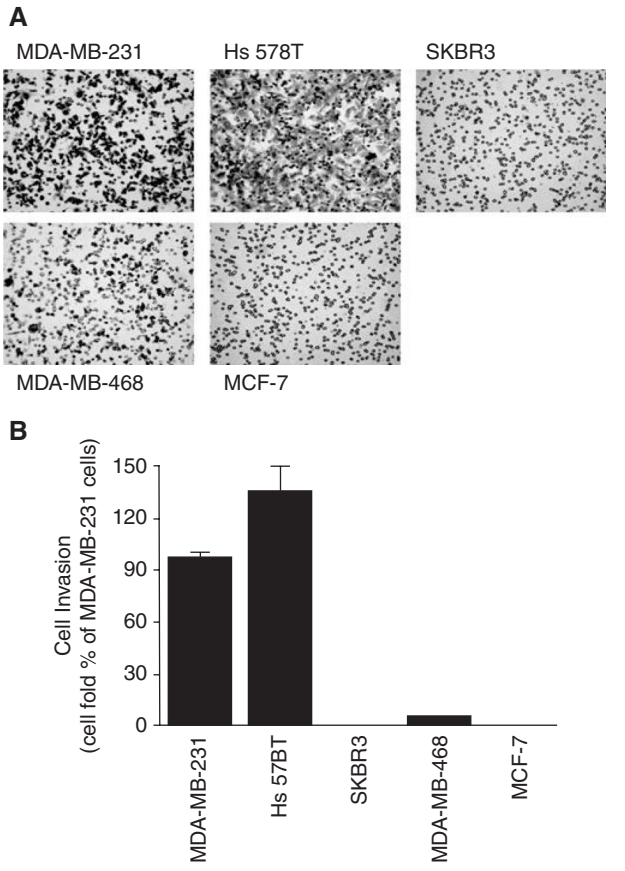

Fig. 3. Cell invasion (A) and statistical analysis (B) in five breast cancer cell lines. The invasion assays were performed on cell cultures as described in Materials and Methods. The data are presented as means ± SE of three replicates from three independent experiments.

three cell lines while PKCα expression in MDA-MB-468 and MCF-7 cells was not detected (Fig. 4). In RT-PCR assays, the mRNA expressions level of PKCα in MDA-MB-231 and Hs57BT cells was also significantly higher than those in the other three cell lines (Fig. 5). Statistical analysis of the above findings indicates that the expression of PKCα in breast cancer cell lines was significantly correlated with the potential of cell migration ($r = 99$, $P < 0.05$) and invasion ($r = 0.99$, $P < 0.05$), but not with the rate of cell proliferation.

The expression levels of Elk-1 and MZF-1 were also highest in MDA-MB-231 and Hs57BT cells (Fig. 4). In RT-PCR assays, the mRNA levels of Elk-1 and MZF-1 were also highest in MDA-MB-231 and Hs57BT cells, similar to the results of PKCα (Fig. 5). These results indicate that high level of expression of the PKCα gene has significant correlation with the expression of the Elk-1 and MZF-1 genes in human breast cancer cells ($r = 0.98$, $P < 0.05$ and $r = 0.74$, $P < 0.05$, respectively).

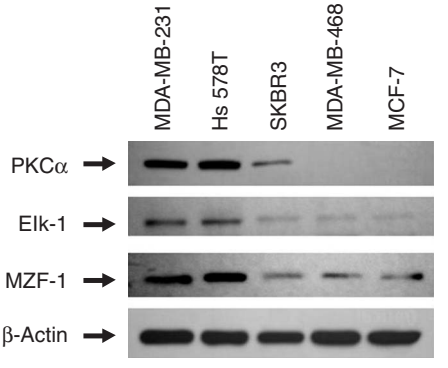

Fig. 4. Expressions of PKCα and two transcription factors in five breast cancer cell lines. The protein levels of PKCα, Elk-1, and MZF-1 were detected by Western blotting. β-actin was used as an internal control.

Discussion

In this study, we found that PKCα expression, cell migration and cell invasion were significantly

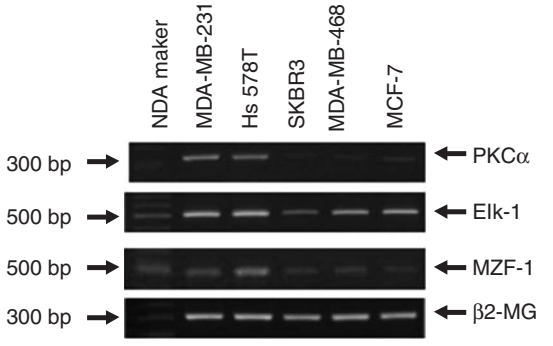

Fig. 5. The mRNA levels of PKCα, Elk-1 and MZF-1 in five breast cancer cell lines detected by RT-PCR. β2-MG was used as an internal control.

higher in MDA-MB-231 and Hs 578T cells than in SKBR3, MDA-MB-468 and MCF-7 cells. These results indicate that the elevated expression of PKCα in MDA-MB-231 and Hs 578T cells may be correlated with the potential of cell migration and invasion. These phenomena are similar to a previous report done on human hepatocellular carcinoma cells which also showed increased PKCα expression and increased cell migration and invasion in the poorly-differentiated HA22T/VGH and SK-Hep-1 cells (11, 36). Moreover, many papers on experiments of the same nature have been published which show that PKCα expression is more pronounced in MDA-MB-231 cells when compared to MCF-7 and SKBR3 cells (18) and that no PKCα is present in MDA-MB-468 cells (23). These results confirm that PKCα level is higher in MDA-MB-231 cells than that in the other breast cancer cell lines (20, 21).

The significance of PKC expression and activity in breast cancers had been demonstrated in a previous study to be higher than that in normal breast tissues (8, 28). It was also shown that an increase in the secretion level of urokinase plasminogen activator (uPA) in MDA-MB-231 cells correlated with cell migration and invasion (5), and can be induced by PKCα activation (24, 30) through the AP-1 and NFκB signal pathways resulting in the cells being estrogen receptor-negative, highly invasive and chemotherapy-resistant. Moreover, when the PKCα gene was transfected to nonmetastatic MCF-7 cells, a more aggressive neoplastic phenotype was produced, and an increase in uPA expression was observed (31, 35). The results of this study revealed that PKCα expression was correlated with cell migration and invasion in breast cancer and was in agreement with the studies mentioned above.

Our previous study showed that Elk-1 and MZF-1 expression was increased in poorly-differentiated HCC cells and demonstrated that these transcription factors were able to regulate PKCα expression (11). Similarly, the present data also shows that the expression of PKCα may be associated with the expression of the transcription factors Elk-1 and MZF-1, as both protein and mRNA expression levels of PKCα, Elk-1 and MZF-1 in MDA-MB-231 and Hs57BT cells were significantly higher than those in the other three cell lines (Figs. 4 and 5). These observations were confirmed by real-time RT-PCR analysis (data dot shown). Moreover, to confirm the connection between PKCα and Elk-1 and MZF-1, a shRNA knockdown assay was performed and the data showed that the reduction of Elk-1 or MZF-1 expression in the MDA-MB-231 cell line decreased the expression of PKCα (data not shown). These results indicate a significant relationship between the expression of the two transcription factors and PKCα in MDA-MB-231 cells and that high expression of PKCα gene in human breast cancer cells may be correlated with the Elk-1 and MZF-1 genes.

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