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Protein kinases C isozymes are differentially expressed in human breast carcinomas

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Abstract

Aims—The protein kinase C (PKC) family of enzymes has been implicated in cellular proliferation, differentiation, and apoptosis. However, the distribution of specific PKC isoforms with varying functions in normal and malignant human tissues remains to be determined. The objective of this study was to investigate the expression of certain PKC isoforms (, I, II,) in human breast cancer specimens relative to adjacent uninvolved tissue ($n = 24$) and in the normal breast tissue obtained from patients undergoing reduction mammoplasty ($n = 12$).

Main methods—Western blot analysis using PKC isoform specific antibodies was performed on tissue extracts from breast tumors, adjacent uninvolved tissues, and reduction mammoplasty tissues.

Key findings—Mean levels of cytosolic and membrane PKC- , PKC- I, and PKC- II were significantly higher in the cancer specimens than in the adjacent uninvolved breast tissues (Wilcoxon signed-ranks test; $P < 0.05$ for each, after adjustment for multiple comparisons). There was a notably higher mean level of membrane PKC- II isozyme in Her-2 positive and in poorly differentiated tumors. No significant differences were observed when normal tissue adjacent to tumor was compared to breast tissue obtained from reduction mammoplasty specimens.

Significance—Higher level of PKC- , PKC- I, and PKC- II in cancer specimens and higher level of PKC- II in Her-2 positive tumors require further exploration of the intracellular pathways involving PKC- and - isoforms in breast cancer because both could be specific targets for the development of new therapies and for the prevention and treatment of this disease.

Keywords

Protein kinase C; Breast cancer; Her-2/neu

Introduction

Breast cancer is the most frequently diagnosed malignancy in women, accounting for 26% of all new cancer cases in women in the USA (Jemal et al. 2008). It is estimated that 182,460 new cases of breast cancer will occur in 2008. Despite the decline in the mortality of this disease more than 40,000 deaths due to breast cancer are expected in 2008.

New strategies to develop potent, yet tolerable systemic therapies, for breast cancer are dependent on the improvements in our understanding of breast carcinogenesis and the mechanisms of resistance of cancer cells to available therapies. Much of the fundamental cancer research over the past several decades focused on identifying the molecular changes that underlie malignant transformation. These abnormalities may be potential targets for the development of drugs that will selectively prevent and treat breast cancer.

Abnormalities of cell signaling pathways are frequently associated with malignant transformation. Pathways that induce cell proliferation, promote invasion, or evade apoptosis are rational targets for the development of new therapies against breast cancer. Protein kinase C (PKC) belongs to the family of serine/threonine kinases that play a key role in the signal transduction of various cellular processes, such as cell survival, proliferation, and apoptosis (Deacon et al. 1997; Mackay and Twelves 2003). PKCs can be classified into three groups of isozymes, based on cofactor requirements for their catalytic activities (Blobe et al. 1994; Ron and Kazanietz 1999). The first group, named “classic” PKCs (PKC- α , β , γ , δ , ϵ , ζ , η , θ , ι , κ , λ , μ , ν , ξ , $\o$, and τ), require both phorbol ester/diacylglycerol (DAG) and calcium for activation. The second group, the “novel” PKCs (PKC- β , δ , ϵ , ζ , and η), only require DAG for activation. The third group or atypical PKCs (PKC- λ and τ) are both calcium and DAG independent (Dekker and Parker 1994; Jaken 1996). The PKC isozymes are subject to different biochemical regulation, intracellular localization, and tissue distribution. In most cases, at least five or more PKC isozymes are expressed in a single cell with overlapping or even opposite functions (Mischak et al. 1993). Consequently, the constellation of PKC isozymes in tissues may determine the overall biologic effect of PKC modulation under various pathophysiological states and during drug treatment (Kazanietz 2000).

PKC isozymes are potential targets for new drug development for cancer because of their role in carcinogenesis (Philip and Zonder 1999). A number of studies have reported that the total PKC catalytic activity was increased in carcinomas of the breast and other common human cancers compared to their normal tissue counterpart (El-Rayes et al. 2008; Sauma et al. 1996). PKC is also one of the targets of tamoxifen that is widely used in the prevention and treatment of hormone-responsive breast cancers (Dem et al. 2004). In addition, increased metastatic capacity of breast cancer cells with elevated PKC levels in part participate in the modulation of integrin expression, was also reported in MCF-7 cells (Yamamoto et al. 1999). Taken together, these findings confirmed that PKC mediated transduction pathways are, at least, partly involved in breast pathogenesis and tumor progression. Nevertheless, the contribution of each PKC isoform to the overall altered activity remains poorly understood. In general, studies addressing the specific roles of PKC isozymes have been undertaken using human cancer cell lines. For example, in the MCF-7 breast cancer cell line, high glucose-induced PKC- II down-regulation, and was associated with the acceleration of cell cycle progression (Yamamoto et al. 1999).

Signaling through the steroid hormone receptors plays important roles in breast carcinogenesis. Approximately two-thirds of all breast cancers express the estrogen receptor (ER) and/or the progesterone receptor (PR). The expression of either of these receptors by the tumor cells influences the biological behavior and response to therapy, especially to endocrine manipulation (Gruvberger et al. 2001; Osborne 1998). In human breast cancer cell lines, an inverse relationship has been demonstrated between total PKC activity and expression of the ER (Stranzl et al. 1997).

In recent years, growth factor pathways have attracted significant interest as targets for therapy. Over-expression of the Her-2/neu protein is seen in 20–30% of human breast cancers and is associated with increased proliferation and worse prognosis (Benz et al. 1992; Gusterson et al. 1992; Paik et al. 1990). The relationship between Her-2/neu receptor

signaling and PKC activity remains poorly understood. In one study over-expression of the constitutively active truncated form of Her-2/neu was up regulated by PKC in breast cancer cells over-expressing this receptor (Esparis-Ogando et al. 1999). PKC has also been implicated in the regulation of the cellular response to the growth factor heregulin in human breast cancer cells (Le et al. 2001; Yao et al. 2001). These results collectively provide evidence in support of the pathogenic role of PKC in many cancers including breast cancer; however the expression of PKCs in human breast tumors compared to normal breast tissues have not been reported. Therefore, in the present study, we determined the level of expression of PKC isozymes (, I, II and) in human breast carcinomas compared to adjacent benign breast tissues as well as normal breast specimens obtained from reduction mammoplasty. Moreover, we also assessed the relationship between the expression of PKC isozymes and the expression of the ER and Her-2/neu proteins in this study.

Materials and methods

Tissue collection

Biopsy material comprising paired tumor and adjacent normal breast tissue from twenty-four patients was analyzed. All patients had a histological diagnosis of breast adenocarcinoma and had undergone a lumpectomy or a modified radical mastectomy. Tissue specimens were labeled and immediately frozen at -80°C pending analysis. Morphologically normal breast tissues furthest away from the tumor were stored separately under similar conditions. In addition, breast tissue was also obtained from 12 women without history of breast cancer, at the time of a reduction mammoplasty. Sample collection and processing were performed according to the regulations of the Human Investigation Committee at Wayne State University.

Western blot analysis of PKC isoforms

Tissue samples were homogenized using a Dounce homogenizer in 500 μl buffer containing (20 mM Tris pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 10 $\mu\text{g/ml}$ leupeptine, 10 $\mu\text{g/ml}$ Aprotine, 0.2 mM PMSF, 1 mM Na_3VO_4 , 5 mM DTT). Homogenization was performed twice with 30 s pulses each. This was followed by sonication twice for 30 s on ice and centrifugation at 7000 rpm for 15 min. The supernatant was transferred into a microtube and centrifuged at 39,000 g for 30 min. After transferring the upper cytosolic layer into a microtube, 50–100 μl of buffer was added to the nuclear membrane pellet before sonication for 30 s. Protein concentration was determined by the BCA protein assay with BSA as a standard. Protein extracts (20 μg) were resolved using 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes at 350 mA for 90 min. The membranes were blocked with 5% nonfat dry milk, washed, and incubated with the following primary antibodies: PKC- , PKC- I, PKC- II, and PKC- (Santa Cruz Biotechnology, Santa Cruz, CA), Horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma, Chemical Co.) was used to detect the bound primary antibody. The probed proteins were detected using the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham, IL). The expression of PKC isozymes was quantified and analyzed against the epithelial marker, cytokeratin 19 in each sample arrayed. The bands were quantified using AlphaEaseFC software tool (Alpha Innotech, San Leandro, CA).

Immunohistochemistry for the detection of estrogen receptor and Her-2 protein

Immunohistochemical staining was performed on fixed tissue using the avidin–biotin–complex procedure. All primary antibodies were obtained from DakoCorp. (Carpenteria, CA). Biotinylated secondary antibody and ABC (Vector Lab., Burlingame, CA) were applied for approximately 10 min each. After development with 3-amino-9-ethylcarbazole, sections were counterstained with hematoxylin. The specimen was considered positive if

>2% of the cells were positively stained for the antibody. Immunohistochemical staining for the Her-2 protein was graded 0 to 3. Only 3+ staining was considered positive for statistical purposes.

Statistical analysis

PKC isozyme levels from breast tumor tissue versus adjacent normal breast tissue (i.e., paired data) were compared using the Wilcoxon signed-ranks test (Sprent and Smeeton 1993). Enzyme levels from normal tissue of breast cancer patients (i.e., normal breast tissue adjacent to the tumor) versus normal tissue from reduction mammoplasty patients (two independent groups) were compared using the Wilcoxon rank sum test (Sprent and Smeeton 1993). Exact methods (via resampling statistics) were used for all comparison testing in light of the modest sample sizes. Assay of 4 PKC isozymes from both cytosol and membrane fractions yielded 8 variables. The Holm multiple comparisons procedure (Holm 1979) was used to control the overall Type I error rate at 0.05 across each set of 8 isozymes. The isozyme levels of the breast cancer patients were examined across the levels of three pathological variables (ER status, Her-2/neu status, and tumor grade) using descriptive statistics only.

Results

Expression of PKC isozymes in breast tumors and adjacent normal tissue

Fig. 1 demonstrates a representative immunoblot for the expression of PKC isozymes in tumor and normal tissues. There were significant inter-individual differences in the expression of PKC isozymes amongst the 24 matched normal/tumor pairs analyzed by Western blot. The mean expression of PKC- α , PKC- β , and PKC- γ was significantly higher in tumor tissue compared to adjacent normal tissue ($p < 0.05$ for each of the paired comparisons, after adjustment via the Holm method) (Fig. 2). In contrast, the mean expression of PKC- δ in both cytosolic and membrane fractions of tumor and adjacent normal tissue was very similar.

Expression of PKC isozymes in non-malignant breast tissues

According to the field cancerization hypothesis, specific aberrations may be present in breast cancers and in the surrounding normal appearing adjacent tissue (Slaughter et al. 1953). We therefore studied the differences in the expression of PKC isozymes in morphologically normal tissue adjacent to breast tumors ($n = 24$) as compared to normal breast tissue from women who underwent reduction mammoplasty ($n = 12$) (Fig. 3). Mean cytosolic PKC-expression was markedly higher in normal tissue adjacent to tumors, but not significantly so, after adjustment for the 8 comparisons involved. The levels of the PKC- α , PKC- β , and PKC- γ isozymes did not differ significantly by the origin of the normal tissue whether in the cytosolic or membrane fractions (Fig. 4).

Expression of PKC isoforms in relation to the tumoral expression of ER, Her-2, and the histologic grade of the tumor

The expression of PKC isozymes in breast tumor tissue was examined with respect to grade, estrogen receptor status, and Her-2 expression levels (Fig. 5). The majority of tumors were poorly differentiated (70%), the rest were either moderately differentiated (17%), or well differentiated adenocarcinomas (13%). Estrogen receptor positivity was observed in 10 patients. Only, 3 patients (13%) were Her-2/neu positive. ER positive tumors had (numerically) lower mean levels of cytosolic and membrane PKC- α , β , γ (Fig. 5A). In ER positive tumors, the mean expression of PKC- δ was lower in cytosol, but higher in the membrane fraction. When Her-2 status and the degree of differentiation were considered,

there were no consistent differences in PKC isozyme levels. However, there was a notably higher mean level of membrane PKC- II isozyme in Her-2 positive (Fig. 5B) and poorly differentiated tumors (Fig. 5C).

Discussion

In recent years there has been a significant interest in the cell signaling abnormalities that are associated with breast carcinogenesis and resistance to therapy. This has been further stimulated by the increasing availability of drugs that may specifically target growth factor receptors and downstream molecules in the activated pathways. Experimental evidence points to a significant role of PKC in the malignant progression of breast cancers and their response to apoptotic stimuli (Sliva et al. 2002; Williams and Noti 2001). The pathways in human cancers that are regulated by the various members of the PKC family of kinases have not been fully determined.

Several studies have addressed the role of specific PKC isozymes in carcinogenesis but these studies have been largely undertaken in experimental systems with very little information from human studies. The current study demonstrated that detectable levels of particular PKC isozymes were differentially expressed in breast carcinomas and the nonmalignant adjacent breast tissue. Of particular interest was the significant increase in PKC- α , I, and II expression in breast cancer specimens. The levels of PKC- α were similar in malignant and normal breast tissue. Given this varied expression of these isozymes between malignant and normal tissue, it would be of much interest to determine the significance of such alterations in the progression of breast cancer and as potential targets for prevention and/or treatment.

A large body of evidence indicates that PKC- α is associated with growth regulation and cell cycle arrest in various cell lines. In vitro data demonstrated that induction of PKC- α over-expression induced a less aggressive biological behavior, which was characterized by reduced in vitro invasiveness and markedly diminished tumor formation and growth in nude mice in one study (Ways et al. 1995). Induction of this isozyme is also crucial for the anti-proliferative action of retinoids on human breast carcinoma cells (Cho et al. 1997). However, another study demonstrated that PKC- α over-expression induced a more aggressive phenotype characterized by enhanced proliferative rate, anchorage-independent growth, and increased tumorigenicity in nude mice (Ways et al. 1995). In support of the latter finding, studies have shown the role of PKC- α in the development of invasive phenotype of MCF-7, glioma-derived, and human renal cell carcinoma cell lines (Engers et al. 2000; Manni et al. 1996; Morse-Gaudio et al. 1998). In both studies, MCF-7-PKC- α cells exhibited a significant reduction in ER expression. The aforementioned contrasting results could be attributed to altered levels of various isozymes in their systems in response to transfection. Similarly, ER negative breast cancer cell lines vary in their endogenous total PKC activity and specifically PKC- α expression (Morse-Gaudio et al. 1998). This underscores the need to evaluate their expression in human tissue and compare it to the existing models.

PKC- II is another potential player in the shift to hormone independent status and the development of a more aggressive phenotype. MCF-7-PKC- β transfected cells exhibited a substantial increase in endogenous PKC- β expression concomitant with the acquisition of more aggressive phenotype (Ways et al. 1995) and the reduction in ER expression (Manni et al. 1996). Consistent with these findings, PKC- II expression was lower in ER positive tumors and higher in Her-2 positive, poorly differentiated tumors. Our PKC- II associations with the different tumor parameters were based on small sample sizes, but could be hypothesis-generating for further testing. Murray et al (Murray et al. 1993) and Sauma et al

(Sauma et al. 1996) have demonstrated that PKC- II promotes cellular proliferation in human leukemia cells and colon cancer cell lines, respectively. Others have shown a positive effect of PKC- I on the growth and proliferation of neuroblastoma cells (Svensson et al. 2000), in addition to its role in anchorage independence and development of metastases (Borner et al. 1995). In summary, this study has provided preliminary evidence demonstrating the expression status of PKC isozymes in breast cancer. Based on the results of this pilot study, future studies could be focused on determining the mechanisms that regulate the expression of these PKC isozymes in breast tissues. The current availability of compounds that interfere with specific PKC isozymes will also help to develop novel therapies to target individual PKC isozymes for the prevention and/or treatment of human breast cancer.

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Fig. 1.

A representative immunoblot of PKC isozyme expression in paired samples of breast tumor (T) and morphologically normal adjacent (NA) mammary tissue. Samples are from three representative patients out of a total of 24 patients examined. Twenty μ g of proteins were separated on a 10% polyacrylamide gel. After transfer to nitrocellulose membrane, the blots were probed with the specific antihuman PKC isozyme antibody followed by standard Western blot detection system, and the protein expression was quantified against cytokeratin using AlphaEaseFC software tool.

Fig. 2. Quantification of PKC isozyme expression in 24 paired tumor (T) and normal adjacent (NA) tissue samples in the cytosol (A) and membrane (B) cellular fractions by AlphaEaseFC software tool. Data are expressed as mean $\pm$ standard error. * $P < 0.05$, after Holm multiple comparisons adjustment.

Fig. 3.

A representative immunoblot of PKC isozyme expression in normal (N) breast tissue ($n = 12$) removed during mammoplasty and normal tissue sample adjacent to tumors (NA). Samples are from three representative subjects out of a total of 12 subjects examined. Twenty micrograms of proteins were separated on a 10% polyacrylamide gel. After transfer to nitrocellulose membrane, the blots were probed with the specific antihuman PKC isozyme antibody followed by standard Western blot detection system, and the protein expression was quantified against cytokeratin using AlphaEaseFC software tool.

Fig. 4. Quantification of PKC isozyme expression in normal (N) breast tissue samples ($n = 12$) removed during mammoplasty and normal tissue samples adjacent (NA) to tumors ($n = 24$) in the cytosol (A) and membrane (B) cellular fractions. Data are expressed as mean $\pm$ standard error. * $P < 0.05$, after Holm multiple comparisons adjustment.

Fig. 5. Quantification of PKC isozyme expression in 24 breast tumor tissue samples according to the tumor estrogen receptor status (A), Her-2/neu receptor status (B), and degree of differentiation of the tumor (C). Tumor grade categories are coded as: 1 = well differentiated; 2 = moderately differentiated; 3 = poorly differentiated. Data are expressed as mean $\pm$ standard error.