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Elevated Protein Kinase C Expression in Human Breast Tumor Biopsies Relative to Normal Breast Tissue[1]

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ABSTRACT

The Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC), is a critical enzyme in the regulation of cell growth. In this report, we demonstrate elevated expression of PKC activity in surgical specimens of eight of nine spontaneous human breast tumors, as compared with the expression of PKC activity in normal breast tissue obtained from the same patients. The mean PKC specific activity in histologically normal breast tissue was 166 ± 63 pmol ^{32}P/min/mg, whereas the mean PKC specific activity in the breast tumors was 460 ± 182 pmol ^{32}P/min/mg ($P = 0.0003$; Student's t test). The low interpatient variability among the PKC levels observed in the histologically normal breast tissue specimens and the significant elevation of PKC levels observed in the tumors indicate that elevated expression of PKC activity in breast tissue is a potential marker for malignant disease in the breast.

INTRODUCTION

PKC,[3] the Ca^{2+}- and phospholipid-dependent protein kinase, represents a family of closely related isozymes (1–4) that play a critical role in cellular growth regulation, according to three major lines of evidence. PKC is specifically activated by phorbol ester and related tumor promoters (5, 6), PKC transduces mitogenic signals of certain growth factors (7, 8), and the specific introduction of PKC-encoding cDNAs into cultured fibroblasts results in an overexpression of PKC activity and a dramatic loss of growth control mechanisms (9, 10).

Studies with cultured human breast cancer cells provide evidence that PKC expression is necessary for their growth. The prolonged exposure of mammalian cells to the tumor promoter TPA generally causes a down-regulation of PKC (11). In several human breast cancer cell lines including MCF-7, down-regulation of PKC by TPA is associated with an inhibition of growth. The removal of TPA from the cultured breast cancer cells results in an up-regulation of PKC that correlates with a resumption of cell growth (12, 13). Furthermore, we demonstrated previously that tamoxifen, a synthetic antiestrogen which antagonizes human breast cancer *in vivo*, is a PKC inhibitor (14, 15). We also showed that the estrogen-irreversible cytotoxic effects of tamoxifen and its metabolites against the human breast cancer cell line MCF-7 correlate with their inhibitory potencies against isolated PKC but not with their affinities for the estrogen receptor or the antiestrogen receptor (16). Taken together, these data strongly suggest a critical role for PKC in human breast carcinogenesis.

In this report, we show an elevated expression of PKC activity in surgical specimens of nine human breast tumors, as compared with the expression of PKC activity in normal breast tissue obtained from the same patients. Our results show that PKC expression is a potential marker for human breast cancer.

MATERIALS AND METHODS

ATP, bovine serum albumin, histone III-S, phenylmethylsulfonyl fluoride, Tris-HCl, 2-mercaptoethanol, DEAE-Sephacel, Triton X-100, leupeptin, soybean trypsin inhibitor type I-S, and phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Corp. (Arlington Heights, IL). Whatman phosphocellulose paper, grade p81, was from Fisher Scientific (Houston, TX). Surgical specimens of human breast tumors and normal human breast tissue were obtained from mastectomies in the operating rooms of the University of Texas M. D. Anderson Cancer Center and were stored at −70°C for no longer than 1 month prior to measurements of PKC activity.

PKC activity was assayed as previously described (17). All procedures were done at 4°C. Specimens of human breast tissue were homogenized by 75 strokes with a Dounce homogenizer at a ratio of 1 g tissue/10 ml homogenization buffer. The homogenization buffer contained 20 mM Tris-HCl, 5 mM EDTA, 5 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor type I-S, 15 mM 2-mercaptoethanol, and 0.1% Triton X-100 at pH 7.5. A 30-min exposure to 0.1% Triton X-100 at 0°C has been demonstrated to extract PKC activity from tissue suspensions of rat brain, liver, and kidney (18). Therefore, homogenates were stirred for 1 h and then centrifuged at 13,800 × g for 15 min. Supernatants (5–10 ml) were applied to 0.5-ml DEAE-Sephacel columns equilibrated in homogenization buffer minus Triton X-100. Columns were washed with 5 ml equilibration buffer, and PKC activity was eluted with 2 ml equilibration buffer containing 0.2 M NaCl. Protein concentrations of the PKC-containing eluants were determined with the Bio-Rad protein assay solution (Richmond, CA). PKC assay mixtures contained 20 mM Tris-HCl (pH 7.5), 15 mM 2-mercaptoethanol, 1 mM $CaCl_2$, 30 μg/ml phosphatidylserine (or none), 10 mM $MgCl_2$, 70 μg/ml histone III-S, 70 μM [γ-^{32}P]ATP, and about 5 μg tissue extract protein. The concentration of Triton X-100 in our PKC reaction mixtures (<0.01%, v/v) has no inhibitory effect on PKC activity (19). Reactions were initiated with [γ-^{32}P]ATP and proceeded for 10 min at 30°C. Reactions were terminated on phosphocellulose paper, and the incorporation of ^{32}P into histone was measured as previously described (17). PKC activity was calculated by subtracting the phosphotransferase activity observed in the presence of 1 mM Ca^{2+} from the activity observed in the presence of 1 mM Ca^{2+} plus 30 μg/ml phosphatidylserine. Each value represents the mean of triplicate determinations. P values were determined using the Student t test to compare PKC specific activities in normal and tumor tissues of each patient.

Estrogen receptor (20) and progesterone receptor (21) measurements were done by methods using sucrose density gradients and dextran-coated charcoal, respectively.

RESULTS

We measured the expression of PKC activity in surgical specimens of nine spontaneous human breast tumors and in specimens of normal breast tissue obtained from the same patients. Clinical characteristics of the patients with breast cancer are summarized in Table 1. The mean PKC specific activity in histologically normal breast tissue of the nine pa-

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[3] The abbreviations used are: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ER^+, estrogen receptor-positive; ER^-, estrogen receptor-negative.

Table 1 *Patient clinical characteristics*

Patient no.	Age	Histology	Tumor grade[a]	Estrogen receptor[b]	Progesterone receptor[b]
1	35	Cystosarcoma phylloides	III	Not done	Not done
2	27	Invasive ductal carcinoma	I	Negative	Negative
3	49	Invasive ductal carcinoma	I	Negative	Negative
4	48	Invasive ductal carcinoma	I	Negative	Negative
5	75	Intraductal comedo carcinoma	I	Positive	Negative
6	71	Invasive ductal carcinoma	II	Positive	Positive
7	54	Tubular carcinoma	III	Positive	Positive
8	32	Invasive lobular and ductal carcinoma	II	Positive	Positive
9	62	Invasive ductal carcinoma	I	Negative	Negative

[a] Grade I is least differentiated; grade II, intermediate; and grade III, most differentiated (24).
[b] A level of 10 fmol receptor/mg or greater is considered clinically positive for both estrogen receptor and progesterone receptor and was used to divide patients into positive and negative groups in subsequent analyses.

tients was 166 ± 63 pmol ^{32}P/min/mg (Table 2). The mean PKC specific activity in the nine corresponding tumor specimens was 460 ± 182 pmol ^{32}P/min/mg (Table 2). The paired difference in PKC specific activities in normal *versus* tumor tissue of the nine patients was 294 ± 48 (Student's t test = 6.16; degrees of freedom = 8; $P = 0.0003$). Thus, expression of PKC activity was significantly elevated in the breast tumors, as compared to PKC expression in normal breast tissue. When a statistical analysis of the expression of PKC activity in tumor *versus* normal breast tissue was done for each patient, significant overexpression of PKC activity was observed in the tumor tissue of eight of nine patients, as indicated by the P values in Table 2. We measured the expression of estrogen receptors and progesterone receptors in the various tumors (Table 1) and found that both ER$^+$ and ER$^-$ tumors expressed elevated levels of PKC activity relative to normal breast tissue (Tables 1 and 2).

We also examined PKC expression in the breast tissue of a 44-year-old woman with a family history of breast cancer. Her mother had bilateral breast cancer diagnosed at ages 42 and 46; she died at age 47 from disseminated disease. Her older sister had breast cancer diagnosed at age 24 and then, in the contralateral breast, at age 28. Her younger sister had breast cancer at age 46. The patient underwent a bilateral prophylactic mastectomy after a breast biopsy showed atypical hyperplasia. Histological examination revealed both ductal and lobular hyperplasia with atypia. The PKC specific activity in the tissue from the mastectomy specimen was 346 ± 63 pmol ^{32}P/min/mg. Thus, an increasing gradient of PKC activity is observed when comparing the histologically normal tissue (166 ± 63 pmol ^{32}P/min/mg) to the tissue with atypical hyperplasia (346 pmol ^{32}P/min/mg) to the malignant tissue (460 ± 182 pmol ^{32}P/min/mg).

DISCUSSION

In this report, we demonstrate that expression of PKC activity in surgical specimens of human breast tumors is consistently elevated, as compared to PKC expression in specimens of normal breast tissue obtained from the same patients. All normal breast tissue specimens had similar levels of PKC activity (mean = 166 ± 63 pmol ^{32}P/min/mg). The fact that little interpatient variability was observed among the PKC levels observed in the normal tissue specimens suggests that the elevated PKC expression observed in the tumors may be useful as a clinical marker for breast cancer. While we cannot rule out the possibility that endogenous PKC inhibitors and/or phosphatases contribute to the differences that we observe between PKC activity levels in normal breast tissue and breast tumors, it is clear that the net level of PKC activity is elevated in the tumor tissue relative to the normal tissue.

PKC expression has been studied extensively in human breast cancer cell lines. Several ER$^-$ human breast cancer cell lines express higher levels of PKC than ER$^+$ human breast cancer cell lines, indicating a negative correlation between PKC and estrogen receptor expression in the cells (22). A similar correlation between phorbol ester receptor and estrogen receptor expression has been observed in a comparison of ER$^-$ and ER$^+$ human breast tumors (23). Studies with cultured human breast cancer cells also provide evidence that PKC expression is necessary for their growth, since the down-regulation of PKC by TPA in cultured human breast cancer cells is associated with an inhibition of growth and the removal of TPA from the cells results in an up-regulation of PKC and a resumption of cell growth (12, 13).

PKC is composed of a family of at least seven isozymes, which differ in their tissue distributions (1–4). We are currently identifying the PKC isozymes expressed in human breast tumors, in order to develop a PKC assay based on immunoreactivity. The development of monoclonal antibodies against human breast tumor PKC isozymes may facilitate the use of PKC expression as a marker in human breast cancer. Such a marker could be particularly sensitive if the PKC isozymes expressed in human breast tumors differ from the isozymes expressed in normal breast tissue. In addition, the elevated expression of PKC activity which we observed in the breast tissue of a patient at high risk for breast cancer and with premalignant disease suggests that PKC expression might also be useful as a marker for certain premalignant breast diseases.

Table 2 *PKC specific activities in normal and malignant breast tissue*

pmol ^{32}P/min/mg represents the pmol ^{32}P transferred from [γ-^{32}P]ATP to histone III-S/min/mg tissue extract protein. P values were determined using the Student's t test to compare PKC specific activities in normal and tumor tissues of each patient.

Patient no.	PKC activity (pmol ^{32}P/min/mg)		
	Normal	Tumor	P
1	90 ± 80	356 ± 107	<0.009
2	156 ± 31	395 ± 53	<0.002
3	119 ± 79	433 ± 109	<0.008
4	142 ± 61	391 ± 50	<0.002
5	91 ± 11	290 ± 58	<0.004
6	231 ± 28	632 ± 82	<0.001
7	288 ± 79	780 ± 157	<0.001
8	164 ± 43	191 ± 42	0.34
9	212 ± 14	670 ± 102	<0.002

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