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Expression of Protein Kinase C Isoforms in Cancerous Breast Tissue and Adjacent Normal Breast Tissue

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Abstract

The role of protein kinase C (PKC) in the carcinogenesis of human breast tissue has been studied at the molecular level for more than two decades. In this study, we employed Western blotting to determine the presence of PKC isoforms in cancerous and normal breast tissues. The results indicate significant expression of a conventional PKC (PKCα) and two atypical PKCs (PKC ζ and λ/ι) in both breast tumors and adjacent normal breast tissue. For the α, ζ and λ/ι isoforms, the expression of individual isoforms was higher in the breast tumors than in the adjacent normal breast tissue. Although the correlation coefficient was low, significant linear correlation was found among the activities of the isoforms. The data suggest a potential new direction in cancer chemotherapy, namely the blockage of the signal transduction pathway of specific PKC isoforms.

Key Words: protein kinase C (PKC), human breast cancer

Introduction

Protein kinase C (PKC), a lipid-regulated and calcium-dependent protein kinase, has 10 isoforms. Based their cofactors, the isoforms can be divided into three main classes: Ca^{++}-dependent or conven-

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tional PKCs (α, βI, βII, and γ), Ca^{++}-independent or novel PKCs (δ, ε, η and θ), and Ca^{++}-independent and DAG- and phosphatidylserine-activated PKCs (ζ and λ/ι) (4, 14). These isoforms may exist in some organs or universally in all organs. In different organs, they may exhibit differences in structure and also differ in cell signaling functions (17).

It has been reported that overexpression of PKC may lead to disorders in cell proliferation and differentiation (9, 11). The expression of PKC in human breast tumor biopsies has been found to be significantly higher than that in adjacent normal breast tissues (16). Overexpression of HER-2/neu in breast cancer has been attributed to overexpression of this enzyme (21). In addition, PKC mediates phosphorylation of membrane-associated oncogene products, such as pp60src and Ki-ras proteins, and also activates nuclear proto-oncogenes such as c-jun and c-fos (1, 5, 8). These findings suggest that PKC may play an important role in the regulation of proto-oncogenes and oncogenes involved in the carcinogenesis of human breast cancer.

Although the role of PKC in the carcinogenesis of human breast cancer has been studied at the molecular level, there is no available information concerning changes in the activities of the isoforms in the breast tissue. In this study, we employed Western blotting to determine the existence of PKC isoforms in cancerous and normal breast tissues. The relationships between the isoforms of PKC and various demographic and clinical factors were also investigated.

Materials and Methods

Specimen Collection

Written consent was obtained from each patient. Surgical specimens of breast tumor (infiltrating ductal carcinoma) and normal human breast tissues were obtained by mastectomy from the operating rooms of Changhua Christian Hospital in Changhua and Chung Shan Medical University Hospital in Taichung, Taiwan. After resection, these specimens were stored at -70°C before being used for the analysis.

PKC Extraction

PKC extraction was performed according to Nishizuka (15) with minor modifications. All operations were carried out at 4°C. The specimen (80 mg) was sectioned into small pieces and washed with homogenized buffer A (pH 7.5, 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 2 mM ethylenedianmine-tetraacetic acid (EDTA), 50 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 50 mM β-mercaptoethanol). The tissue was then mixed with 200 µl buffer B (pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 50 mM PMSF, 10% glycerol, 0.1% Triton X-100). After homogenization, 200 µl homogenized buffer B was added to the homogenate before repeating the homogenization step. The homogenate was then transferred to another vial and a further 330 µl homogenized buffer B was added. This mixture was incubated for 1 h with stirring at intervals of 5-10 min. The homogenate was then centrifuged at 15,000 g for 3 h. The supernatant (1.5 ml) was then transferred into vials and stored at -70°C for further experiments.

Determination of Protein Contents

Protein contents of the sample preparation were determined by the Bradford protein assay (7). The protein assay reagents were purchased from Bio-Rad Lab (Richmond, CA, USA). Coomassie brilliant blue G-250 was used for staining and bovine serum albumin (BSA) was employed as the standard. Changes in optical density were monitored at 595 nm.

Western Blotting

PKC isoforms in the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10, 13). The extracts were standardized to the same volume with phosphate buffer solution (PBS). The extracts were standardized to the same volume with PBS. After adding a treatment buffer containing 0.125 M Tris-HCl buffer, pH 6.4, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and 0.001% bromophenol blue (final pH of the treatment buffer was 6.8) in a ratio of 1 to 5 to the extract. The mixture was boiled for 10 min and then rapidly placed in an ice bath. The mixture was then spun down in a centrifuge and loaded onto slab polyacrylamide gels, using a 4% stacking gel (pH 6.8) and 10% separating gel (pH 8.8). Electrophoresis was run on a mini vertical slab gel unit (Biorad Scientific Instruments, Richmond, CA, USA) at 140 V and 35 mA for 3.5 h. After electrophoresis, the gels were equilibrated in a cold transfer buffer and proteins were transferred onto nitrocellulose membranes (Amersham, Hybond-C Extra Supported, 0.45 m) using a Hoefer Scientific Instruments Transphor Unit at 100 mA overnight. The nitrocellulose membranes were washed with a washing buffer and incubated in 50 ml 3% FBS blocking buffer (3% FBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) at room temperature for 1 h. Primary antibodies against individual PKC isoforms (Transduction Laboratory, Lexington, KY, USA) (1:100 dilution) in 20 ml 3% FBS blocking buffer were then added and incubated at room temperature for 3 h. The nitrocellulose membranes were washed in the washing buffer for 10 min

in triplicates and then immersed in the secondary antibody of the corresponding isoform (1:1,000 dilution) containing 20 ml 3% FBS blocking buffer. The membranes were washed in triplicates with the washing buffer for 10 min. Color was developed using 20 ml of a color developing substrate in color reagents (700 µl nitrobluetrazolium and 780 µl 5-bromo-4-chloro-3-indolyl-phosphate at 10 mg/ml). The reaction was terminated by addition of deionized water. Color changes on the nitrocellulose membranes were determined using a densimeter (Alphalmager 2000 version 3.2, Alpha Innotech Corp., San Leandro, CA, USA).

Statistical Analysis

The protein expression levels of the PKC isoforms were calculated from quantified data. One patient, number 17, was designated as the benchmark with figures set at 100% of norm because the evenness of her isoform markers had made her a good candidate for normalization, and all other samples were measured against the benchmark. Data were expressed as means ± SEM. We used an unpaired *t*-test to analyze the differences in PKC isoform expression levels between cancerous and normal breast tissues. To analyze correlation between the PKC isoforms, differences in the data between isoforms were evaluated using the linear regression test. To analyze correlation between PKC isoforms and clinicopathological parameters, differences in the data between the two groups were evaluated using the Fisher exact test. $P < 0.05$ was considered to be statistically significant.

Results

To determine whether PKC isoforms were associated with breast cancer development, we scanned for ten PKC isoforms (α, βI, βII, γ, δ, ε, η, θ, ζ and λ/ι) using the Transduction Laboratory antibodies in both breast tumor tissues and the adjacent normal breast tissues taken from mixed tissue samples of 10 patients (Fig. 1). Results show significant immunostaining in both cancerous and adjacent normal breast tissues by the PKC α and γ antibodies at 80 kDa, and the PKC ζ, λ, and ι antibodies at 72 kDa, whereas the PKC β (both βI and βII), δ, ε, η, and θ antibodies did not show any immunoreactivity.

The positive finding of PKCγ raised some questions, as it had only previously been found in the brain. It could be that the antibody used cross-reacted with PKCα. A different PKCγ antibody (purchased from Gibco BRL, Grand Island, NY, USA) was used and it confirmed that the PKCγ was a false finding. A non-specific band at 47 kDa was detected with all tested PKC isoforms reacting to the antibodies. The

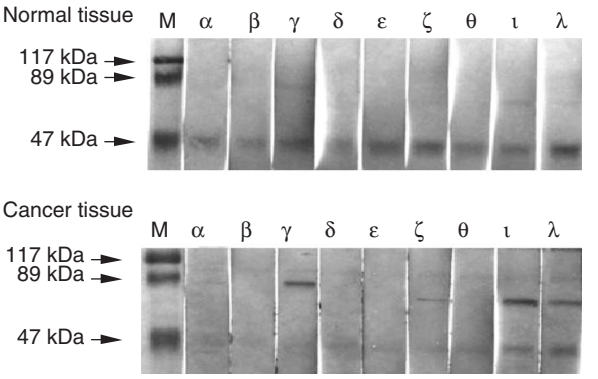

Fig. 1. Immunoblot analysis of protein kinase C isoforms in an adjacent normal breast tissue (A) and breast tumor (B) taken from mixed tissue samples of 10 patients (ID No. 1-10). Specimens were prepared as described in "Materials and Methods". Aliquots of the homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose membranes. The blots were stained with PKC isoenzyme-specific antibodies. M, molecular weight marker.

appearance of the band was related to the antibody used, and had also appeared in a previous test with brain cell lysates (data not shown).

From the above data, the following Western blotting tests focused on the detection of the most abundant isoforms, α, ζ, λ and ι, in the breast cancer tissues. The results revealed significant expression of conventional PKCα and atypical PKCs (PKC ζ, λ, and ι) in both the tumorous breast tissue (Fig. 2 and Table 1) and adjacent normal breast tissue. For the isoforms α, ζ and λ/ι, the expression of the individual isoforms were significantly higher in the breast tumors than in the adjacent normal breast tissues (α: 149.36 ± 10.63% *vs*. 23.75 ± 15.48%, ζ: 173.76 ± 10.38% *vs*. 27.63 ± 5.21%, λ: 124.52 ± 11.92% *vs*. 19.80 ± 7.47%, ι: 103.45 ± 7.35% *vs*. 16.45 ± 10.76%). Although the correlation coefficient was low, significant linear correlation was found among the activities of the isoforms (Table 2). However, there were no significant correlations in the activities of individual isoforms attributable to lymphatic metastasis, stage of carcinoma, age of the patient, or the presence of estrogen and progesterone receptors, which are prognosis markers for breast cancer (18) (Table 3). The similarities between the expression of PKCλ and PKCι in our data confirm that they are the same gene (17), and, hence, are regarded as one gene in this report.

Discussion

It has been reported that the activity of PKC is elevated significantly in human breast tumor than in

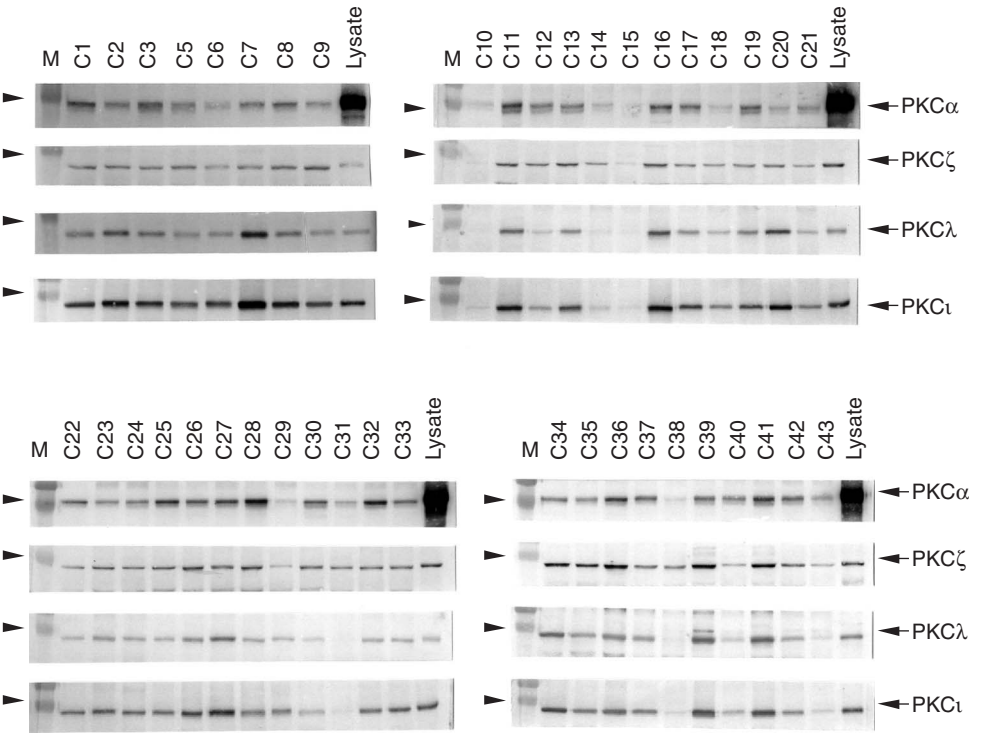

Fig. 2. Immunoblot analysis of protein kinase C isoforms in breast tumors. Forty-two specimens (from patient number 1 to patient number 43 except number 4; C, cancer tissue) were prepared as described in "Materials and Methods". Aliquots of the homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose membranes. The blots were stained with PKC isoenzyme-specific antibodies. M, molecular weight markers. Lysate indicates a brain cell lysate. The arrow (▶) indicates the marker of 89 kDa molecular weight.

the adjacent normal breast tissues (16). Using Western blotting, we not only confirmed these reports but also identified the three endogenous isoforms (α, ζ, λ/ι) that exist in breast tumor tissues. These findings suggest that some PKC isoforms may be associated with the development of human breast cancer.

In this study, PKCα in breast tumor was found to be overexpressed. Similar overexpression of PKCα in the breast cancer cell line MCF-7 was previously shown to lead to the generation of an aggressive neoplastic phenotype with increased tumorigenicity in nude mice (22). Therefore, PKCα may be an important factor in the carcinogenesis of human breast tumor. Overexpression of PKC isoforms α, βI, δ, ε and ζ have been reported in uterine cancer cells where the proteins increase the rate of proliferation of uterine tumor cells (6). In this study, we demonstrated that the expression of PKCζ was significantly higher in the breast tumor tissues than in the adjacent normal breast tissues. This finding suggests the role of PKCζ as an important enzyme in the proliferation of breast cancer cells. PKCλ/ι and PKCζ belong to the atypical PKC isoform group and show 86% similarity in their amino acid sequences (20). These two isoforms exist universally in numerous tissues. The presence of PKCλ/ι has been demonstrated in an undifferentiated mouse embryonal carcinoma cell line, P19. In P19 cells, expression of PKCα and PKCε is elevated and that of PKCλ/ι is significantly decreased after differentiation (2). Overexpression of PKCλ/ι not only leads to a higher degree of undifferentiation in breast cancer cells but can also be used as a prognostic marker. Although PKCλ/ι does not affect the proliferation of cells, this isoform has been considered to have a significant association with drug resistance (12). This phenomenon has been demonstrated in the apoptosis of leukemia cells where inhibiting PKCλ/ι lessened drug resistance. The presence of PKCλ/ι in our specimens suggests that breast tumors may have chemopreventative effects against anti-cancer drugs. However, this speculation requires further investigation.

Although PKCα, δ, ζ, and λ/ι are age-dependent in the development of kidney (19), we did not find significant differences in the expression of these PKC isoforms in breast tumor tissues by age group. Moreover, there was no significant association between the levels of expression of PKCα, ζ, and λ/ι in breast tumor tissues at the stage of carcinoma, nor at the stage of lymphatic metastasis. There was also no significant association to the presence or absence of estrogen or progesterone receptors.

Table 1. Clinical characteristics of the patients with breast tumor (infiltrating ductal carcinoma) analyzed in this study

Patient ID	*PKCα	PKCζ	PKCλ	PKCι	LN	Grade	ER	PR	Histology	Age (years)
1	256	163	159	166		3	–	–	IDC	
2	235	163	196	181		3			IDC	
3	246	124	153	154		3			IDC	
5	214	163	116	133		2	+	+	IDC	63
6	107	101	104	133		1	+	+	IDC	56
7	256	147	294	217		2	–	–	IDC	58
8	150	202	165	136		2			IDC	46
9	150	240	116	102		3	+	+	IDC	74
10	65	63	18	30	1	3	–	–	IDC	46
11	175	188	200	144	1	2	+	+	IDC	63
12	104	169	36	52	1	3	–	–	IDC	46
13	91	200	100	106	1	3	+	+	IDC	75
14	78	94	5	29	1	3	–	–	IDC	48
15	58	19	5	16	0	2	–	–	IDC	67
16	150	194	232	163	0	3	+		IDC	
17	100	100	100	100	1	2	+	+	IDC	82
18	71	88	45	57	0	2	+		IDC	57
19	136	119	141	88	0	2	+	–	IDC	49
20	80	125	255	132	1	2	+		IDC	43
21	129	131	86	57	1	2	–	–	IDC	58
22	85	169	67	88	1	2	+		IDC	40
23	85	219	153	101					IDC	51
24	85	183	92	105	0	2	–		IDC	45
25	111	219	55	92	0	2	–	–	IDC	66
26	170	261	123	123	1	2	+		IDC	64
27	196	233	233	167	0	2	–		IDC	55
28	179	268	86	107					IDC	63
29	60	106	61	78	0	3	+		IDC	49
30	119	219	37	53	0	3	+		IDC	55
31	94	176	4	25	0	3	+		IDC	55
32	196	226	110	94	1	3	–		IDC	76
33	94	247	104	96	1	2	+	+	IDC	48
34	238	261	199	136	1	3	–	–	IDC	31
35	162	220	164	99	1	2	–	–	IDC	56
36	264	318	216	123	0	3	+	+	IDC	26
37	153	153	150	102	0	3	–	–	IDC	38
38	102	148	66	45	0	2			IDC	58
39	221	294	258	163	0	1	+		IDC	72
40	187	85	94	60	0	2	–		IDC	23
41	340	245	251	154	0	2	+		IDC	62
42	179	148	129	93	0	3	+		IDC	40
43	102	107	52	45	1	2			IDC	43

*Staging and histology were determined independently of the PKC isoform data. Patient 17 was designated as the benchmark. Grade I was least differentiated; grade II intermediate; grade III most differentiated. LN, lymph node metastases; ER, estrogen receptor; PR, progesterone receptor; +, positive; –, negative; ND, non-detect; IDC, infiltrating ductal carcinoma. The level of 10 fmol receptor/mg or greater is considered clinically positive for both the estrogen and progesterone receptors.

Table 2. Correlation in coefficience (*P* value) among immuno-activities of PKCα, PKCζ, PKCι and PKCλ in the human breast tumor tissues

	PKCα	PKCζ	PKCι
PKCζ	r = 21 (< 0.002)		
PKCι	r = 0.47 (< 0.001)	r = 0.19 (< 0.003)	
PKCλ	r = 0.48 (< 0.001)	r = 0.17 (< 0.004)	r = 0.78 (< 0.001)

Table 3. Comparison of immuno-activities of PKCα, PKCζ, PKCι and PKCλ by lymphatic metastasis, grade of carcinoma, age of the patient and the presence of the estrogen receptor

	PKCα	PKCζ	PKCι	PKCλ
Lym. N + (n = 15)	124.4 ± 13.7	170.6 ± 17.4	108.0 ± 19.0	88.7 ± 10.2
– (n = 17)	148.4 ± 19.1	173.3 ± 19.5	121.7 ± 22.0	93.3 ± 12.0
Grade 3 (n = 15)	146.2 ± 18.2	181.9 ± 17.8	104.8 ± 20.5	99.2 ± 12.5
2 (n = 17)	144.3 ± 18.9	163.9 ± 16.9	126.8 ± 19.5	102.6 ± 10.6
Age > 45 (n = 29)	138.2 ± 12.4	175.9 ± 13.1	111.6 ± 14.9	96.4 ± 9.1
< 45 (n = 9)	152.4 ± 24.5	172.2 ± 26.3	139.4 ± 25.0	98.4 ± 10.9
ER + (n = 20)	144.8 ± 16.5	185.0 ± 15.6	133.0 ± 17.7	107.7 ± 8.5
– (n = 15)	151.5 ± 18.3	157.7 ± 18.6	113.3 ± 23.0	94.8 ± 15.4

PKCα, βI and βII have been reported to be associated with human breast tumors (3). The identification of PKC α, ζ, and λ/ι in human breast tumors in this study suggests that chemotherapeutically blocking the signal transduction pathway of these specific isoforms may be an additional direction in the treatment of breast cancer.

Acknowledgments

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