Dysregulation of protein kinase C activity in chemoresistant metastatic breast cancer cells

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This study was performed to evaluate the role of protein kinase C (PKC) activity in the development of chemoresistance in clinical breast cancer cells. To simulate the clinical situation, native tumor cells derived from 10 patients with advanced breast cancer were brought into short-term cultures, and treated with anthracyclines (doxorubicin, mitoxantrone), paclitaxel and combinations, respectively. After 3 days of incubation, we determined total PKC activity relative to each control incubated with blank medium. Furthermore, we determined the chemoresistance against these drugs from each cell population separately. Relative PKC activity ranged from 14 to 249%; 64% (37 of 58) of the breast cancer cell suspensions were considered chemoresistant. There was a non-significant trend to a higher relative PKC activity in resistant cells compared to non-resistant cells ($p=0.058$), regardless of the antineoplastic agent investigated. The individual variability in both PKC activity and chemoresistance pattern revealed that dysregulated PKC activity mediates resistance to antineoplastics. In order to achieve clinical value, evaluation of more data concerning the PKC signal-transduction pathway is necessary. New protocols of cancer treatment will require this information in order to be successful. *Anti-Cancer Drugs* 15:265–268 © 2004 Lippincott Williams & Wilkins.

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Introduction

Much effort has been undertaken to explore the role of protein kinase C (PKC) in carcinogenesis and cancer progression in order to create new PKC-tailored therapy regimen. Both altered expression and localization of PKC-ε occurs in human breast cancers [1]. The chemopreventive effects of retinoic acids might be mediated via PKC-δ activation [2]. Additionally, PKC-α modulation leads to a partial reversal of the multidrug resistance phenotype in breast cancer cell lines [3]. Activated PKC-α is a transient negative regulator of breast cancer cell spreading and motility [4]. If metastasis occurs after primary application of chemotherapy, metastatic breast cancer cells derive from primary tumor cells surviving the therapy due to developing chemoresistance. This might be traced back to activation of Akt [5], a member of the signal-transduction pathway intensifying cell survival in breast cancer [6]. Furthermore, Stat3-dependent overexpression of Bcl-2 contributes to the chemoresistance of metastatic breast cancer [7].

However, changes in overall PKC activity probably contribute to breast cancer progression. PKC is able to adjust the multidrug resistance phenotype conveyed by the PKC isotypes -α, -β, -γ, -ε and -ϕ [8]. Resistance of breast cancer cells against adriamycin is accompanied by activated PKC -α, -δ, -θ and -ε, whereas PKC-δ mediates early-stage resistance [9,10]. Due to its central role in signal transduction, PKC is one of the most promising targets in developing new therapeutic approaches against cancer. However, the main investigations in tumor cells of the varying entities were performed using established cell lines. To more closely mimic the clinical situation, this study uses cancer cells from patients with advanced breast cancer to more closely evaluate the role of PKC activity in chemoresistance. To this end, we examined PKC activity and resistance pattern against commonly used anticancer regimen using two different *ex vivo* assays.

Methods

Patients

Breast cancer cells were collected from 10 patients with metastatic breast cancer. The median age was 53.9 ± 7.7 years (range 44–74 years). Following surgery of the primary tumor, all patients received an adjuvant cytotoxic

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regimen consisting either of cyclophosphamide, methotrexate and 5-fluorouracil (600/40/600 mg/m^2) or epirubicin and cyclophosphamide (60/600 mg/m^2), respectively. The patients were included in this study at the first evidence of progressive disease. The median time to progression was 5.3 ± 5.8 years (range 1–19 years).

Isolation of tumor cells

The tumor cells were collected from pleural effusions and enriched by centrifugation (800 *g*, 20 min). After passing through a 60-μm gauze filter, the tumor cells were pooled from the interphase of a discontinuous Percoll density gradient centrifugation (55–66%, 800 *g*, 20 min). Tumor cells were washed in Complete Assay Medium (CAM) (DCS, Innovative Diagnostik Systeme, Hamburg, Germany) and then immediately processed for all subsequent examinations. Quality and viability of the resulting single-cell suspension were determined by Trypan blue dye exclusion and cytological examination using standard Papanicolau stains. Only suspensions with a tumor cell content greater than 90% were further included into the study.

Cell culture

Tumor cells were grown in CAM medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 g/ml streptomycin. Cell cultures were maintained at 10^5 cells/ml and 37°C in a humidified 95% air, 5% CO_2 atmosphere. Cells were exposed for 72 h to either blank medium or different antineoplastic regimen comprising anthracyclines, paclitaxel or a combination. Drug concentrations used *in vitro* corresponded to the clinically achievable plasma peak concentration (C_{max}) after administration of a standard dose: doxorubicin 0.5 μg/ml corresponding to 60 mg/m^2 i.v., mitoxantrone 0.65 μg/ml corresponding to 12 mg/m^2 i.v. and paclitaxel 13.6 μg/ml corresponding to 175 mg/m^2 i.v.

Chemosensitivity assay

Using cell suspensions isolated from corresponding tumor specimens, population-based sensitivity against cytotoxic agents was determined using the *ex vivo* ATP-based tumor chemosensitivity assay (ATP-TCA) (TCA 100; DCS Innovative Diagnostik Systeme) as described previously [11]. Results of the assay are given as 'resistant' or 'non-resistant', the latter group comprising both sensitive and partially sensitive tumors according to a semiquantitative score published earlier [11].

Protein isolation

Cells were pelleted (2 min, 10000 *g*), washed in PBS and resuspended in 50 μl of extraction buffer (20 mM Tris–HCl, 1 M glycine, 2.5%. SDS, 1 mM β-mercaptoethanol, 0.5 mM dithiothreitol, 0.3 U/ml aprotinin, pH 7.5). Cells were lysed by ultrasound for 5 min and cell debris was directly removed by centrifugation (10 min, 16 000 *g*). Aliquots of 1 μl of the supernatant were immediately used for the PKC activity assay.

PKC activity assay

PKC activity was determined using the PepTag assay (Promega, Madison WI) as described previously [12]. In brief, a PKC-specific fluorescent peptide was phosphorylated by the active PKCs of the cell lysate. The assay was performed in a total volume of 25 μl, consisting of 1 μl cell lysate, 0.4 mg/ml peptide, 100 mM HEPES, pH 7.4, 6.5 mM $CaCl_2$, 5 mM dithiothreitol, 50 mM $MgCl_2$, 5 mM adenosine triphosphate, 10 μM leupeptin and 1 mg/ml phosphatidylserine. Subsequently, phosphorylated and unphosphorylated peptide molecules were separated by 0.8% (50 mM Tris–HCl, pH 8.0) agarose gel electrophoresis for 15 min at 100 V. Resulting bands were visualized under UV light and documented quantitatively by densitometry (GS-690 Imaging Densitometer; Bio-Rad, Munich, Germany).

For each cell population a separate control without drugs was co-incubated. For presentation of data, each control was set equal to 1 and PKC activities are indicated as the ratio PKC activity^{assay}:PKC activity^{control}.

Statistical analysis

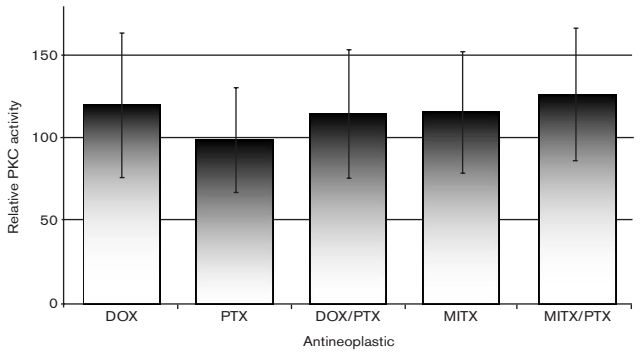
Statistical analyses were carried out using Student's *t*-test. The SPSS statistical package was employed for these as well as to generate descriptive statistics of the data. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

Relative PKC activity ranged from 14 to 249% relative to each individual control (= 100%). Figure 1 illustrates the relative PKC activity of the breast cancer cells investigated after exposure to the antineoplastics for 3 days. The relative PKC activity shows a highly individual distribution indicated by a high standard deviation, regardless of the drugs examined. The results of all 58 drugs assayed are given in Table 1. The tumor cells could be divided into 37 resistant (64%) and 21 non-resistant (36%) cell suspensions.

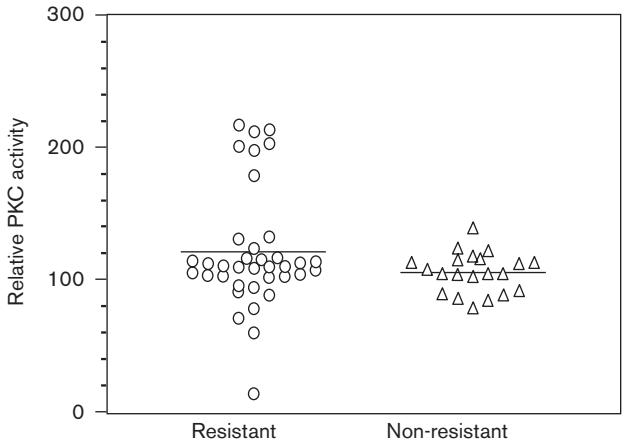
There was a trend towards a higher mean relative PKC activity in resistant patterns (121% relative PKC activity) compared to non-resistant patterns (105%). However, this trend did not reach statistical significance ($p = 0.058$). Statistical analyses of the anthracycline-based assays revealed similar results (anthracycline group, resistant 122%, non-resistant, 107%, $p = 0.28$; anthracycline + paclitaxel, resistant 133%, non-resistant 107%, $p = 0.13$). The tests investigating paclitaxel showed the reverse results, but also without statistical significance (resistant 91%, non-resistant, 107%, $p = 0.48$).

Fig. 1



Relative PKC activity of breast cancer cells exposed to anticancer drugs *in vitro*.

Fig. 2



Dot-plot of the single results of the PKC activity assays with respect to the corresponding chemoresistance.

Table 1 ***Ex vivo*** **chemoresistance of the breast cancers investigated against anthracyclines (doxorubicin or mitoxantrone, respectively), paclitaxel and combinations, respectively**

	No. of assays	Non-resistant	Resistant	*p*
Anthracyclines	19	4	15	0.2878
Paclitaxel	10	5	5	0.4862
Anthracyclines + paclitaxel	18	7	11	0.1271
All drugs assayed	58	21	37	0.0577

Figure 2 illustrates the heterogeneous distribution of relative PKC activities in resistant breast cancer cells in comparison to non-resistant cells. Although the assays exhibit a comparable mean value between resistant and non-resistant cells, chemoresistance is accompanied by dysregulation of PKC activity.

Discussion

Overcoming chemoresistance is a major concern in cancer therapy. Although involvement of PKC as a modulator of drug resistance is evident, its definite role in breast cancer is not well known. This work was performed to further investigate PKC activity as an effector of chemoresistance in clinical breast cancer. To simulate the clinical situation as closely as possible with the most stringent substrate, experiments were performed using human breast cancer cells from patients with advanced breast cancer. In our experiments, cells were brought into a short-term culture in the presence of antineoplastic agents. The relative ratio of PKC activity was calculated from cell extracts and the *ex vivo* chemoresistance was determined using an ATP-based luminometrical assay.

PKC-tailored therapy is considered a promising novel strategy in clinical oncology. It is known that improved gemcitabine-induced apoptosis works via PKC activation in ovarian cancer [13]. Bryostatin, an effective PKC activator, potentiates the effect of chemotherapies in the treatment of leukemias and lymphomas [14,15]. On the other hand, PKC inhibition also improved cytostatic regimens. Platinum resistance was reversed by PKC inhibiting drugs in non-small cell lung cancer [16]. PKC-β inhibition had favorable antitumor effects in various entities including glioblastoma, gastric and colon cancer [17–19]. Tamoxifen inhibits PKC and decreased breast tumor severity [20]. Furthermore, PKC inhibition resulted in increased radiosensitization [21] and acted preventatively against colon cancer [22].

Thus, major efforts were undertaken to improve cytostatic therapy regimens by addition of PKC-modulating agents. However, it is common to all studies that no clear correlation exists between the biological effect observed and the corresponding PKC activity. In our study, PKC activity was deregulated in resistant tumor cells. Both increase and decrease of PKC activity occurred in chemoresistant cells. On the other hand, a 'homeostasis' of PKC activity was observable in chemosensitive cells. Although further studies are needed with larger numbers, the data suggest that chemoresistance is accompanied by dysregulation of PKC activities. It is tempting to speculate that the dysregulation of PKC affects its down-stream effectors. It is well recognized that intracellular signal transduction is abnormal in neoplastic cells including the PKC-mediated pathway [23]. In conclusion, this study might explain the lack of a clear correlation between PKC activity and the corresponding clinical effects. Further investigations are warranted to carefully address this issue prior to offering a clinically useful PKC-targeted therapy regimen.

References

1 Masso-Welch PA, Winston JS, Edge S, Darcy KM, Asch H, Vaughan MM, *et al.* Altered expression and localization of PKC-ε in human breast tumors. *Breast Cancer Res Treat* 2001; **68**:211–223.

2 Kambhampati S, Li Y, Verma A, Sassano A, Majchrzak B, Deb DK, *et al.* Activation of protein kinase C-delta (PKC-δ) by all-*trans*-retinoic acid. *J Biol Chem* 2003; **278**:32544–32551.

3 Gupta KP, Ward NE, Gravitt KR, Bergman PJ, O'Brian CA. Partial reversal of multidrug resistance in human breast cancer cells by an *N*-myristoylated protein kinase C-α pseudosubstrate peptide. *J Biol Chem* 1996; **271**:2102–2111.

4 Gauthier ML, Torretto C, Ly J, Francescutti V, O'Day DH. Protein kinase Cα negatively regulates cell spreading and motility in MDA-MB-231 human breast cancer cells downstream of epidermal growth factor receptor. *Biochem Biophys Res Commun* 2003; **307**: 839–846.

5 Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L, *et al.* HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* 2003; **22**:3205–3212.

6 Mills GB, Lu Y, Fang X, Wang H, Eder A, Mao M, *et al.* The role of genetic abnormalities of PTEN and the phosphatidylinositol 3-kinase pathway in breast and ovarian tumorigenesis, prognosis, and therapy. *Semin Oncol* 2001; **28**(**suppl 16**):125–141.

7 Real PJ, Sierra A, De Juan A, Segovia JC, Lopez-Vega JM, Fernandez-Luna JL. Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. *Oncogene* 2002; **21**:7611–7618.

8 Yang JM, Chin KV, Hait WN. Interaction of P-glycoprotein with protein kinase C in human multidrug resistant carcinoma cells. *Cancer Res* 1996; **56**:3491–3494.

9 Ratnasinghe D, Phang JM, Yeh GC. Differential expression and activity of phosphatases and protein kinases in adriamycin sensitive and resistant human breast cancer MCF-7 cells. *Int J Oncol* 1998; **13**: 79–84.

10 Budworth J, Gant TW, Gescher A. Co-ordinate loss of protein kinase C and multidrug resistance gene expression in revertant MCF-7/Adr breast carcinoma cells. *Br J Cancer* 1997; **75**:1330–1335.

11 Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, Harel G, *et al.* Chemosensitivity testing of human tumors using a microplate adenosine triphosphate assay: clinical correlation for cisplatin resistance of ovarian carcinoma. *Cancer Res* 1995; **55**:5276–5282.

12 Schöndorf T, Becker M, Kolhagen H, Kurbacher CM, Göhring U-J. *Ex vivo* assays to evaluate the role of protein kinase C in tumor cells of patients with breast cancer. *Anticancer Res* 2000; **20**:5073–5076.

13 Cartree L, Kucera GL. Gemcitabine induces programmed cell death and activates protein kinase C in BG-1 human ovarian cancer cells. *Cancer Chemother Pharmacol* 1998; **41**:403–412.

14 Cragg LH, Andreef M, Feldman E, Roberts J, Murgo A, Winning M, *et al.* Phase I trial and correlative laboratory studies of bryostatin (NSC 339555) and high-dose 1-β-D-arabinofuranosylcytosine in patients with refractory acute leukemia. *Clin Cancer Res* 2002; **8**:2123–2133.

15 Mohammad RM, Wall NR, Dutcher JA, Al-Katib AM. The addition of byrostatin 1 to cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy improves response in a CHOP-resistant human diffuse large cell lymphoma xenograft model. *Clin Cancer Res* 2000; **6**:4950–4956.

16 Lara Jr PN, Gandara DR, Longmate J, Gumerlock PH, Lau DH, Edelmann MJ, *et al.* Activity of high-dose toremifene plus cisplatin in platinum-treated non-small-cell lung cancer: a phase II California Cancer Consortium Trial. *Cancer Chemother Pharmacol* 2001; **48**:22–28.

17 Teicher BA, Menon K, Alvarez E, Galbreath E, Shih C, Faul M. Antiangiogenic and antitumor effects of a protein kinase C β inhibitor in human T98G glioblastoma multiforme xenografts. *Clin Cancer Res* 2001; **7**:634–640.

18 Teicher BA, Menon K, Alvarez E, Liu P, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase C β inhibitor in human hepatocellular and gastric cancer xenografts. *In Vivo* 2001; **15**:185–193.

19 Teicher BA, Menon K, Alvarez E, Galbreath E, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase C β inhibitor in human HT-29 colon carcinoma and human CaKi1 renal cell carcinoma xenografts. *Anticancer Res* 2001; **21**:3175–3184.

20 Boyan BD, Sylvia VL, Frambach T, Lohmann CH, Dietl J, Dean DD, *et al.* Estrogen-dependent rapid activation of protein kinase C in estrogen receptor-positive MCF-7 breast cancer cells and estrogen receptor-negative HCC38 cells is membrane-mediated and inhibited by tamoxifen. *Endocrinology* 2003; **144**:1812–1824.

21 Zaugg K, Rocha S, Resch H, Hegyi I, Oehler C, Glanzmann C, *et al.* Differnetial p53-dependent mechanism of radiosensitization *in vitro* and *in vivo* by the protein kinase C-specific inhibitor PKC412. *Cancer Res* 2001; **61**:732–738.

22 Rao CV, Simi B, Hirose Y, Upadhyaya P, El-Bayoumy K, Reddy BS. Mechanisms in the chemoprevention of colcon cancer: modulation of protein kinase C, tyrosine protein kinase and diacylglycerol kinase activities by 1,4-phenylenebis-(methylene)selenocyanate and impact of low-fat diet. *Int J Oncol* 2000; **16**:519–527.

23 Kohn EC, Liotta LA. Molecular insights into cancer invasion: strategies for prevention and intervention. *Cancer Res* 1995; **55**:1856–1862.