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Lung Cancer Cells Often Express High Levels of Protein Kinase C Activity

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We analyzed protein kinase C (PKC) activity in twenty-two tumor cell lines derived from lung, pancreas, stomach, tongue and vulva, and found that lung cancer cells often (9 out of 13) exhibit significantly higher PKC activity than other types of cancer cells. The PKC in these lung cancer cells was separated into one major and one minor peaks by a Mono Q column chromatography. The PKC in the major peak had an absolute requirement for Ca^{2+}, phosphatidylserine and 12-O-tetradecanoylphorbol-13-acetate (TPA), as expected. However, the PKC in the minor peak did not require TPA for its activation. Hydroxyapatite column chromatography revealed that the PKC in the major peak is type III. These results indicate that in lung cancer cells type III PKC activity is often elevated compared to other types of cancer cells. The growth of many lung cancer cell lines was inhibited by TPA.

Key words: Protein kinase C — Lung cancer — Tumor promoter — Phospholipid

Protein kinase C (PKC) is a Ca^{2+}/phospholipid-dependent serine/threonine protein kinase and is thought to play an important role in the signal transduction of a wide variety of mitogens.[1)] Recent analysis of the PKC cDNA indicated the presence of several subspecies[2–5)] which are expressed in a tissue-specific fashion.[6)] These subspecies of PKC may have different physiological functions that are not yet well understood.

PKC is a receptor for 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter.[1)] PKC overexpressed by rat fibroblasts transfected with PKC cDNA caused anchorage-independent growth in these cells.[7, 8)] Thus, PKC is believed to be involved in some steps of tumor promotion. Several types of cancer cells and tissues overexpress various oncogenes, often by gene amplification.[9–11)] In this study, we tested the possibility that PKC is overproduced in some cancer cells.

First, we examined the levels of PKC activity in twenty-two cancer cell lines of different tissue origins. Cytosol fractions were prepared from these cells and PKC activity was estimated by measuring ^{32}P-incorporation into a substrate, histone IIIS. As shown in Fig. 1, high levels of PKC activity were frequently observed in lung cancer cells with only a few exceptions. There was an obvious difference compared with the average activity of non-lung cancer cell lines. Cell lines derived from squamous cell carcinoma and adenocarcinoma exhibited particularly high PKC activities. A cell line derived from small cell lung carcinoma also exhibited a high level of PKC. Squamous cell carcinoma of tongue and vulva and adenocarcinoma of stomach expressed only low levels of PKC activity. Two cell lines derived from adenocarcinoma of pancreas expressed intermediate levels of PKC activity. The relative amounts of PKC activity in membrane fractions were similar to those in the cytosol fractions (data not shown).

Next, we analyzed the type of PKC expressed in three lung cancer cell lines (A549, RERF-LC-MS and Calu-1) and a pancreatic cancer cell line (UCVA-1) by chromatographic procedures using a Mono Q column. As shown in Fig. 2, PKC in A549 cells was eluted as two peaks, one major and one minor. PKC in Calu-1 and RERF-LC-MS cells was similarly eluted (data not shown). PKC in UCVA-1 cells was eluted in one peak corresponding to the major peak in lung cancer cells (data not shown). Because three different types of PKC are eluted in one peak by Mono Q column chromatography, PKC contained in the major peak of A549 cells was further analyzed by hydroxyapatite column chromatography. On this column, PKC can be separated into three subspecies, types I, II and III,[15)] which correspond to the γ, β and α subspecies of PKC, respectively. As shown in Fig. 3, PKC in A549 cells appeared as a single peak, corresponding to the type III PKC (α-subspecies). PKC in UCVA-1 cells was also identified as type III (data not shown). These results indicate that the type III PKC is responsible for the high level of PKC activity observed in lung cancer cells.

Recently, Ono *et al.* reported an additional member of the PKC family,[5)] which does not show an absolute requirement for phospholipid and diacylglycerol. Thus, we examined whether the PKC in the minor peak observed in lung cancer cells corresponds to this new PKC by testing its dependency on Ca^{2+}, phospholipid and

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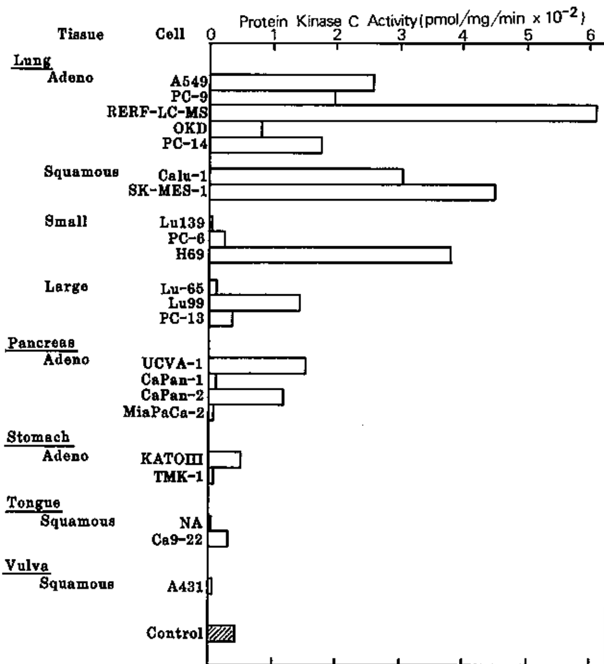

Fig. 1. Protein kinase C activities in various cancer cells. Lung cancer cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 μg/ml kanamycin and 1 μg/ml Fungizone. Other cancer cells were maintained in DMEM with the same supplements. Cells grown to confluence in 15-cm dishes were washed twice with phosphate-buffered saline (PBS)/1 m*M* EDTA containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF), scraped with a Teflon tape-coated razor blade and collected into 15-ml tubes. Cell pellets were washed once with 0.25 *M* sucrose, and then lysed in 1 ml of SEAT buffer (0.25 *M* sucrose, 1 m*M* EDTA, 10 m*M* acetic acid, 10 m*M* triethanolamine, pH 7.4) containing 1 m*M* PMSF by pipetting 40 times with a Gilson P1000 Pipetman.[12] The lysates were centrifuged at 1,000 rpm for 10 min to remove nuclei and cell debris. The supernatants were then centrifuged at 100,000 rpm for 15 min in a Beckman TLA100.2 rotor. The supernatants were used as the cytosol fraction. All procedures for preparation were carried out at 4°C. PKC activity in cell lysates was assayed using histone IIIS (Sigma) as a substrate.[13] The standard reaction mixture (final volume: 100 μl) contained 20 m*M* Tris-HCl (pH 7.5), 10 m*M* $MgCl_2$, 40 μg of histone IIIS, 9 μM [γ-^{32}P]ATP (1.1 Ci/mmol), cytosol fraction (1.2–10 μg proteins) in the presence of 0.2 m*M* $CaCl_2$, 25 μg/ml phosphatidylserine, 4 μg/ml TPA or 2 m*M* EGTA. The reaction was initiated by the addition of ATP and was incubated for 15 min at 30°C. The reaction was terminated by applying 75 μl of reaction mixture onto a Whatman P81 filter washing with four changes of water. ^{32}P-incorporation into histone IIIS was quantified by Cerenkov counting. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.[14] Results were the average values of triplicate assays. The average of PKC activity in non-lung cancer cell lines was used as an arbitrary control.

TPA. As summarized in Table I, the PKC in the minor peak did not require TPA whereas the PKC in the major peak showed the typical requirement. These results indicate that the PKC in the minor peak may correspond to the new member of the PKC family found by Ono *et al.*[5]

We then analyzed the effect of TPA on the growth of lung cancer cells. As shown in Fig. 4, there were no cell lines whose growth was stimulated by TPA. Instead, the growth of many cell lines was inhibited by TPA. Cells in group A possessing a relatively high level of PKC activity (>200 pmol/mg/min) tended to be more sensitive to TPA than cells in group B possessing a lower level of PKC activity, although the correlation was not perfect.

Hyperexpression of various oncogene products was observed in a variety of cancer tissues and cell lines[9–11] and was thought to be one of the most important causes of cancer development. PKC is a receptor for the tumor promoter TPA.[1] In addition, rat fibroblasts overproducing PKC exhibit disordered growth control.[7] Thus, PKC appears to play a critical role in growth control, and its

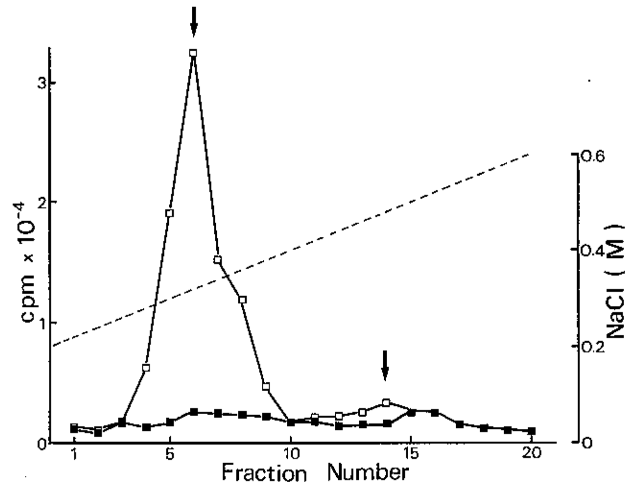

Fig. 2. Mono Q column chromatography of protein kinase C from A549 cells. A549 cells from ten 15-cm dishes were homogenized in buffer A (20 m*M* Tris-HCl pH 7.5, 0.25 *M* sucrose, 10 m*M* EGTA, 2 m*M* EDTA, 20 μg/ml of leupeptin) with a Teflon homogenizer. The homogenates were centrifuged at 100,000 rpm for 15 min in a Beckman TLA100.2 rotor. The supernatant was diluted 2–3 times with buffer B (20 m*M* Tris-HCl pH 7.5, 0.5 m*M* EGTA, 0.5 m*M* EDTA, 10 m*M* 2-mercaptoethanol) and applied to a Mono Q column (HR5/5, Pharmacia) pre-equilibrated with buffer B. PKC was eluted with a linear gradient of NaCl (0.2–0.6 *M*) at a flow rate of 1 ml/min. Protein kinase C activity in each fraction (0.5 ml) was assayed in the presence of either Ca^{2+}, phosphatidylserine and TPA (□) or EGTA (■). Arrows indicate the major and minor activity peaks.

activation may be of central importance in the process of carcinogenesis.

The mouse cell line B82 was reported to produce the highest level of PKC.[16] In this study, we demonstrated that the PKC activities in several lung cancer cell lines are several times higher than in B82 cells (estimated to be 98 pmol/mg/min). The elevation of PKC activity in these lung cancer cell lines was observed even after purification by Mono Q and hydroxyapatite column chromatographies (data not shown). In contrast, squamous cell carcinoma of tongue and vulva have considerably reduced PKC activity. Thus, elevated levels as well as reduced levels of PKC may have significance in carcinogenesis.

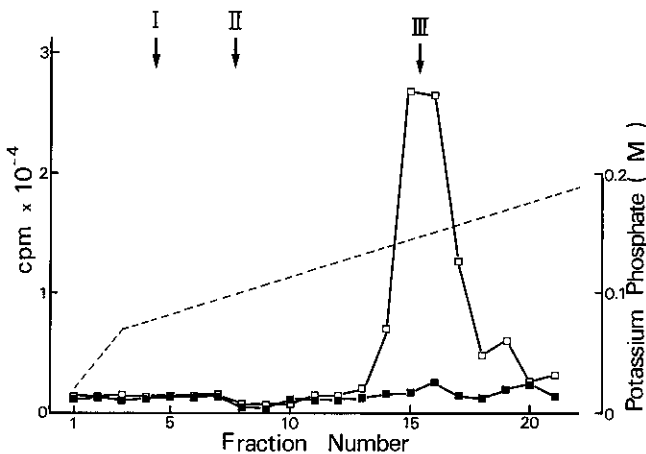

Fig. 3. Hydroxyapatite column chromatography of protein kinase C from A549 cells. Fractions containing PKC activity (the major peak in Fig. 2) were combined, diluted with one volume of buffer C, then applied to a hydroxyapatite column (type S, KOKEN, Tokyo) pre-equilibrated with buffer C (20 mM potassium phosphate buffer pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol). PKC was eluted with a linear gradient of potassium phosphate (20–280 mM) at a flow rate of 0.4 ml/min. Protein kinase C activity in each fraction (2 ml) was assayed in the presence of either Ca^{2+}, phosphatidylserine and TPA (□) or EGTA (■).

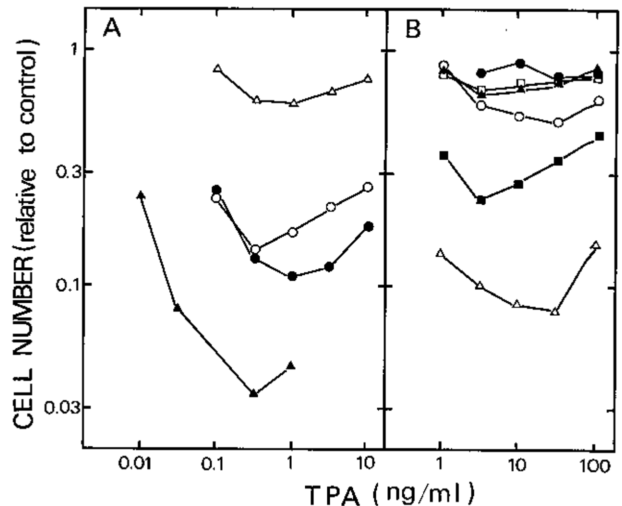

Fig. 4. Effect of TPA on growth of lung cancer cells. Cells (5 $\times 10^4$) were plated in 35-mm dishes and incubated for 1 week. TPA was added at various concentrations on days 1, 3 and 5. Cells were detached with trypsin/EDTA and counted on day 7 using a hemocytometer. (A) Cells possessing a relatively high level of PKC activity (>200 pmol/mg/min). (△) SK-MES-1; (○) A549; (▲) RERF-LC-MS; (●) Calu-1. (B) Cells possessing a relatively low level of PKC activity (<200 pmol/mg/min). (●) PC6; (○) PC9; (▲) PC14; (△) Lu99; (■) Lu65; (□) PC13.

Table I. Ca^{2+}, Phosphatidylserine (PS) and TPA-dependency of Protein Kinase C Purified from Lung Cancer Cells

	RERF-LC-MS		A549		Calu-1	
	Major	Minor	Major[a]	Minor	Major	Minor
	cpm (%)	cpm (%)	cpm (%)	cpm (%)	cpm (%)	cpm (%)
Ca^{2+}	6251 (21.2)	5721 (26.7)	2307 (20.7)	7606 (96.2)	7553 (15.9)	8124 (61.3)
Ca^{2+}, PS	13275 (44.9)	22962 (107.3)	6580 (58.9)	6862 (86.8)	23564 (49.7)	13337 (100.6)
Ca^{2+}, PS, TPA	29546 (100)	21408 (100)	11169 (100)	7906 (100)	47459 (100)	13257 (100)

a) Purified by hydroxyapatite column chromatography.
Protein kinase C was purified from RERF-LC-MS, A549 and Calu-1 by Mono Q or hydroxyapatite column chromatography and assayed as described in the text. Major and minor represent the two activity peaks shown in Fig. 2. The results are the average values of triplicate assays.

The mechanism of elevation of PKC activity is not yet known. We previously postulated three mechanisms by which epidermal growth factor (EGF) receptor level may be regulated.[17, 18] These mechanisms involve gene amplification, enhanced transcription, and reduced rate of metabolic turnover of gene products. Increased specific activity may also be considered. Which mechanisms are involved in the elevation of PKC activity remains to be elucidated.

EGF inhibits the growth of EGF receptor-overproducing tumor cells *in vitro*.[19] This inhibitory effect of EGF is well correlated with the number of EGF receptors on the cell surface.[20] The excess activity of the receptor tyrosine kinase is postulated to be involved in the inhibition of cell proliferation.[21] TPA also inhibited the growth of some of the lung cancer cells with elevated PKC activities. However, unlike in the EGF receptor system, the inhibitory effect of TPA showed only a weak correlation with the level of PKC. This may reflect the complexity of signal transduction via TPA-activated PKC.

To date, several subspecies of PKC have been reported, but their exact nature is not yet completely understood. Lung cancer cells with elevated PKC levels will be useful to clarify the physiological function of some of these PKC subspecies.

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