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High Activities of Protein Kinases C and M in Fresh Human Stomach and Breast Tumors

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The activities of Ca^{+2} –PL dependent protein kinase (PKC) and independent protein kinase (RKM) were measured in human stomach and breast tumors and in the respective counterparts of normal tissue. Expression of c-fos and c-myc of the tissues were also measured. RNAs of c-fos and c-myc were unexpectedly high in the tissue from normal stomach and breast as well as in all cancer tissue. On the other hand, cytosolic and particulate PKC activities were higher in the tumors as compared to those of normal controls. Notably, some cases exhibited altered activities of PKC i.e. increased PKM activities as high as RKC, which might be related to the autocrine control of growth in the tumor mass.

Key Words: PKC and PKM activities, c-myc and c-fos expression, human stomach and breast cancer.

Calcium-PL dependent PKC is currently recognized as a mediator in signal transductions (Berridge 1984; Nishizuka 1984) by the action of c-onc genes, growth factors and tumor promoters (Heldin 1984; Rabin *et al.* 1986).

PKC is widely distributed in animal tissues (Kuo *et al.* 1986) and is also found in certain plants (Schafer *et al.* 1985; Olah and Kiss 1986). Modulation of PKC activity was induced by calcium ions in human neutrophils (Melloni *et al.* 1985), by tumor-promoting phorbol esters in PYS-2 cells (Kraft and Anderson 1983) and in human platelets (Tapley and Murray 1984), by the gonadotropin-releasing hormone (Naor *et al.* 1985), by ACTH in the bovine adrenal cortex (Vilgrain *et al.* 1984) and by antigen IgE in the mouse mast cell line-PT18 (White *et al.* 1985). These data suggested that the redistribution of PKC from the cytosol to membrane fraction was caused by the above cofactors, and that 80K-dalton PKC was cleaved to the 50K-dalton form of the enzyme (PKM) by a calcium requiring protease. PKM reenters to the cytoplasm and phosphorylates the substrates required for RNA synthesis prior to DNA synthesis, and that may be the key signal transduction event in B cell mitogenesis (Guy *et al.* 1986). Moreover PKC can induce the expression of c-myc (Kaibuchi *et al.* 1986) and c-fos (Tsuda *et al.* 1986) in Swiss 3T3 cells. The PKC monomer binds to mixed micelles in four or more molecules of PS and one molecule of Ca^{+2} in the presence of one molecule of DAG (Bell 1986) and forms a quaternary complex on the membrane surface (Farrar and Anderson 1985). The lipid binding regulatory domain of PKC contains the phorbol diester binding activity, and using gel filtration it has been completely separated from the catalytic domain which contains protein kinase activity (Lee and Bell 1986).

Therefore, PKM is a product of the irreversible activation of PKC, and it can induce cellular proliferation independently from Ca^{+2} and PL. However, PKC and PKM activities have not yet been studied in human tumor tissues, although PKC activities in various normal tissues were reported in rats (Kuo *et al.* 1980).

This study reports that PKC activities in human stomach and breast tumors were markedly increased in the particulate fraction as well as in the cytosolic fraction. In some cases, PKM activities were as high

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The abbreviation used are: PL. phospholipid; PKC, protein kinase C; PS, phosphatidylserine; DAG diacylglycerol; TPA, 12-0-tetradecanoylphorbol-13-acetate; EGTA, ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

as PKC activities in both fractions and were significantly increased in tumors compared to the normal controls. However, we did not observe any significant differences in the expression of c-myc and c-fos between the tumors and the corresponding normal tissues.

MATERIALS AND METHODS

Materials.

Leupeptin, phenylmethylsulfonylfluoride(PMSF), EGTA, EDTA, ATP, PS and diolein were purchased from the Sigma Chemical Co. (St. Louis, MO); DEAE-Sephacel was obtained from Pharmacia Fine Chemicals; 2-mercaptoethanol, diethylether, DMSO, chloroform, trichloroacetic acid and isoamyl alcohol were purchased from Merck (E. Merck, Darmstadt); Protosol, Aquasol, (α-^{32}P) dCTP and nick translation kit (NEK-004A) were NEN products (DU PONT); (γ-^{32}P) ATP was purchased from Amersham (England); E. coli MC1061 containing human c-fos was purchased from ATCC (Rockville, MD, USA); pHBR-1 harboring human c-myc was generously donated by Dr. Bishop at the University of California, San Francisco.

Preparation of tumor samples.

Adenocarcinoma of the stomach and infiltrating ductal carcinoma of the breast in moderately to poorly differentiated tissues were obtained from the operating room, and necrotic or fatty tissue was removed on ice. Normal stomach mucosa and breast ductal tissue were collected immediately after resection. Protein kinase and RNAs were extracted from 11 stomach cancer, 10 breast tumors and from each of 6 corresponding normal tissues.

Partial purification of PKC.

PKC was partially purfified from the normal and tumor tissues by modification of published methods (Niedel *et al.* 1983). One gram of tumor tissue was homogenized in 10 ml of cold homogenization buffer (20mM Tris-HCl, pH 7.5, 0.25M sucrose, 10mM EDTA, 1mM EGTA, 50mM 2-mercaptoethanol, 2mM PMSF and 0.01% leupeptin) using the polytron homogenizer (Brinkman Instrument Co., N.Y.) for 5 min and sonicated for 3 min by a sonicator (Fisher Scientific Co.). All subsequent steps were performed at 4°C. After centrifugation at 1,000×g for 20 min (Sorvall RC-5) and at 100,000×g for 1 hour (Beckman LB-M), the particulate fraction was sonicated with 2 ml of the homogenization buffer containing 0.3% Triton-X-100 and gently stirred for 1 hour at 4°C. The suspension was centrifuged at 24,000×g for 40 min. The 100,000×g and 24,000×g supernatants were applied to DEAE-Sephacel column (0.8×5cm) and eluted with 150 mM Nacl in buffer A (20mM Tris-HC, pH 7,5., 0.5 mM EDTA, 0.5 mM EGTA and 10mM 2-mercaptoethanol) for the cytosolic and particulate fractions, respectively.

Enzyme assay.

PKC was assayed by measuring the incorporation of ^{32}P from (γ-^{32}p) ATP into calf thymus histones (type III-s) (Hannun *et al.* 1985). The reaction mixture (0.1 ml) contined 2 μmol of Tris-HCl, pH 7.5, 25 μg of histone, 2.5 μmol of $MgCl_2$, 10 mM 2 – mercaptoethanol, 4 nmol of cold ATP, 1 μCi of (γ-^{32}p) ATP (0.5-3 Ci/mmol) and either 50 nmol of $CaCl_2$ or 500 nmol of EGTA. Triton X-100, 20 μg PS and 2 μg diolein mixed micellar solution were added to the reaction mixture. The reaction was started with the addition of 20μg of the enzyme fraction at 37°C and terminated after 10 min by the addition of 0.1 ml of cold 50% trichloroacetic acid(TCA) and 300 μg of bovine serum albumin. Precipitates were washed 3 times with 500 μl of 5% TCA, 2 times with 500 μl of 95% ethanol, and one time with 500 μl of diethylether by centrifugation at 6,000 rpm (HS-4 rotor, Sorvall) for 15 min. Final precipitates were solubilized with Protosol, and the radioactivity was measured in 10 ml of Aquasol using a liquid scintillation counter (Packard Tricarb 4530).

Dot blot hybridization.

Cellular RNAs from 0.5-1.0g of tumor and normal tissue were purified using 4M guanidine thiocyanate and 5.7M CsCl by centrifugation in a type 65 rotor (Beckman) for 16 hours. Human c-fos and c-myc fragments were purified from MC1061 and HB101 harboring pc-fos (human)-1 and PHSR-1, respectively, and DNA probes wee prepared using (α-^{32}p) dCTP. Hybridization was performed using between 1.5-3.0 ug of isolated RNAs and probe DNAs according to the method of Müller et al.(1982).

RESULTS

Changes in PKC activity in the cytosol and particulate fractions of human stomach and breast cancers.

The PKC activities of the partially purified fractions were assayed from tumor preparations and from normal stomach mucosa and breast ductal tissue (Fig. 1). PKC in the normal tissue was very soluble and rarely particulate. Ca^{2+}-PL dependent protein kinase activities

were markedly increased in the tumors in both the cytosol and particulate fractions. PKC activity in the particulate fraction of the normal tissue was negligible, whereas the activity in tumor tissue was prominent as compared to that in normal tissue. There was no significant difference in the change in activity between stomach and breast.

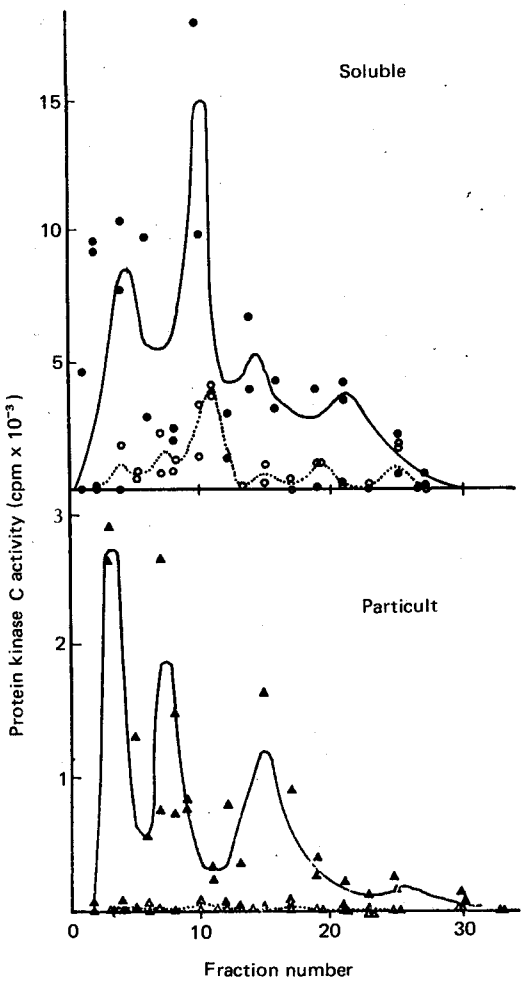

Fig. 1. DEAE-Sephacel chromatography of human stomach adenocarcinoma and suitable normal mucosa. 100,000×g supernatant (upper panel) and 24,000×g superatant (lower panel) were applied to the column and washed with 50 ml of buffer A containing 150 mM Nacl. Fractions were collected and 20μl of sample were assayed for Ca^{2+}-PL dependent protein kinase in the presence of 0.5mM $CaCl_2$ or EGTA. PKC activity was calculated from the differences between the two activities with $CaCl_2$ or EGTA. The solid and dotted lines indicate the mean PKC of the tumor and the normal, respectively. Each point represent the individual activity of 11 stomach cancers.

PKM activities in tumors.

When PKC activities were measured, Ca^{2+}-PL dependent activities usually changed with the amount of the enzyme (Fig. 2). However, Ca^{2+}-PL independent PKM activities were not decreased even at low concentrations of the proteins in some turmos. Especially, PKM activity was maintained at a higher level than

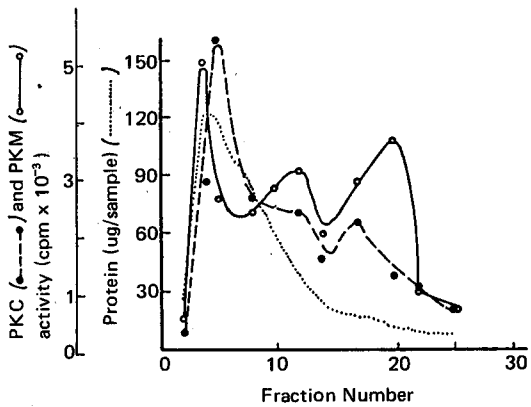

Fig. 2. Changes of PKC and PKM levels in some stomach cancer cytosols. PKM activity represents the incorporation of (γ^{32}-P) ATP into histone IIIs in the reaction mixture of 5mM EGTA and 25mM $MgCl_2$ and PKC represents the incorporation of radioactivity in the presence of 0.5mM $CaCl_2$, PS and diolein.

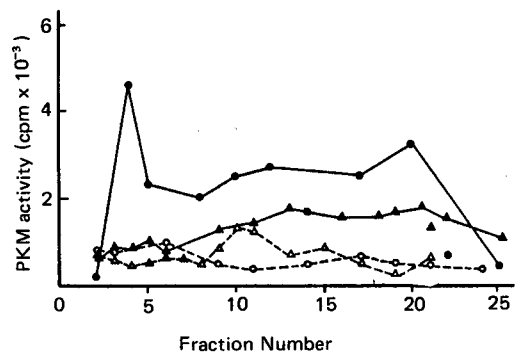

Fig. 3. PKM activities in cytosol and particulate fractions of the tumor (solid line) and normal (dotted line) tissues. Circles and triangles indicate the PKM activities of the cytosols and the particulate fractions, respectively. The other legends are same as in Fig. 1. and 2.

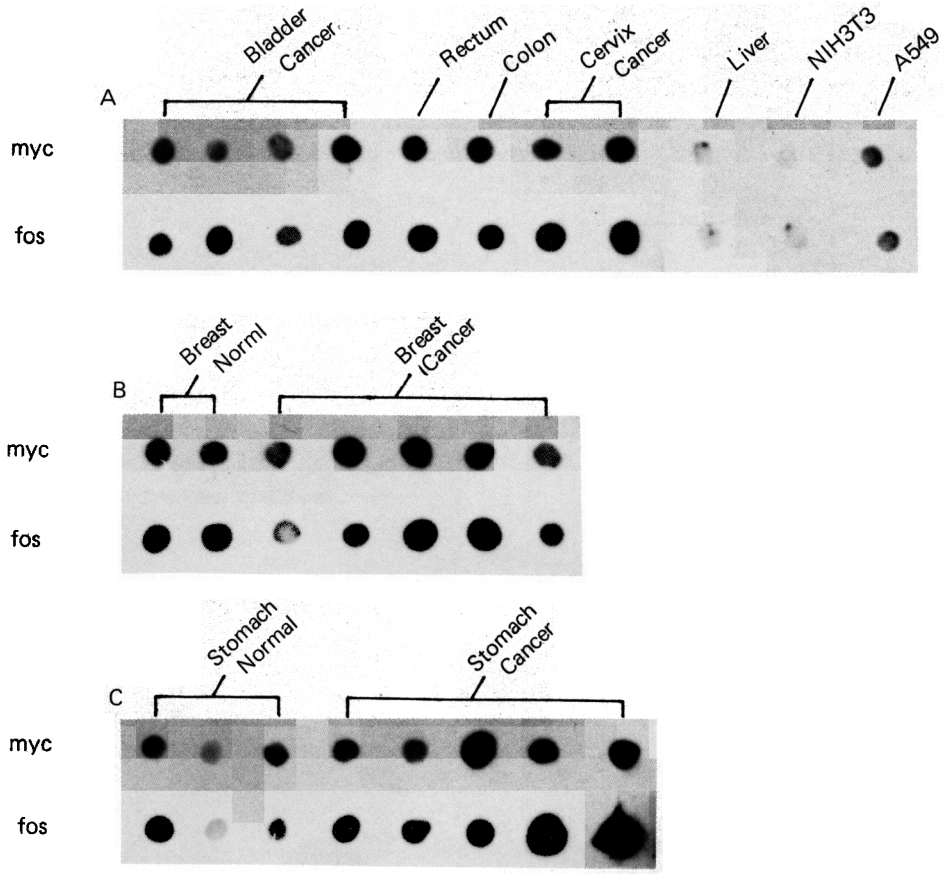

Fig. 4. c-myc and c-fos expressions in fresh human bladder, rectum, colon, cervix, breast, and stomach cancers and in their counterpart normal tissues. Liver indicates the normal liver tissue of the A/J mouse. NIH3T3 indicates NIH3T3 fibroblast, and A549 indicates human lung cancer cell line used for the technical control.

PKC activity in those fractions beyond fraction number 10 (Fig. 2). PKM activities obtained from tumor cytosols were significantly increased in the first 20 fractions and a slight increased activity in the particulate was observed in the fractions beyond number 10 as compared to the normal values (Fig. 3).

Cellular oncogene expression in human stomach and breast cancers.

RNAs purified from human tumors of the stomach and breast were examined. The expressions of c-myc and c-fos were increased in the tumors (Fig. 4). Expressions of c-fos and c-myc were prominent in all tumors and the expression of these genes in their counterpart normal tissues were also unexpectedly high especially in the stomach mucosa and breast ductal tissue (Fig. 4).

DISCUSSION

During the past few years, a novel protein phosphorylation system has been characterized in bovine cerebellum and rat brain (Inoue *et al.* 1977; Takai *et al.* 1977). This Ca^{2+}-activated PL-dependent protein kinase (PKC) may generate a Ca^{2+}-PL independent form (PKM) upon the action of Ca^{2+}-activated proteases (Kishimoto *et al.* 1983; Tapley and Murray 1984).

When we assayed the activity of PKC from fresh

human stomach and breast tumors, increased activities were observed in both the cytosol and particulate fractions as compared with the normal (Fig. 1). Notably, the prominent PKC activity in the particulate fraction of tumors compared to the low activity in normal tissue is in good agreement with the findings of PKC redistribution from the soluble fraction to the particulate fraction after treatment of the cells with phorbol esters (Kraft and Anderson 1983; Tapley and Murray 1984; White *et al,* 1983). However, total PKC activity was not changed by these exogenous stimulants. But we observed a remarkable increase in total PKC activity in tumor tissues (Fig. 1), indicating that the intracellular tumor was producing a signal.

Many investigators have reported indirect evidence of the involvement of PKC in the intracellular regulation of some growth factors (Fearn and King 1985), c-oncogenes (Coughlin *et al.* 1985; Kaibuchi *et al.* 1986; Tsuda *et al.* 1986) and in a possible role in the transformation of cultured cells as well as in tumorigenesis in animals (Macare *et al.* 1984; Whitman *et al.* 1985).

A more interesting result is shown in Fig. 2, which suggests a possible increase in PKM, as much as PKC, in tumors, and the separation of PKM from PKC on DEAE-Sephacel chromatography. Decreased PKC and increased PKM activities in the cytoplasm and in the particulate fraction have been reported in Go human B cells after treatment with TPA for 4 hours (Guy *et al.* 1986).

PKM was increased mainly in the cytosol of stomach and breast tumors (Fig. 3), but the particulate fraction of the tumor contained higher PKM activities than that normal tissues had in the later part of fractions on DEAE-Sephacel chromatography.

From the above results, we agree with the concept of the possible role of increased PKC and PKM in the autocrine control of tumor growth (Stiles 1984), although the data are not sufficient to prove it. Expressions of c-myc and c-fos were prominent in all tumors with a moderate expression of these genes in the control tissues. Expressions of c-myc and c-fos in normal tissues might be due to the continuous differentiation and regeneration of the gastrointestinal mucosa. However, we can not explain the expression of c-myc and c-fos in the normal ductal tissue of the breast.

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