The Effect of Phorbol Esters on Cell Growth and Epidermal Growth Factor Receptor Modulation in a Human Gastric Carcinoma Cell Line TMK-1

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ABSTRACT

Tumor promoting phorbol esters, 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12, 13-dibutyrate (PDBu), significantly enhanced the growth of human gastric cancer cell line TMK-1, whilst activating protein kinase C. The time course of ^{125}I-epidermal growth factor (EGF) binding to TMK-1 cells after TPA treatment showed a decrease in the number of EGF receptors on TMK-1 cells within 3 hr. Autophosphorylation of EGF receptor decreased in accordance with the decrease of EGF binding by TPA treatment. Scatchard plot analysis of TMK-1 cells after TPA treatment showed that high affinity EGF receptor disappeared at 3hr but the number of EGF receptors increased at 24 hr. These findings suggest that tumor promoting phorbol esters stimulate the cell growth through activation of protein kinase C and modification of EGF receptor of human gastric cancer cell line TMK-1.

Key words: Phorbol ester, Protein kinase C, Gastric cancer cell, EGF receptor

Phorbol ester derivatives are focused on their growth promoting and differentiating effects on a variety of cells[27]. These effects are assumed to be mediated through activation of protein kinase C[5,16]. Once activated by these exogenous agents or endogenous diacylglycerol activator, the effects of activated protein kinase C are pleiotropic[6,7,21]. We have previously reported that activity of protein kinase C in gastric cancer is significantly higher than in normal mucosa[24]. The physiological significance of protein kinase C in gastric cancer, however, has not been fully elucidated. On the other hand, we have found that many gastric cancers contain an increased level of epidermal growth factor (EGF) receptor[25,26] and that EGF stimulates cell growth and expression of mRNA for EGF receptor in gastric cancer cell lines[18,27]. In normal cells, the signal transduction system of EGF receptor appears to be tightly controlled. Negative control can occur through increase in serine and/or threonine phosphorylation of EGF receptor by protein kinase C[4,11,12]. Activation requires EGF binding followed by autophosphorylation of tyrosine residues. Since gastric cancer shows increased levels of both protein kinase C and EGF receptor expression, it is of interest to know whether the control mechanism found in normal cells is present in gastric cancer cells. We report here the effects of phorbol esters on cell growth, activation of protein kinase C and the modification of EGF receptor using human gastric carcinoma cell line TMK-1.

MATERIALS AND METHODS

Chemicals

12-O-tetradecanoylphorbol-13-acetate (TPA), phorbol-12, 13-dibutyrate (PDBu) and phorbol-13-monoacetate (P13A) were purchased from Iwai Chem. Co. (Tokyo). Human EGF was kindly supplied by Wakunaga Pharm. Co. (Hiroshima).

Cell Culture

Human gastric carcinoma cell line TMK-1 was establised from poorly differentiated adenocarcinoma in this laboratory. The characteristics of the cells have been described in detail elsewhere[17]. TMK-1 cells were maintained in RPMI 1640 (Nissui Seiyaku Co., Tokyo) containing 10% fetal bovine serum (Witterker, Maryland, USA), 100 U/ml penicillin (GIBCO), and 0.1 mg/ml streptomycin (GIBCO) under the condition of 5% CO_2 in air at 37°C.

Cell Growth

Cells were seeded in 35 mm dishes in serum free medium containing 10 μg/ml transferrin and 1 μg/ml insulin. Two days after seeding, phorbol ester derivatives were added at the concentration indicated in figure legends. The medium was changed every third day and cell number was counted using Neubauer type counting plate. All experiments were done in triplicate.

EGF Receptor Assay

EGF was ioninated by chloramine-T method using Na^{125}I-EGF[9]. The specific activity of ^{125}I-EGF ranged from 200 to 220 μCi/mg). EGF receptor was assayed as described elsewhere[18]. Numbers of

binding site and dissociation constant (Kd) was calculated by the method of Scatchard[20].

Protein Kinase Assay

Sample preparation was done by a modified method described previously[24]. About 3-5 × 10^7 cells were homogenized in 4 ml of 20 mM Tris-HCl pH 7.5 containing 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA and 0.5 mM PMSF and centrifuged at 100,000g for 60 min. The supernatant thus obtained was used as a soluble fraction. Protein kinase C was assayed by a modification of the method of Minakuchi et al[15]. The reaction was initiated by adding 5 μl of 1.25 nmol [γ-^{32}P]ATP (1.2-1.8 × 10^6 cpm/nmol) to 120 μl of a solution containing 20 mM Tris-HCl pH 7.5, 5 mM magnesium acetate, 25 μg of H1 histone, 10 μg of phosphatidylserine (Sigma), 0.3 μg of diolein (Sigma), 0.5 mM CaCl$_2$ and an enzyme fraction, and incubated at 30°C for 4 min. The acid-precipitated radioactivity was determined with a liquid scintillation counter.

EGF Receptor Phosphorylation

Membrane fraction of TMK-1 cells was prepared by the method of Usui et al[23]. Final precipitate was suspended in 20 mM Hepes pH 7.4 at a concentration of 2-5 mg protein/ml. Phosphorylation was carried out as described by Carpenter et al[3]. The reaction mixture (60 μl) containing 20 mM Hepes pH 7.4, 50 μg membrane protein, 1 μM MnCl$_2$, 10 nM NaVO$_3$ and 15 μM [γ-^{32}P]ATP (3 × 10^3 cpm/pmol) was incubated in the presence or absence of 10 nM EGF for 5 min at 0°C. The reaction was terminated by boiling for 3 min in SDS-sample buffer containing 5 mM EDTA. The sample was subjected to 8% SDS-PAGE followed by autoradiography.

RESULTS

Growth Promoting Effect of Phorbol Esters

The effect of phorbol esters on cell growth of TMK-1 is shown in Fig. 1. The cell growth was significantly stimulated by both TPA and PDBu on the 6th and 9th day at a concentration of 1 μg/ml (p < 0.01). P13A did not stimulate the cell growth. The dose dependent effect of phorbol esters was then studied. TPA and PDBu stimulated cell growth in a dose dependent manner up to 10 μg/ml (Fig. 2). P13A, however, did not show significant stimulatory effect on cell growth at any concentration.

Tumor promoting phorbol esters, TPA and PDBu are known to bind directly to and activate protein kinase C substituting for endogenous diacylglycerol but P13A does not[5]. We then examined whether protein kinase C is surely present in TMK-1 cells and phorbol esters can activate protein kinase C. As expected, TPA and PDBu increased protein kinase C activity (Fig. 3). In contrast, protein kinase C in TMK-1 cells did not respond to P13A at concentration of up to 100
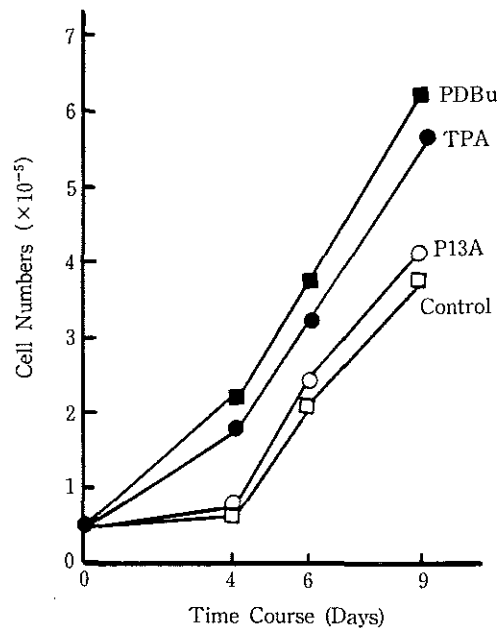

Fig. 1. Effect of phorbol esters on cell growth of TMK-1 cells. Phorbol esters (1 μg/ml) were added to about 5 × 10^4 cells in serum free medium containing transferrin (10 μg/ml) and insulin (1 μg/ml). Cell number was counted on 4th, 6th and 9th day after the addition.
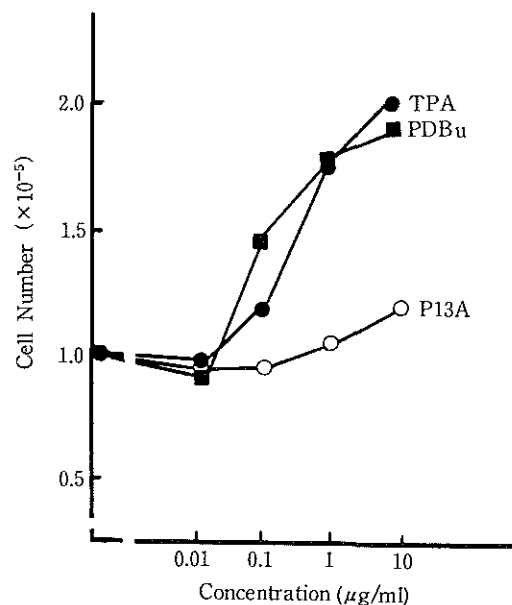

Fig. 2. Dose dependent effect of phorbol esters on cell growth of TMK-1 cells. About 5 × 10^4 cells were received phorbol esters at concentrations as indicated. Cell number was counted on 4th day after the addition.

μg/ml. Therefore, activation of protein kinase C by phorbol esters was thought to cause growth promotion of TMK-1 cells.

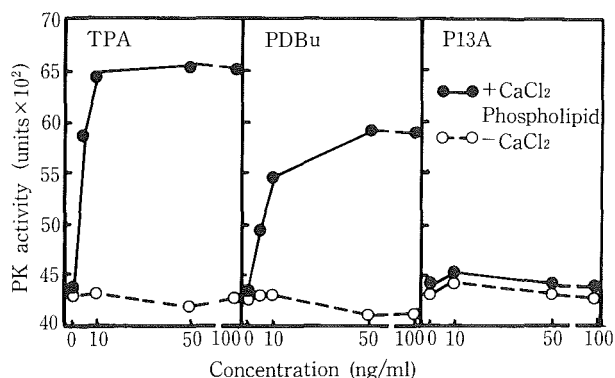### Modification of EGF Receptor Binding by Phorbol Esters

Fig. 3. Effect of phorbol esters on protein kinase C in TMK-1 cells. Enzyme fraction of TMK-1 cells was prepared and its activity was determined as described in Materials and Methods. One unit is an amount which catalyzed the incorporation of 1 nmol phosphate into H1 histone in 1 min at 30°C. Closed circles, protein kinase activity measured in the presence of $CaCl_2$ and phosphatidylserine; open circles, the activity measured in the presence of EGTA instead.
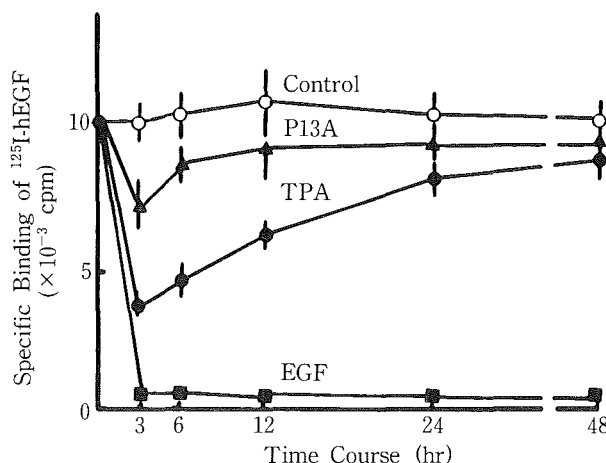

Fig. 4. Time course of effect of phorbol esters and EGF on ^{125}I-EGF binding of TMK-1 cells. The cells were treated with 1 μg/ml phorbol esters or 10 nM EGF in serum free medium for the periods as indicated. Specific binding of EGF was determined as described in Materials and Methods.
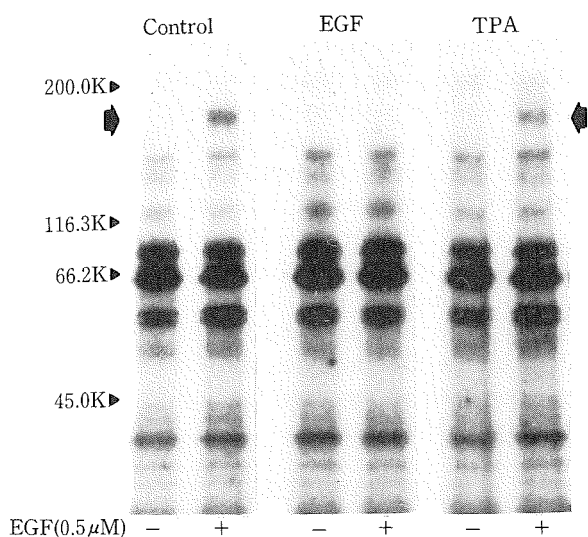

EGF(0.5μM)   −   +   −   +   −   +

Fig. 5. Effect of TPA and EGF on autophosphorylation of EGF receptor. The cells were treated with 1μg/ml TPA or 10 nM EGF for 3 hr in serum free medium. Membrabe fraction was prepared and phosphorylation was carried out as described in Materials and Methods in the presence or absence of 0.5 μM EGF.
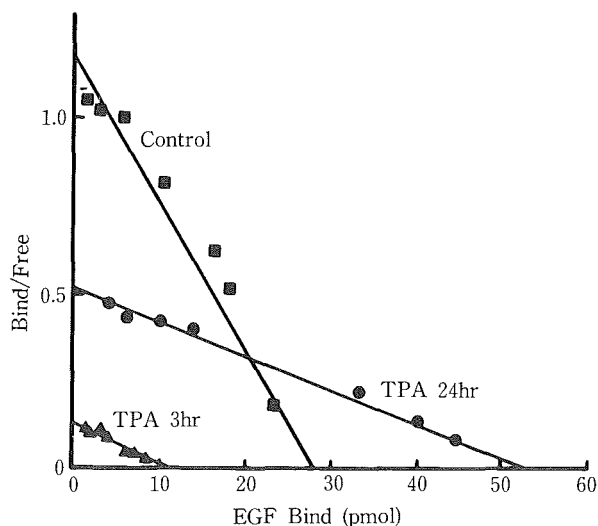

Fig. 6. Scatchard plot analysis of EGF receptor of TMK-1 cells after TPA treatment. The cells were treated with 1 μg/ml TPA for 3 and 24 hr. Specific binding of EGF was determined in Materials and Methods using ^{125}I-EGF at concentrations ranging from 12.5 pM to 1nM and analyzed by Scatchard's method[20].

It has been reported that EGF receptor is negatively regulated by protein kinase C under controlled cell proliferation[4,11,12]. We studied the time course of ^{125}I-EGF binding to TMK-1 cells after the treatment of phorbol esters or EGF (Fig. 4). TPA produced about 60% reduction in binding activity in comparison with EGF binding in control at 3hr after TPA treatment. The decrease of EGF receptor continued to at 24 hr and returned to the control level at 48 hr. P13A reduced EGF binding slightly only at 3 hr after the treatment. In con-

trast, EGF treatment dramatically decreased EGF binding at 3 hr and a very low level of EGF binding continued during EGF treatment.

Consistent with the binding data described above, autophosphorylation of EGF receptor changed on treatment with EGF or TPA for 3 hr (Fig. 5). That is, TPA treatment significantly decreased the kinase activity, while EGF treatment erased EGF receptor kinase activity completely. We confirmed that this phosphorylation of EGF receptor was mainly on tyrosine residues on the basis of sensitivity to KOH treatment[23] (data not shown). Den-

sitometric tracing showed that the intensity of EGF receptor band from TPA treated cell was 52% of the control. Autophosphorylation of EGF receptor at 24 hr after addition of TPA was nearly the same as that of control whereas that was still low when treated with EGF for 24 hr (data not shown).

We have previously reported that affinity of EGF receptor changes in growth status of TMK-1 cells[18]. We then analysed the effect of TPA on the affinity of EGF receptor of TMK-1 cells using Scatchard plot. As shown in Fig. 6, the Kd of the control cells was 0.2×10^{-9}M. Kd value of the cells increased to 1.0×10^{-9}M and 0.88×10^{-9}M at 3 hr and 24 hr after TPA treatment, respectively. Interestingly, the total binding site per cell at 24 hr after the treatment increased beyond the control level.

DISCUSSION

Many mitogen including growth factors induce a rapid turnover of phosphatidylinositol[1]. Protein kinase C is activated by diacylglycerol produced from membrane phosphoinositides upon activation of certain receptors by their agonists. It is also directly activated by tumor promoting phorbol esters such as TPA[5]. Pleiotropic effects of phorbol esters on growth of various cells may be involved in the facts that phorbol ester modulates mitogenesis through pathways converging with other mitogenic ligands and the protein kinase C represents a point of convergence in the action of a variety of mitogenic agents[6,7]. We reported here the stimulatory effect of phorbol esters on cell growth of gastric cancer cells through activation of protein kinase C. It seemed to be a direct effect of protein kinase C because we used the medium free of serum which contains lots of growth factors. However, even if the conditioned medium is serum-free, a certain concentration of transferrin and insulin was added to make cells alive. It is known that protein kinase C phosphorylates and modulates the receptor for insulin and transferrin negatively[8,14,22]. Therefore, it is difficult to assume that the stimulatory effect of phorbol ester on cell growth reported here solely depends on the modification of receptors for insulin and transferrin. Although there is a possibility that these receptors are regulated positively by protein kinase C especially in gastric cancer cells, we have to be aware that protein kinase C may activate the transcription of myc and fos oncogenes which are closely related to mitogenic response. Recently, Bjorge et al[2] have found that TPA as well as EGF stimulates concurrent accumulation of mRNA for EGF receptor and transforming growth factor a (TGFa) in breast cancer cells and they speculated that TPA may enhance autocrine growth. It is possible that this may have happened in our case.

We have previously reported that many gastric cancers contain an increased level of EGF recep-

tor and that cell growth of gastric cancer cells are stimulated by EGF[18,26]. In the present study, EGF receptor seemed to be negatively regulated by phorbol ester through protein kinase C as in cells under controlled proliferation, normal cells for instance. That is, TPA treatment reduced EGF binding to TMK-1 cells and decreased autophosphorylation of EGF receptor (see Fig. 4 and 5). We also confirmed that protein kinase C phosphorylated EGF receptor of TMK-1 cells (data unpublished). In addition, we found in this study a unique phenomenon that TPA treatment changed the affinity of EGF binding as well as the number of binding sites. TPA decreased affinity of EGF binding, whereas the number of binding sites increased at 24 hr after the treatment (see Fig. 6). It is likely that TMK-1 cells initially possess a high level of high affinity receptor and an undetectable level of low affinity receptor and that TPA erases high affinity receptor by phosphorylation at threonine-654 of EGF receptor through protein kinase C[4,12]. As aforementioned above, induced accumulation of EGF receptor mRNA by TPA reported by Bjorge et al[2] may account for this increase of low affinity receptor.

Although much remains speculative, protein kinase C and its pathway are closely related to oncogene products and growth factors, and its receptors are the key elements of the mitogenic pathway[10,13,19]. It was of interest to note in the present study that protein kinase C mediated the alterations of affinity of EGF receptor and stimulated the growth of gastric cancer cells. Further studies should be conducted to examine whether protein kinase C stimulates mRNA expression for oncogenes such as myc and fos and growth factors including EGF and TGFa.

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