Herbimycin A Inhibits Phorbol Ester-Induced Morphologic Changes, Adhesion, and Megakaryocytic Differentiation of the Leukemia Cell Line, MEG-01 (43902)

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Abstract. **12-O-Tetradecanoylphorbol 13-acetate (TPA) induces rapid changes in the morphology of the human megakaryoblastic leukemia cell line, MEG-01, as well as changes in adhesion and megakaryocytic differentiation. To investigate the signal transduction pathway of these three phenomena, we studied the effect of herbimycin A, an inhibitor of tyrosine kinase (TK) and the effects of calphostin C, a specific inhibitor protein kinase C (PKC) on TPA treated MEG-01 cells. Both herbimycin A and calphostin C inhibited all three TPA-induced phenomena, suggesting that both pathways are required for these phenomena. Herbimycin A but not calphostin C blocked the tyrosine phosphorylation of cellular proteins. Immunohistochemical staining of PKC using an anti-PKC monoclonal antibody showed that herbimycin A did not interfere with the translocation and subsequent down regulation of PKC induced by TPA, suggesting that the TPA-induced effect on PKC (translocation and probably its activation) is not dependent on TK. Induction of c-*fos* and c-*jun* expression by TPA was inhibited by both herbimycin A and calphostin C, suggesting that both PKC and TK pathways are necessary for the induction of the TPA-induced transcription factor AP1, which is a known TPA-inducible early immediate gene product. Taken together, our results show that the tyrosine kinase signal transduction system as well as the PKC pathway is indispensable for the TPA-induced phenomena of morphologic change, cell attachment, early immediate gene expression, and lineage-specific phenotypic expression in the MEG-01 cell line.** [P.S.E.B.M. 1995, Vol 209]

The process of megakaryocytic differentiation includes an increase in DNA ploidy, the expression of unique glycoproteins such as GP IIb/IIIa on the cell surface and the production of various coagulation factors. Using the established megakaryoblastic leukemia cell line, MEG-01 (1), we previously found that TPA induced-MEG-01 cells could mimic a commitment process to megakaryocytic differentiation, and that aphidicolin, a specific inhibitor of DNA polymerase-α blocked this process (2). Aphidicolin also induces the erythroid differentiation of a human erythroleukemia cell line, K562 (3). These results indicate a close relationship between DNA replication and the selection of differentiation lineage of established leukemia cell lines.

Investigation of EGF and PDGF receptors (4, 5) has shown that tyrosine phosphorylation is an important step in the signal transduction pathway during cell proliferation. The signal transduction pathways of cy-

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tokines and their receptors have come to be better understood (6, 7), but the precise differentiation mechanism of the megakaryocytic lineage remains to be determined.

Another recent advance in signal transduction analysis has been the development of specific inhibitors of various kinases. Herbimycin A is one of these agents and is a specific inhibitor of tyrosine kinase (TK) (8). Inhibition of tyrosine phosphorylation by herbimycin A can block the transforming activity of oncogenes such as *src, abl, fps,* and *yes* (9, 10). It also causes phenotypic reversion of Rous sarcoma-transformed cells (11) and induces the erythroid differentiation of K562 and Friend cells (12, 13).

In the present study, we investigated the effect of herbimycin A on various TPA-induced phenomena (rapid shape change, attachment, and GP IIb/IIIa expression) in MEG-01 cells. We also studied some of these effects in two human erythroleukemia cell lines, HEL and K562, which have the potential for megakaryocytic differentiation.

Protein kinase C (PKC) is reported to be the receptor for TPA, and we, as well as others, have previously shown that PKC undergoes translocation in MEG-01 cells treated with TPA (14). To determine the relationship between the PKC signal and tyrosine kinases, the effect of a specific protein kinase C inhibitor, calphostin C (15), also was investigated using MEG-01 cells after TPA induction.

We show here that the involvement of the tyrosine kinase signal transduction system in TPA-induced events in MEG-01 cells, including early immediate gene expression, cytoskeletal changes, DNA replication and differentiation.

Materials and Methods

Cells Lines and Reagents. MEG-01 (2), HEL, and K562 cells were maintained as previously described (3). TPA and phorbol were purchased from Sigma Chemical Co. (St. Louis, MO). Herbimycin A was a generous gift from Dr. Y. Uehara (National Institute of Health, Tokyo, Japan). The PKC inhibitor, calphostin C, was the generous gift of Dr. T. Tamoaki, (Kyowa Hakko Co., Tokyo, Japan). Mouse mono-clonal antibodies against PKC isozymes (clone MC-1a for type I, MC-2a for type II and MC-3a for type III) were obtained from Dr. H. Hidaka (The Department of Pharmacology, Nagoya University School of Medicine).

Morphologic Change of MEG-01 Cells. MEG-01 cells were washed twice with phosphate buffered saline (PBS), resuspended in PBS at 1×10^6/ml, and incubated with 10^{-7} *M* TPA for 30 min. Then, an equal volume of 8% paraformaldehyde in PB was added and the cells were left at room temperature for 20 min for fixation. To analyze the effect of various inhibitors, cells were pretreated with each of the inhibitors for 2–4 hr before addition of TPA. Cells with pseudopods were counted as described previously (16). Data are expressed as the mean ± SD of three separate experiments.

Cell Adhesion Assay. MEG-01 cells (3×10^5/ml) were plated for 12–24 hr in 25-mm plastic dishes in triplicate, and inhibitors (0.1 or 1 μg/ml of herbimycin A or 10 or 100 n*M* of calphostin C) were added 2–4 hr before the addition of TPA, and was present in the whole culture period. Twelve to twenty-four hours after TPA addition, the floating cells were collected carefully. After gentle washing with PBS, adherent cells were recovered with trypsin + EDTA in PBS. The number of cells in each sample was determined, and the percentage of adherent cells was calculated.

Detection of Glycoprotein IIb/IIIa and IIIa. MEG-01, HEL, and K562 cells (1×10^5 cells/ml) were cultured with or without TPA (10^{-7} *M*) for 5 days. Inhibitors (1 μg/ml of herbimycin A or 100 n*M* calphostin C) were added 4 hr before the addition of TPA, and were present in the whole culture period. In some culture, 4-phorbol 12,13 didecanoate was used instead of TPA. After collecting the cells, cytospin slide were made. Expression of glycoprotein (GP)IIb/IIIa (in MEG-01 and HEL cells) and IIIa (in K562 cells), a characteristic of the megakaryocytic lineage, was detected using Dako's LSAB-staining kit (Dako, Carpinteria, CA) as described previously (2). Mouse monoclonal antibody against human GP IIb/IIIa, TP80, was purchased from Nichirei Co. (Tokyo, Japan). It recognizes human GP IIb/IIIa complex and was authorized in the Fourth International Workshop and Conference on Human Leukocyte Differentiation Antigens (Vienna, 1989). It does not cross-react with $\alpha v\beta_3$. Mouse monoclonal antibody against GP IIIa was purchased from Dako (Dako, Carpinteria, CA).

Immunocytochemical Detection of PKC. MEG-01 cells were plated on coverslips. Each coverslip was rinsed in 10 m*M* PBS and moved into the reaction medium (145 m*M* NaCl, 1 m*M* $MgSO_4$, 5 m*M* KCl, 1 g/liter glucose, 20 m*M* Hepes, pH 7.4, 1 m*M* $CaCl_2$). Herbimycin A (1 μg/ml) was added to TPA + herbimycin samples 3 hr before the addition of TPA. After treating the coverslip with TPA (1×10^{-7} M) or TPA + herbimycin A (1 μg/ml) at 37°C for the appropriate incubation period, the coverslips were immersed immediately in 4% paraformaldehyde/0.1 *M* phosphate buffer (PB) for 30 min, rinsed in PB, and subsequently incubated in 0.1% Triton X100/PB for 15 min, then rinsed three times with PBS. After incubation with 5% normal goat serum for 20 min to block to nonspecific binding sites, the coverslips were incubated with antibody solution against PKC (1:100 dilution of mouse ascitic fluid) for 60 min. According to the previous report describing the characterization of these anti-

bodies against PKC isozymes using MEG-01 cells (14), antibody against PKC isozyme type II (MC-2a) was mainly used. Then they were rinsed three times with PBS, incubated with the second antibody, fluorescein-conjugated goat F(ab)'$_2$ anti-mouse IgG (Tago, Inc., Burlingame, CA) (1:50 dilution) for 45 min, rinsed, and then mounted with glycerol containing 5% n-propyl galate. All these procedures were carried out at room temperature. A confocal laser scanning microscope MC 500 (Bio-Rad Inc., Richmond, CA) was used for the observation as well as taking pictures.

Immunoblotting with Anti-phosphotyrosine Antibody. MEG-01 cells were cultured for 1 day in the same way as described in the section of the detection of GP IIb/IIIa. Herbimycin A (1 μg/ml) or calphostin C (100 n*M*) were added several hours before the addition of TPA (10^{-7} *M*). Serum deprivation was not performed before addition of TPA to the culture medium. After collecting cells, the detection of phosphotyrosine-containing proteins was done as described previously (17). In brief, cell pellets were suspended in Sol buffer (10 m*M* Tris hydrochloride, pH 7.4, 1% sodium dodecyl sulfate (SDS), 1 m*M* sodium orthovanadate, 0.1 m*M* sodium molybdate, 1 m*M* phenylmethyl-sulfonyl fluoride) and immediately heated in boiling water for 5 min. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA), and 100 μg of protein was analyzed by SDS-7.5% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose filter using a transfer buffer (25 m*M* Tris glycine, pH 8.6, 20% methanol), and were stained with an anti-phosphotyrosine antibody (anti-PTYR) followed by incubation with [^{125}I]-labeled protein A (Amersham Corp., Buckinghamshire, England). Autoradiography was performed using x-ray film and an intensifying screen at −80°C for 24 hr unless otherwise indicated.

***De novo* DNA synthesis.** Cultured MEG-01 cells were labeled with [^{3}H]thymidine for 2 hr. The uptake of radioactivity was analyzed as described previously (3).

Northern Blotting. Northern blotting was performed as described previously (18). The culture condition of MEG-01 cells was the same as those described in the section of GP IIb/IIIa detection method. Total RNA was extracted from cells according to the method of Chomczynski *et al.* (19). Ten micrograms of total RNA was electrophoresed on 1% agarose gel and Northern blotting was performed using cDNA probes for c-*fos* ($EcoR_1$ digest), c-*jun* (Bam H_1-$EcoR_1$ digest), and β-actin (Pst_1 digest). c-*fos* cDNA was obtained from the Japan Cancer Research Resources Bank (Yokohama, Japan), while c-*jun* cDNA was the generous gift of Dr. R. Tjian (UC Berkeley, Berkeley, CA). The cDNA probes were labeled with ^{32}P using a Boehringer Mannheim random labeling kit (Mannheim, Germany).

Results

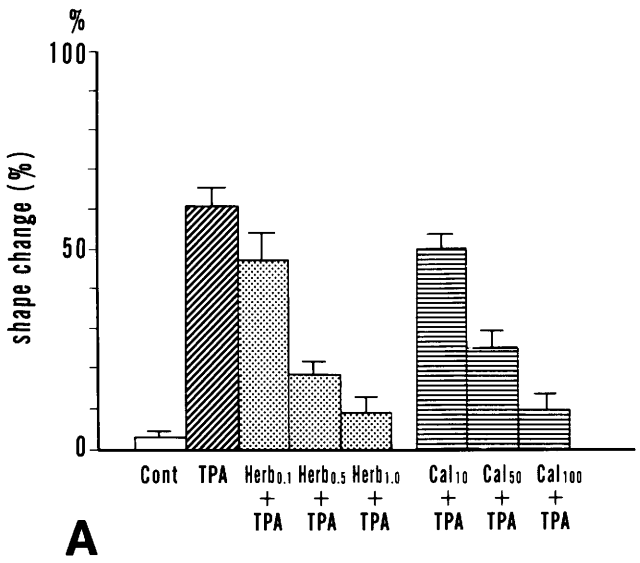
Effect of Herbimycin A and Calphostin C on TPA-Induced Rapid Change in Morphology. TPA treatment induced a remarkable shape change (pseudo-pod like) in around 60% of cells under our experimental conditions. However, pretreatment with herbimycin A strongly suppressed the induction of shape changes in MEG-01 cells by TPA (Fig. 1A). Calphostin C also inhibited TPA-induced shape changes. Figure 1B shows typical photomicrographs of control, TPA-treated, and kinase inhibitor + TPA-treated MEG-01 cells 30 min after the addition of TPA. Cells treated with TPA alone showed many pseudo-pod-like structures on their surface.

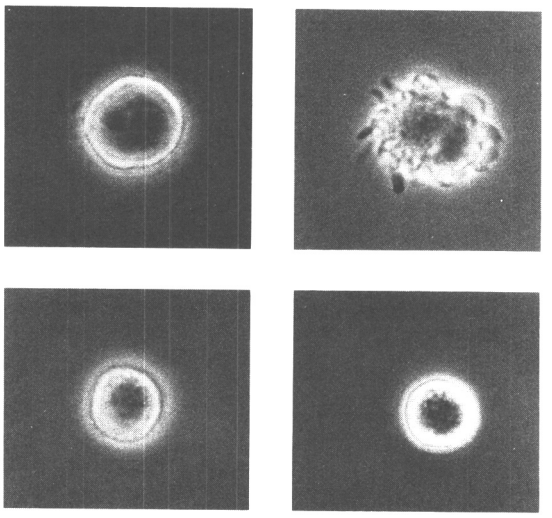
Effect of Herbimycin A and Calphostin C on TPA-Induced Adhesion of MEG-01 Cells. Control cells mostly remained in suspension, although some cells (30%) showed weak adhesion (Fig. 2A). TPA treatment reverted most of MEG-01 cells (80%) to adherent state, and they became firmly attached to the culture flask or plate. Pretreatment of cells with either herbimycin A or calphostin C inhibited TPA-induced attachment (Fig. 2A). Figure 2B shows photomicrographs of control, TPA-treated, and TPA + kinase inhibitor treated MEG-01 cells. The round cells with white halos are free cells lying on the adherent cell layer.

Inhibition of TPA-Induced Megakaryocytic Differentiation by Herbimycin A and Calphostin C. TPA induced GPIIb/IIIa or IIIa expression in MEG-01, HEL and K562 cells (Fig. 3). Herbimycin A, and calphostin C both strongly inhibited TPA-induced glycoprotein expression in all cell lines tested (Fig. 3). Although TPA was a strong inducer, its derivatives, 4-phorbol 12,13 didecanoate (4PDD) and 4-α phorbol, which show no tumor-promoting activity, did not induce glycoprotein expression.

Effect of Herbimycin A on Phosphotyrosine-Containing Proteins. Within 1 min of treatment with 10^{-7} M TPA, tyrosine phosphorylated bands, p110, increased significantly, and the increase in p110 persisted thereafter (Fig. 4). The p160 band increased slightly. Another band, p130, showed a slight decrease of intensity in most of the experiments. Herbimycin A pretreatment almost completely abolished the tyrosine phosphorylated bands, while pretreatment with calphostin C did not affect the general pattern of TPA-induced changes in the tyrosine phosphorylated protein bands (Fig. 4).

Effect of Herbimycin A on TPA-Induced PKC Translocation and Subsequent Downregulation Induced with TPA. In preliminary experiments, we confirmed our previous observation (14) that only an

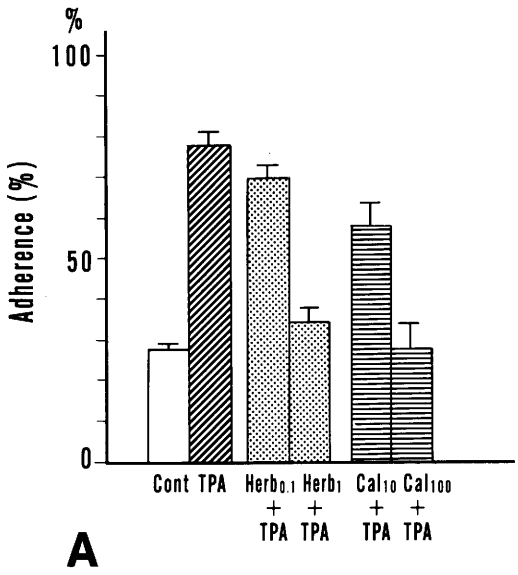


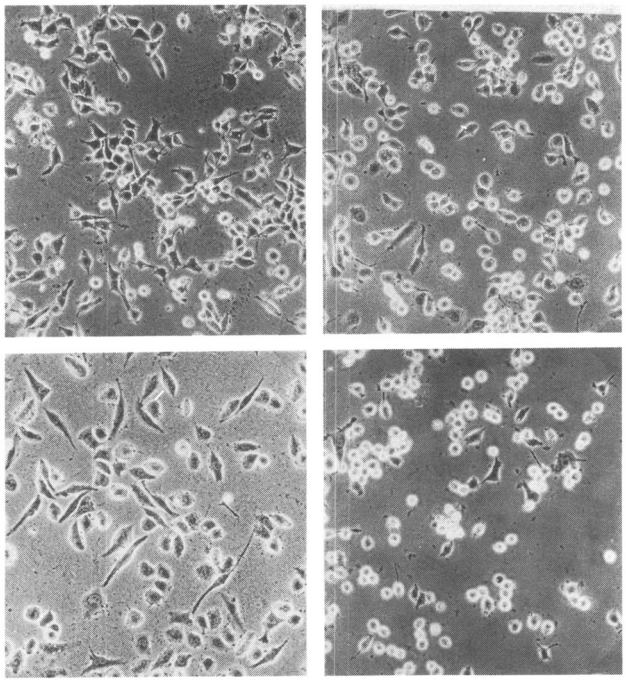


B

Cont	TPA
TPA + Herb	TPA + Cal

Figure 1. Effect of various kinase inhibitors on TPA-induced morphologic changes in MEG-01 cells. (A) After pretreatment of MEG-01 cells with herbimycin A (0.1, 0.5, or 1.0 μg/ml), or calphostin C (10, 50, or 100 n*M*) for 2 hr, TPA (1×10^{-7} *M*) was added and incubation was continued for 30 min. After fixation, cells showing the characteristic morphologic response were counted. Data are the mean ± SD from three separate experiments. Cont = untreated cells: TPA = MEG-01 cells treated with 10^{-7} *M* TPA; $Herb_{0.1,\ 0.5,\ or\ 1.0}$ + TPA, cells treated with 0.1, 0.5, or 1.0 μg/ml herbimycin A + 10^{-7} *M* TPA. $Cal_{10,\ 50,\ or\ 100}$ + TPA denotes 10, 50, or 100 n*M* calphostin C + 10^{-7} *M* TPA. (B) Photomicrographs of typical morphologic changes of MEG-01 cells. Cont, TPA, TPA + Herb, TPA + Cal denote untreated, TPA-treated, TPA + herbimycin A 1.0 μg/ml-treated, TPA + calphostin C 100 n*M*-treated MEG-01 cells, respectively. Only cells treated with TPA alone show a morphologic change.





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TPA	TPA + Cal

Figure 2. Effects of kinase inhibitors on TPA-induced adhesion of MEG-01 cells. (A) MEG-01 cells were treated with herbimycin A (0.1 or 1 μg/ml) or calphostin C (10 or 100 n*M*) for 2 hr. Twenty-four hours after the addition of 10^{-7} M TPA, cells in suspension and adherent cells were collected separately and the percentage of adherent cells was determined. (B) Photomicrographs of adherent MEG-01 cells. After incubation with TPA for 24 hr, floating cells were removed and the adherent cell layer was gently washed with PBS. In order to adjust the confluence of adherent cells for photomicrographic presentation, the initial cell concentration was increased 2-fold in the control and TPA + herbimycin A-treated groups compared with the others only in this experiment.

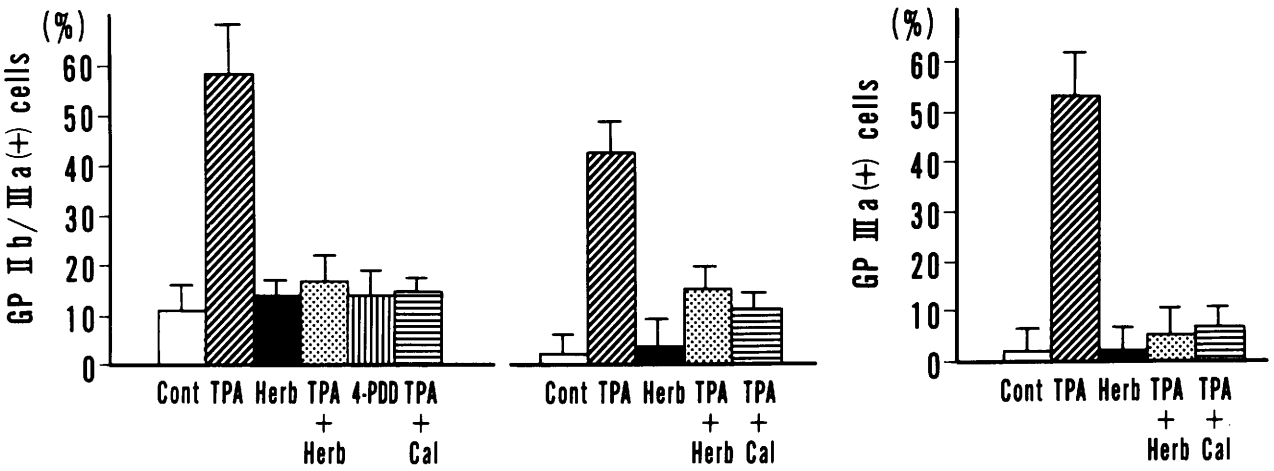

Figure 3. Glycoprotein IIb/IIIa (GP IIb/IIIa) or GP IIIa expression by MEG-01, HEL, and K562 cells. Cells (1×10^5/ml) were cultured with 1×10^{-7} *M* TPA, 1 μg/ml herbimycin A (Herb) or both TPA + Herb. Control cells were cultured without reagents. GP IIb/IIIa or GP IIIa expression was measured on Day 5. 4PDD denotes 1×10^{-7} *M* 4-phorbol 12,13 didecanoate-treated cells. TPA + Cal denotes 10^{-7} *M* TPA + calphostin (100 n*M*)-treated cells. The ordinate shows the percentage of GP IIb/IIIa-positive (MEG-01 and HEL) or GP-IIIa-positive (K562) cells. Results represent the mean ± SD from three separate experiments.

antibody against PKC type II (clone MC-2a) gave positive staining with MEG-01 cells. Antibodies against PKC type I and III did not give an significant staining. Control cells showed weak and almost homogeneous staining of the cytoplasm with anti PKC type II antibody (Fig. 5). Within 5 min after cells were treated with 100 n*M* TPA, the immunofluorescein increased on the plasma membrane in more than 80% of cells. The membrane staining gradually decreased and only a slight staining remained after 12 hr (Fig. 5D). The addition of herbimycin A did not affect these TPA-induced changes in PKC localization (Fig. 5 C and E).

Inhibition of c-*fos* and c-*jun* Gene Expression by Herbimycin A. Expression of both genes began to increase 1 hr after TPA stimulation, persisted for several hours, and declined after 6 hr (Fig. 6). Herbimycin A as well as calphostin C inhibited this induction process completely. The pattern of β-actin mRNA is shown as an internal control.

Discussion

TPA, a tumor-promoting phorbol ester has been used in a wide variety of *in vitro* and *in vivo* systems, and produces numerous effects on target cells (20, 21). TPA is one of the few agents that induce megakaryocytic phenotypes in established leukemia cell lines (1, 22, 23). There have been few analyses of the signal transduction pathways involved in megakaryocytic differentiation (24, 25).

TPA caused sequential changes of MEG-01 cells (i.e., rapid and reversible formation of pseudopod-like structures on the cell surface within minutes, adhesion to the culture vessel within hours, and subsequent megakaryocyte lineage-specific phenotypic expression of GP IIb/IIIa), while phorbol analogs lacking tumor-promoting activity could not induce these phenomena. PKC is known to be the receptor for TPA (26), and TPA induces the rapid translocation of PKC (bII) from the cytoplasm to the cell membrane in MEG-01 cells (14). However, it is too simple to propose that all these phenomena are caused solely by the PKC signal transduction system, and several recent reports have suggested that this is not so. Differential translocation and

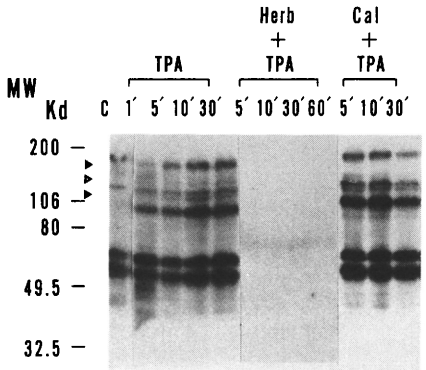

Figure 4. Changes of phosphotyrosine in MEG-01 cells. MEG-01 cells were treated with TPA (1×10^{-7} M), TPA + herbimycin A (1 μg/ml) (T + herb), TPA + 100 n*M* calphostin C (T + Cal). After the indicated times, cells were collected and their extracts were electrophoresed, transferred onto a membrane, and stained with anti-phosphotyrosine antibody. Molecular weights are given on the ordinate. Solid arrowheads (p160, p110) denote the bands showing increased intensity compared with the control. The open arrowhead (p130) denotes a band with a slight decrease of intensity shortly after TPA addition in most experiments compared to the control.

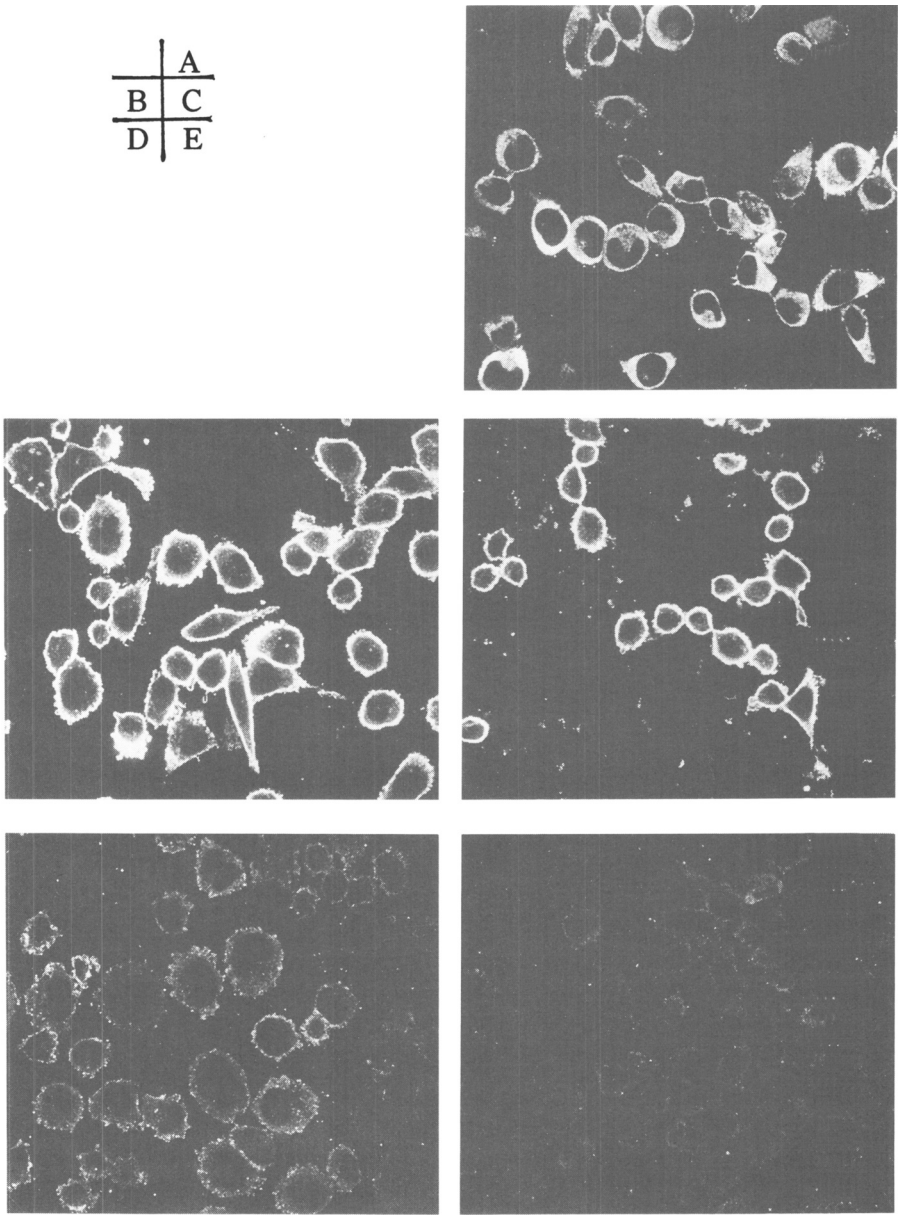

Figure 5. Immunohistochemical detection of PKC type II. MEG-01 cells were treated with or without herbimycin A for 3 hr and TPA was added after the medium was changed to reaction buffer A described in Materials and Methods. Herbimycin A was present in the TPA + herbimycin A group during TPA treatment. After the appropriate incubation period, coverslips were processed for microscopic observation as described in Materials and Methods. (A) Untreated cells; (B and D) TPA treatment for 5 min and 12 hr; (C and E) TPA + herbimycin A treatment for 5 min and 12 hr.

the different roles of PKC isozymes have been reported (27). Jalava *et al.* (28) and Yen *et al.* (29) have reported that staurosporine, a powerful but less specific PKC inhibitor, induces neuronal differentiation of human neuroblastoma cells through a PKC-independent pathway and the megakaryocytic differentiation of K562 cells.

In experiments using enzyme inhibitors, their specificity is most important for evaluating the result. According to a previous publication (30), the IC_{50} of calphostin C for PKC is 0.05 μM, for protein kinase A (PKA) is more than 50 μM, and for tyrosine kinase p60v-*src* is more than 50 μM. In the case of staurosporine, the IC_{50} values were 0.0027 μM (for PKC) and 0.0054 μM (for PKA). As herbimycin A is readily inactivated by the reducing agents required to maintain the activities of the purified kinases, the determination of its IC_{50} is practically difficult (31). However, several methods have been developed for the detection of PKC, PKA, TK, and calmodulin-dependent protein kinase activity without the use of reducing agents (32, 33). Using these methods, herbimycin A

has been proven not to be inhibitory to PKC and PKA. Immuno-histochemical detection using an anti-PKC type II antibody (Fig. 6) showed that herbimycin A causes no apparent inhibition of the initial phase of PKC translocation from the cytoplasm to the membrane and subsequent down regulation induced by TPA (Fig. 6).

Morphologic changes induced by TPA (Fig. 2B) suggest that the signal is connected to changes in the cytoskeleton. Herbimycin A and calphostin C could both inhibit the rapid shape changes induced by TPA, which resemble the platelet shape changes induced by thrombin or TPA (34). Several reports have described the relationship between phosphotyrosine-containing proteins and intracellular signal transduction systems, such as the elevation of Ca^{++} and PKC activation (35). The relationship between tyrosine phosphorylation of proteins and cell adhesion remains unclear, but a possible candidate is focal adhesion kinase (FAK), a tyrosine kinase localized at focal adhesion plaques (36).

The c-*fos* and c-*jun* complex, also known as the AP-1 transcription factor, represents early immediate genes inducible by various agents such as serum factor and TPA (37). In other TPA-inducible leukemia cell lines, c-*fos*/*jun* expression is reported to be correlated with differentiation induction (38, 39). Normal immature megakaryocytes are reported to express more c-*fos*/*jun* protein than mature megakaryocytes (40). Our Northern blotting analysis shows that herbimycin A and calphostin C inhibited TPA-induced c-*fos* and c-*jun* gene expression. This result suggests that tyrosine phosphorylation is located upstream of *fos*/*jun* gene expression, and that these gene expression are one of the putative candidates for the common target of the PKC and tyrosine kinase signal pathways.

Herbimycin A inhibited more than 80% of *de novo* DNA synthesis by MEG-01 cells (data not shown) and cell growth was almost completely inhibited by herbimycin A. These results support our previous observation that DNA replication is necessary for the megakaryocytic differentiation of these cell lines (2, 41). Okabe *et al.* (42) have reported that the inhibitory effect of herbimycin A on the growth of established cell lines is dependent upon the presence of abnormal bcr/abl TK activity. K562 and MEG-01 cells are Ph_1-positive, but HEL cells are not. Herbimycin A inhibited the growth of HEL cells to the same extent as that of K562 and MEG-01 cells (data not shown), so the phenomena observed were not solely *bcr*/*abl* related.

According to our immunohistochemical detection method, the inhibitory effect of herbimycin A is not due to changes in the PKC activation step itself induced by TPA. Under the present experimental conditions, herbimycin A but not calphostin C abolished the tyrosine phosphorylated bands in whole cell lysates, although both agents inhibited TPA-induced rapid shape changes. There are various alternative models potentially suggested by the present data. The simplest model might be that TPA is upstream of both PKC and TK and that both of these (the PKC and TK) are needed to cause changes in fos and jun transcription, and subsequent cellular changes in shape, adherence and GP IIb/IIIa expression.

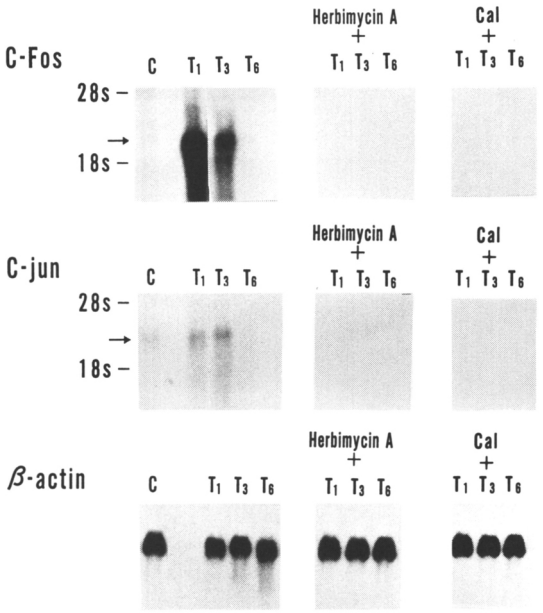

Figure 6. Effect of herbimycin A on c-*fos* and c-*jun* gene expression by MEG-01 cells treated with TPA. MEG-01 cells were cultured with TPA or TPA + herbimycin A (1 μg/ml) or TPA + calphostin C (100 n*M*). After 1, 3, and 6 hr, RNA was extracted and Northern blotting was performed. Arrowheads show the position of c-*fos* and c-*jun* RNA, while 28s and 18s indicate ribosomal RNA. β-actin mRNA is the internal control.

Considering a previous report that herbimycin A can induce the erythroid differentiation of K562 and Friend cells (12, 13), and our present result that herbimycin A inhibits TPA-induced megakaryocytic differentiation, suggests that the TK system is very important for, or the determinant of, the selection of differentiation between the erythroid and megakaryocytic lineages. Although we can not point out which protein (or tyrosine phosphorylated protein) is the most important for lineage determination at present, one of the putative candidates is lineage-specific transcription factors, the GATA families. GATA-1 has been reported to be present in cells of in both erythroid and megakaryocytic lineage, including untreated MEG-01 cells, and is thought to play an essential role in their differentiation process (43). Little is known about quantitative changes in GATA-1 and 2 or the phosphorylation status of these factors with different inducers. Future analysis of the exact link between the PKC system and the TK system in MEG-01 cells may reveal the real commitment factors in each differentiation lineage.

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