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Effect of Various Factors on Early THP-1 Cell Adhesion Induced Phorbol 12-Myristate 13-Acetate (PMA)

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We evaluated the effects of various factors (e.g., serum, inhibitors of protein synthesis, and cytoskeleton and protein kinases) on early PMA-induced THP-1 cell adhesion using an adhesion assay with Sulforhodamine B (SRB) staining, which was used to assess the proliferation of the attached cells. THP-1 cell adhesion to a plastic substrate was detected 1 hr after exposure to Phorbol 12-Myristate 13-Acetate (PMA) and peaked after 18 hr. At concentrations > 25 nM PMA, the level of adhesion did not change. Based on our preliminary results, we used 25 nM PMA and 5 hr of culture as standard assay conditions. Early PMA-induced cell adhesion was not affected by the presence of serum or PD 98059 in the culture medium, but was affected by the addition of PKC inhibitors and cycloheximide. In the presence of actin inhibitor with PMA, the cell adhesion increased when comparing with PMA treatment only. Thus, early PMA-induced adhesion of THP-1 cells does not require serum in the culture medium, MAP-kinase activation, or actin polymerization, but does require *de novo* protein synthesis and PKC activation. Our SRB-based cell adhesion assay may be used to screen other PKC inhibitors.

Key words : Cell adhesion, monocytic cells, Phorbol 12-myristate 13-acetate (PMA), protein kinase C

Introduction

Protein kinase C (PKC), a serine-threonine protein kinase, is present in most cell and tissue types of the body [28,36]. At least 11 isotypes of PKC have been identified; these play important roles in cellular proliferation, differentiation, and apoptosis [12,22,25,27,29]. Recent studies have shown a correlation between the activation of specific PKC isotypes and certain diseases, including cancer and diabetic vascular complications [11,15,20,21]. Based on these data, several specific and partially specific PKC inhibitors have been developed as therapeutic agents [16,39]. The PKC isoforms can be divided into three subfamilies, i.e., conventional, novel, and atypical, based on their particular second messenger requirements. Conventional PKCs depend on calcium and diacylglycerol (DAG), novel PKCs depend on DAG, and atypical PKCs function independently of calcium and DAG [2,6,17,18]. Phorbol 12-myristate 13-acetate (PMA), an analog of DAG, is widely used as an activator of PKC to investigate the roles of the kinase in cellular processes such as proliferation, differentiation, and apoptosis [28].

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In vitro exposure to PMA causes monocytes to differentiate into macrophages via a PKC-dependent mechanism that involves the activation of multiple downstream kinases, including c-Raf and MAP-kinase [3,5,19,32]. During this process, monocytes in suspension adhere to the substratum of a culture dish and subsequently spread [37]. Most studies of PMA-induced monocyte differentiation have focused on diverse features of differentiated macrophages, including the ability to perform phagocytosis, metabolic activity, the presence of surface markers, and the ability to interact with endothelial cells [7,23,24,31,32,37,41]. However, little is known about the regulatory mechanism of early PMA-induced adhesion of monocytic cells to the substratum. Thus, we examined the effects of various factors, including serum, protein synthesis inhibitors, actin inhibitors, and protein kinase inhibitors, on early PMA-induced monocytic cell adhesion.

Materials and Methods

Chemicals and materials

PMA, nocodazole, cytochalasin D, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PD 98059, Go 6983, and bisindolemaleimide-1 (Bis I) were obtained from Calbiochem (San Diego, CA, USA). All

chemicals were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. In all experiments, the final concentration of DMSO was ≤0.1%. Mouse monoclonal phospho-ERK1/2, rabbit polyclonal ERK2, and goat polyclonal actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal phospho-c-Raf antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA, USA). SRB was obtained from Sigma Chemical Co. and dissolved 0.1% (v/v) acetic acid.

Cell culture

The human monocytic leukemia cell line THP-1 was purchased from the ATCC (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium supplemented with L-glutamine and penicillin/streptomycin plus 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 37°C.

Cell adhesion assay

To test for cell adhesion, 5×10^4 cells were added to polystyrene flat-bottomed 96-well plates (Nunc, Roskilde, Denmark). The cells were incubated with various chemical inhibitors for 1 hr and then stimulated with PMA. At the end of the culture period, the cells were washed once with D-PBS and fixed in 3.7% formalin for 1 hr. The plates were vigorously washed five times in tap water to remove non-adherent cells and thoroughly oven-dried at 37°C. Subsequently, 100 μl of 0.4% (w/v) SRB was added to each well, and the cells were incubated for 1 hr at room temperature and then quickly rinsed four times with 0.1% acetic acid to remove any unbound dye. The plate was then completely oven-dried at 37°C. For elution of the dye, 100 μl of 10 mM Tris base solution (pH 10.5) was added to each well, and the plate was rotated on a gyratory shaker for 1 hr. Subsequently, the optical density (OD) was measured at 540 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) [35]. The results are expressed as percentages based on the highest and lowest OD, which were compared against a blank well (i.e., without cells). The maximum OD was defined as 100%.

Immunoblotting

THP-1 cells (5×10^5) were precultured in serum-free RPMI for 1 hr and then cultured with the indicated inhibitors and 25 nM PMA in serum-free RPMI in a six-well plate. The suspended and attached cells were harvested using a rubber policeman after 1, 3, and 5 hr of culture. The cells were then washed with D-PBS and sonicated in lysis buffer (10 mM Tris-HCl, pH 7.6, 137 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM EDTA, 1% (v/v) NP-40, and a protease inhibitor cocktail [Sigma Chemical Co.]). The lysates were then centrifuged at 10,000× *g* for 30 min at 4°C. The protein concentration of each sample was adjusted using a colorimetric assay. The supernatant was subsequently boiled for 5 min in 2 x Laemmli sample buffer. After denaturing, the proteins were separated on 10 or 12% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was incubated in blocking solution (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 5% skim milk) for 1 hr and then incubated with a 1:200 antibody dilution. Immunoreactivity was detected using a Blotting Detection Kit (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions.

Results

Effect of PMA concentration, serum, and culture period on early cell adhesion

Cells were cultured with various concentrations of PMA (3.1 to 100 nM) for 18 hr and evaluated for cell adhesion by SRB-staining. Cell adhesion was detected from 6.3 nM PMA and peaked at 50 nM (Table 1). To determine the time frame for adhesion, cells were treated with 25 nM

Table 1. Effects of PMA concentrations, incubation times, and the presence of serum on early cell adhesion

Effect of PMA concentration	Concentration (nM)	% of cell adhesion
Various concentration of PMA	0	4.3±2.1
	3.1	8.7±1.9
[10% FBS, 5h incubation time]	6.3	26.3± 5.2
	12.5	62.3±5.8
	25	96.3±1.8
	50	99.7±1.1
	100	97.7±2.3
Effect of incubation time	**Incubation time (h)**	
Various incubation time	0	3.7±1.5
	1	28.0±6.7
[10% FBS, 25 nM PMA]	3	60.3±6.2
	5	85.3±4.3
	18	97.0±2.2
	24	95.3±2.0
Effect of serum concentration	**Concentration (%)**	
Various FBS concentration	0 (without PMA)	6.0±1.7
	0	97.7±1.5
	1.3	94.3±3.0
[5 h incubation time, 25 nM PMA]	2.5	92.7±2.8
	5	89.0±3.4
	10	89.3±4.8

THP-1 cells were cultured with (A) the indicated concentrations of PMA for 5 hr, 25 nM PMA for the indicated times, or the indicated serum concentrations for 5 hr, and subjected to a cell adhesion assay. The values are the percentage ± standard error, n=6.

PMA and adhesion was evaluated after 1, 3, 5, 18 and 24 hr. Adhesion was detected by 1 hr, gradually increased, and peaked after 18 hr. Based on these results, we used 25 nM PMA and 5 hr of culture as standard assay conditions. We then tested the effect of serum on cell adhesion. Cells were washed twice with serum-free RPMI medium and seeded on a 96-well plate before treatment with 25 nM PMA plus various concentrations of serum. After 5 hr, cell adhesion was assayed. Our results indicate that serum did not require the adhesion of THP-1 cells, rather showed a tendency to increase early cell adhesion in the absence of serum (Table 1).

Effect of various inhibitors on early cell adhesion

We used cycloheximide, which is a general protein synthesis inhibitor, to examine whether *de novo* protein synthesis is required for early PMA-induced cell adhesion. As can be seen in Table 2, early cell adhesion was inhibited by cycloheximide, indicating that the early PMA-induced adhesion of THP-1 cells requires *de novo* protein synthesis. We also tested the effect of cytochalasin D, an inhibitor of actin polymerization, on early cell adhesion; the results showed additive effect rather than inhibition effect on cell adhesion. PMA activates PKC, which in turn activates several MAP kinases (ERK1/2) via c-Raf and MEK kinase signaling. Thus, we examined the effect of an MEK kinase inhibitor, PD98059, and the PKC inhibitors Go 6983 and Bis I on early cell adhesion. PD98059 did not inhibit early PMA-induced cell adhesion, even at 40 μM, whereas Go 6983 and Bis I successfully inhibited early cell adhesion in a concentration-dependent manner (Table 2). We confirmed the phosphorylation of MAP-kinase and c-Raf in PMA-exposed THP-1 cells following inhibitor treatment by Western blotting. The treatment of THP-1 cells with PMA resulted in c-Raf and ERK phosphorylation; however, pretreatment with 10 μM Bis I completely blocked the phosphorylation of c-Raf and ERK. In contrast, pretreatment with PD 98059 inhibited the phosphorylation of ERK1/2, but not of

c-Raf (Fig. 1). Thus, both kinase inhibitors operated in the THP-1 cells as expected.

Table 2. Effects of various inhibitors on early cell adhesion

Protein synthesis inhibitor	Concentration (nM)	% of cell adhesion
Cycloheximide	0 (without PMA)	6.7±3.2
	0	97.2±4.0
[10% FBS, 25 nM PMA, 5h incubation time]	2.5	34.3±4.1
	5.0	29.1±6.3
	10	23.3±4.2
Actin polymerization inhibitor	**Concentration (μg/ml)**	
Cytochalasin D	0 (without PMA)	5.0±2.7
	0	81.3±2.9
[10% FBS, 25 nM PMA, 5h incubation time]	0.5	90.7±2.3
	1	95.3±2.5
	2	98.3±1.9
MEK inhibitor	**Concentration (μg/ml)**	
PD 98059	0 (without PMA)	6.0±3.2
	0	97.7±4.2
[10% FBS, 25 nM PMA, 5 h incubation time]	10	92.7±2.8
	20	89.0±5.2
	40	89.3±6.1
PKC inhibitor	**Concentration (μg/ml)**	
Go 6983	0 (without PMA)	6.3±2.2
	0	95.3±3.5
[10% FBS, 25 nM PMA, 5h incubation time]	0.25	94.3±3.7
	0.5	69.7±4.8
	1	22.0±3.4
PKC inhibitor	**Concentration (μg/ml)**	
Bis I	0 (without PMA)	10.7±1.8
	0	95.7±1.5
[10% FBS, 25 nM PMA, 5 h incubation time]	2.5	82.0±8.7
	5	57.3±2.2
	10	29.7±7.6
	20	4.0±2.8

THP-1 cells were pretreated with the indicated doses of cycloheximide, cytochalasin D, PD 98059, Go 6983, or Bis I for 1 hr, then cultured with 25 nM PMA for 5 hr, and subjected to a cell adhesion assay. The values are the percentage ± standard error, n=6.

Discussion

We examined the effects of various factors on early PMA-induced THP-1 cell adhesion. Cell adhesion did not require serum in the culture medium, actin polymerization, or MAP-kinase activation (e.g., ERK1/2 phosphorylation), but did require *de novo* protein synthesis and PKC activation (e.g., c-Raf phosphorylation).

Cell adhesion studies focus on three basic types of adhesion: to extracellular proteins, to other cells, and to the substratum of a culture dish. Substratum adhesion studies, which usually involve the detachment of cells using trypsin /EDTA [8,10,34], have shown that cell adhesion is affected

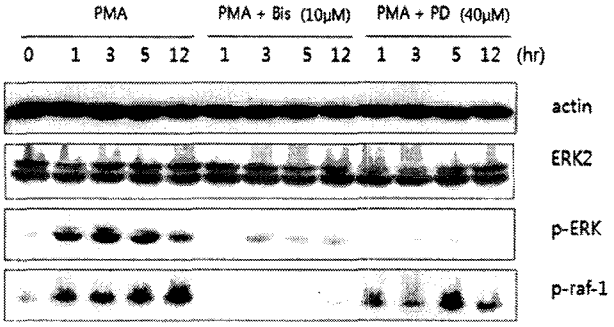

Fig. 1. Effects of kinase inhibitors on the phosphorylation of ERK2 and c-Raf. THP-1 cells were incubated with 25 nM PMA and kinase inhibitors for the indicated times. Whole-cell lysates were then prepared and analyzed using the indicated antibodies (see Materials and Methods). Actin was used to indicate equal protein loading. The data are representative of three independent experiments.

by various factors, including the concentration of serum in the medium and the temperature [9,26,40]. We examined the effects of various factors on cell substratum adhesion during early PMA-induced monocyte differentiation. Using suspension cell differentiation, we confirmed that early cell adhesion was not affected by an actin inhibitor or serum starvation. In the presence of cytochalasin D, our results showed additive effect comparing with PMA treatment alone. Actually, treatment of cytochalasin D alone can induce early cell adhesion of THP-1 (data not shown). This indicates that PMA induces monocytic cell adhesion by more than one pathway in attached cell lines.

PMA induces the differentiation of monocytic cells into macrophage-like cells via a PKC-dependent mechanism that involves the activation of several protein kinases, including PKC/c-Raf/MAP-kinase [5,33]. To address the effects of PKC and/or MAP-kinase activation on early cell adhesion, we used the PKC inhibitors Go 6983 and Bis I, as well as the MAP-kinase inhibitor PD 98059. Our results show that the activation of PKC and/or subsequent c-Raf phosphorylation are required for early cell adhesion, but not for MAP-kinase activation. Although we did not investigate the adhesion molecule(s) involved in early cell adhesion, they may be newly synthesized protein(s) that are regulated by PKC and/or c-Raf and not by MAP-kinase.

Recent studies have shown a correlation between abnormal PKC signaling and various diseases [11,14]. For example, PKC-α/β is associated with the proliferation of human cancer cells [1,4,20], and PKC-β, which is produced in excess when blood glucose levels are high, increases the formation of new blood vessels [13,15]. Therefore, PKC isotypes are considered to be valuable therapeutic targets in the treatment of cardiovascular disease, pain, inflammation, metabolic diseases, angiogenesis, and cancer. In fact, numerous companies and researchers are devoted to the discovery and development of PKC inhibitors [14]. SRB-based assays are used to determine attached cell densities, based on the ability of SRB to bind the basic residues of cellular proteins. Such assays are also useful for large-scale screening of anticancer drug candidates from natural or chemical libraries because they are simple, reproducible, and the end-point measurement is not time-critical [30,38]. However, SRB-based assays are not suitable for use with suspension cell cultures. We used an SRB-based assay to measure the PMA-induced adhesion of cells in suspension culture. Our method allows a large number of samples to be tested within a few days and requires only simple equipment and inexpensive reagents. Thus, we suggest that SRB-based assays are an efficient and highly cost-effective method for PKC inhibitor screening.

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초록 : Phorbol 12-myristate 13-acetate (PMA) 처리로 유도되는 THP-1 세포의 초기 부착에 관한 다양한 인자의 효과

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본 실험에서는 THP-1 세포의 PMA에 의하여 유도되는 초기 세포부착에 관한 메카니즘을 이해하기 위하여 다양한 요인(혈청, 신규 단백질의 합성, 세포 골격 저해제, 단백질 인신화 저해제)들의 효과를 조사하였다. 또한 본 실험에서는 이들 세포부착의 정도를 일반적으로 세포증식 분석에 사용되고 있는 SRB염색법을 도입하여 세포부착 분석에 간편한 방법의 조건을 확립하였다. PMA에 의한 초기 세포부착에는 배양액중의 혈청의 유무는 영향이 없었으나, 신규 단백질의 합성이 요구되는 것을 확인하였다. 또한 이들 초기 세포부착에 PMA처리에 의한 PKC의 활성화는 필수적이나, 그 하류 활성화 인자로 잘 알려진 MAP-kinase (erk1/2)의 인산화는 필요치 않음을 알 수 있었다. 흥미롭게도 액틴 중합 저해제인 cytochalasin D의 PMA와 공 처리는 오히려 세포부착을 PMA 단독 처리시 보다 증가시켰다. 또한 본 실험에서 사용된 SRB 염색법을 통한 세포부착 분석법은 최근 암 등 다양한 질환의 신약 표적 분자로 주목을 받고 있는 PKC 저해제의 초기 세포 기반 분석에 매우 유용하리라고 생각된다.