Volume 304, number 2,3, 153–156 FEBS 11138 June 1992

Increase in protein kinase C activity is associated with human fibroblast growth inhibition

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Received 10 February 1992; revised version received 17 April 1992

Protein kinase C (PKC) activity and DNA synthesis were measured in human fetal bone marrow fibroblasts following treatment with tumor necrosis factor alpha (TNFα) (500 U/ml) or conditioned media containing natural cell proliferation inhibitor (CM-NCPI). Treatment with TNFα led to growth stimulation (120 ± 7% of control in 24 h, 141 ± 6% in 72 h). At the same time particulate PKC activity diminished, reaching 55 ± 8% of control in 24 h and remaining at this level at 72 h. CM-NCPI treatment of the cells resulted in a decrease in DNA synthesis (by 39 ± 6% in 2 h, by 58 ± 5% in 24 h, and by 78 ± 8% in 72 h). This was accompanied by a significant rise in particulate PKC activity which increased over 3-fold in 2 h, over 5-fold in 24 h, and up to 11-fold in 72 h. This 11-fold elevation was maintained after 2 week exposure of the fibroblasts to CM-NCPI. The PKC inhibitor neomycin abolished CM-NCPI induced growth inhibition, whereas PKC activator 12-*O*-tetradecanoylphorbol 13-acetate intensified it. These results suggest that CM-NCPI acts as PKC activator and that negative growth regulation by extracellular agents may involve stimulation of PKC activity.

Protein kinase C; Growth inhibitor; Tumor necrosis factor; Fibroblast

1. INTRODUCTION

Study of protein kinase C (PKC)–phospholipid-dependent serine–threonine protein kinase appears to be of particular importance for the understanding of cell growth regulation [1–4]. It is accepted that this family of enzymes plays a 'pivotal role' in intracellular signal transduction [2]. PKC-dependent signalling is a complex multifactorial process which is only partly understood. Initially, most of the researchers associated the increase in PKC activity with the stimulation of cell growth [5]. Recently, however, it was proven that PKC can inhibit cell growth and tumor promotion [6]. For that reason the study of the effects of negative regulators on PKC seems likely to provide a deeper insight into mechanisms of cell growth inhibition.

In the present work we have studied the cytokine influence on PKC activity and its correlation with DNA synthesis in human fetal fibroblasts. Two agents were used: tumor necrosis factor alpha (TNFα), which is a negative regulator for many cell types [7], and fibroblast-derived conditioned medium containing a negative regulator named NCPI (CM-NCPI) that has been described by us previously [8]. In the research performed we observed that induced inhibition of fibroblast growth is associated with a long-term increase in particulate PKC activity.

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2. MATERIALS AND METHODS

2.1. *Preparation of conditioned medium containing NCPI*

Human fetal bone marrow fibroblasts (1×10^6) were seeded in 175 cm^2 flasks in RPMI-1640 medium with 20% fetal bovine serum (FBS). Afterwards, cells were incubated for 2–4 weeks. The ability of conditioned media to suppress fibroblast proliferation was periodically tested by DNA synthesis assay. Conditioned media were collected, filtered through 0.45 μm filters, and either used immediately or frozen at −70°C for later use.

2.2. *TNFα*

TNFα was obtained from Hayashibara Laboratories Inc. (Okayama, Japan), lot no. 005001.

2.2.2. Fibroblast cultures

Human fetal bone marrow fibroblasts were maintained in RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% carbon dioxide:95% air. Confluent cells were harvested and re-seeded in 12-well plates or 175 cm^3 flasks as needed. The medium was changed every 3 days and 24 h prior to all studies. The human bone marrow fibroblast cell line, BM 5.1, was used in all experiments.

To study the effects of negative regulators 10^6 cells were seeded in 175 cm^2 flasks in RPMI-1640 medium with 20% FBS and grown to 20–30% confluence. The medium was then replaced with fresh medium (control) or one containing TNFα (500 U/ml) or CM-NCPI diluted 1:2.

2.3. *Preparation of cellular fractions*

Cells grown in 175 cm^3 flasks were washed twice with Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS) and resuspended in

buffer A containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 μg/ml leupeptin. Cells were disrupted with 50 strokes in a tight-fitting glass–glass homogenizer. The homogenate was centrifuged at 50,000 × g for 60 min and the cytosol was removed. The membrane pellet was washed with buffer A and resuspended in the same buffer. Aliquots were taken for protein determination by the Lowry method [9]. Triton X-100 was added to the resuspended particulate fraction to 0.2% final concentration. After 45 min incubation at 4°C the membrane suspension was centrifuged at 50,000 × g for 60 min. The supernatant containing the solubilized membrane fraction was collected.

2.4. *PKC activity assay*

The PKC enzyme assay system (Amersham, UK) was used to determine PKC activity of cellular fractions. The determination was based on the transfer of ^{32}P from [γ-^{32}P]ATP to a synthetic peptide. The assay mixture (50 μl) was prepared by the addition of 25 μl of diluted cellular extracts, containing 5 μg of protein, to 25 μl of freshly prepared reagent mixture (3 mM calcium acetate, 2 mole % L-phosphatidyl serine, 6 μg/ml phorbol 12-myristate acetate, 7.5 mM dithiothreitol (DTT) and 225 μM of peptide in 50 mM Tris-HCl, pH 7.5). The reaction was initiated by the addition of 1 μCi of [γ-^{32}P]ATP in 25 μl of 150 μM ATP, 45 mM magnesium acetate, 50 mM Tris-HCl, pH 7.5, into the assay mixture. After 20 min incubation the reaction was terminated by adding 100 μl of stop reagent (PKC enzyme assay system, Amersham), and 125 μl aliquots were transferred to 2.5 × 2.5 cm squares of binding paper. Papers were washed twice with 5% acetic acid and counted in 5 ml of Scintiverse (Fisher, Pittsburg, PA). Kinase activity was determined by subtracting ^{32}P incorporation into peptide in the absence of activators from incorporation in the presence of activators. To ensure complete absence of free calcium 3 mM EGTA was substituted for calcium acetate in controls.

2.5. *DNA synthesis assay*

Exponentially growing human fetal bone marrow fibroblasts were trypsinized, washed and seeded onto 12-well plates (25,000 cells/well) in 1.5 ml of RPMI-1640 medium with 20% FBS. After 24 h the medium was replaced with fresh medium and the cells were cultured for an additional 24 h. The medium was then replaced again with fresh medium (control) or with one containing the agent of interest in the desired concentrations. After 2, 12, 24 or 72 h of treatment 50 μl of [^3H]thymidine was added and cells were cultured for an additional 3 h. After 3 h incubation with [^3H]thymidine the cells were washed with ice-cold 5% trichloroacetic acid (TCA). TCA-insoluble material was solubilized in 0.6 ml NaOH, transferred into scintillation vials with ASC scintillation fluid (Packard, CT) and counted using a Tri-Carb Liquid Scintillation Analyser 1900CA (Packard, CT).

2.6. *Materials*

PMSF, DTT, Triton X-100, leupeptin and neomycin were obtained from Sigma Chemical Co. (St. Louis, MO). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was purchased from LC Services Corp. (Woburn, MA). TNFα was obtained from Hayashibara Laboratories Inc., no. 005001 (Okayama, Japan).

3. RESULTS

PKC activity in human fetal bone marrow fibroblasts BM 5.1, following treatment with TNFα or CM-NCPI, was studied at different intervals. The cell proliferation rate was measured by a [^3H]thymidine incorporation assay. PKC activity was measured separately in cytosol and membrane-derived fractions.

TNFα treatment of fibroblasts resulted in a slow and gradual decline in both cytosolic and particulate PKC activity. Particulate PKC activity was 83 ± 7% of control in 30 min, 72 ± 5% in 2 h, 55 ± 8% in 24 h and 63 ± 8% in 72 h (Fig. 1). Cytosolic PKC activity dropped to 81 ± 9% of control in 30 min, 61 ± 5% in 2 h, 56 ± 8% in 24 h and 62 ± 4% in 72 h (Fig. 1). DNA synthesis in TNFα-treated cells was suppressed by 31 ± 5% in 2 h, however, the suppression was followed by a rise in DNA synthesis to 120 ± 7% of control after 24 h and 141 ± 6% after 72 h (Fig. 1). CM-NCPI caused substantial elevation in both particulate and cytosolic PKC activity. Particulate PKC reached 122 ± 9% in 30 min, 322 ± 36% in 2 h, 559 ± 23% in 24 h and 1090 ± 154% in 72 h (Fig. 2). Cytosolic PKC activity was 94 ± 11% in 30 min, 121 ± 8% in 2 h, 201 ± 27% in 24 h and 218 ± 12% in 72 h (Fig. 2). These elevated levels of PKC activity were still present after 2 weeks exposure of fibroblasts to the CM-NCPI (data not shown on the graph). DNA synthesis in CM-NCPI-treated fibroblasts was suppressed by 39 ± 6% in 2 h, 58 ± 5% in 24 h, and 78 ± 8% in 72 h (Fig. 2).

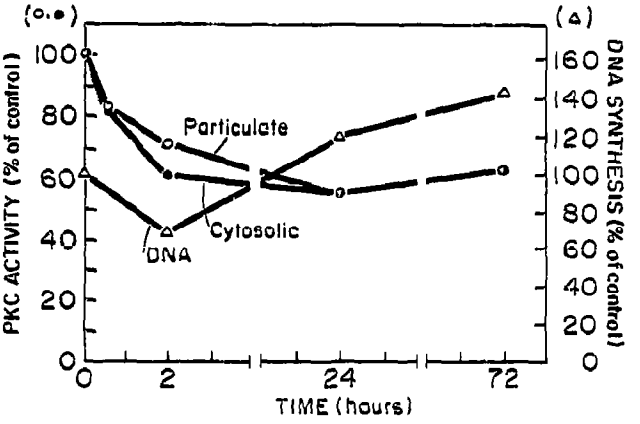

Fig. 1. PKC activity and DNA synthesis in human fetal bone marrow fibroblasts following treatment with 500 U/ml TNFα. Each set of data points represents the means of at least three separate experiments.

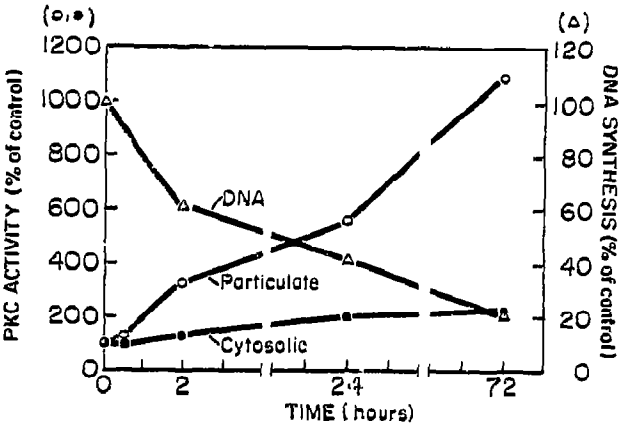

Fig. 2. PKC activity and DNA synthesis in human fetal bone marrow fibroblasts following treatment with CM-NCPI (dilution 1:2). Each set of data points represents the means of at least three separate experiments.

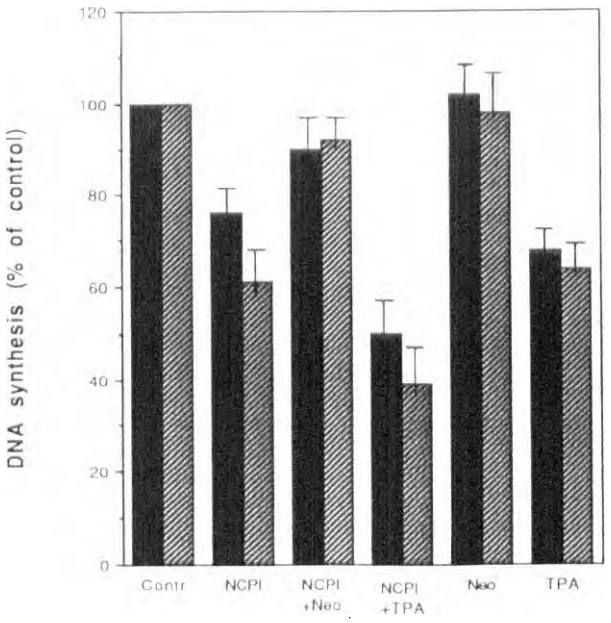

Fig. 3. DNA synthesis in human fetal bone marrow fibroblasts after 2 h (■) and 12 h (▨) treatment with: NCPI, conditioned medium containing NCPI (dilution 1:4); TPA, 12-*O*-tetradecanoylphorbol 13-acetate 10^{-8} M; Neo, neomycin 5 mM; NCPI + TPA, conditioned medium containing NCPI and TPA 10^{-8} M; NCPI + Neo, conditioned medium containing NCPI and neomycin 5 mM.

To verify the involvement of PKC in the process of cell growth inhibition we studied the influence of the PKC inhibitor, neomycin, and the PKC activator, TPA, upon the CM-NCPI or TNFα-induced alteration of fibroblast growth rate. Treatment with 10^{-8} M TPA suppressed DNA synthesis in cells by 32 ± 5% in 2 h, by 36 ± 4% in 12 h (Fig. 3) and by 48 ± 8% in 24 h. Simultaneous treatment with 10^{-8} M TPA and 500 U/ml TNFα caused weaker growth inhibition than TPA alone: by 15 ± 4% in 2 h, 21 ± 6% in 12 h and 27 ± 5% in 24 h. The opposite effect was observed for CM-NCPI. Simultaneous treatment with 10^{-8} M TPA and 1:4-diluted CM-NCPI synergistically inhibited DNA synthesis by 50 ± 6% in 2 h, 61 ± 7% in 12 h (Fig. 3) and 65 ± 6% in 24 h.

Neomycin at 5 mM had no significant effect on DNA synthesis within 12 h, however, it virtually abolished the inhibitory effect of CM-NCPI (90 ± 6% of control in 2 h, 92 ± 4% of control in 12 h, Fig. 3). The effects of TNFα on fibroblast proliferation rate were not altered by neomycin.

4. DISCUSSION

An unexpected correlation between PKC activity and cell growth inhibition was observed after the treatment of fibroblasts with NCPI-containing conditioned media (CM-NCPI). We decided to use conditioned medium as a crude source of the growth factor, as is common practice in similar circumstances [10–12], since the purpose of this work was to study the correlation of PKC activity with growth regulation. Moreover, the use of PKC inhibitor and PKC activator provided an opportunity to verify the involvement of PKC in CM-NCPI-induced cell growth inhibition.

We observed significant elevation of both cytosolic and particulate PKC activity in association with growth inhibition by CM-NCPI. CM-NCPI-induced cell growth inhibition was significantly reversed by neomycin. Neomycin is an inhibitor of phospholipase C (PLC) [13,14]; it was shown to bind to phosphatidyl-inositol biphosphate (PIP_2) and thereby prevents its hydrolysis by PLC. As a result, production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG) is decreased, which leads to PKC activity downfall. On the other hand we found that TPA, a well-known PKC activator [5,6], significantly inhibited fibroblast growth. These observations support the idea that PKC activation may in some cases lead to growth inhibition. Simultaneous treatment of fibroblasts with CM-NCPI and TPA potentiated growth inhibition. This may be due to the ability of both agents to enhance PKC activity.

The possibility of negative regulation via PKC activation has been brought up in the literature [3]. The first direct evidence of PKC-mediated cell growth inhibition was obtained in Weinstein's laboratory [6]. A retrovirus-derived vector system was used to obtain a PKC-overproducing human colon cancer cell line which exhibited growth inhibition in response to TPA.

Our results indicate that extracellular signals which produce growth inhibitory effects can also cause a significant PKC activation. One could suppose that this phenomenon reflects a special PKC-mediated mechanism of cell growth inhibition. In the case of human fibroblasts this may be a normal physiological process.

Effects of TNFα on fibroblasts were different from those of CM-NCPI in terms of both growth rate alteration and PKC activity. TNFα is known to be a negative regulator for some cell lines and a stimulator for others [7]. The present work shows its mitogenic properties for human fetal bone marrow fibroblasts. Our results are in accord with the data obtained by Vilcek et al. [15]. In addition we found that TNFα-induced stimulation of fibroblast growth was preceded by a short-term growth suppression lasting for about 12 h. Following TNFα treatment both cytosolic and particulate PKC activities dropped to about half of control levels within 2 h, but were not restored afterwards. Thus there seems to be no linear correlation between TNFα-induced alteration of fibroblast growth rate and PKC activity. However, one cannot rule out that PKC may be involved in this process in a more complex manner. We also observed that TPA-induced cell growth inhibition was partially reversed by TNFα. This effect was observed not only at 24 h, when TNFα acted as a mitogen, but also at 2 h, when it acted as growth inhibitor. We therefore suppose

that this effect may be a result of TNFα-induced reduction of total PKC available for activation by TPA. This is consistent with the notion that PKC activation may be responsible for the fibroblast growth inhibition caused by TPA.

The complexity and incomplete understanding of the PKC signal transduction system allow us only to speculate about the PKC-dependent mechanisms of negative regulation. However, several working hypotheses may provide a basis for further experiments. So far at least seven isoforms of PKC have been identified [3]. Activation of one or more PKC isoforms may result in cell growth inhibition. PKCα has been reported to phosphorylate EGF receptor thereby making it insensitive to EGF [16]. Hence it is possible that excessive phosphorylation of growth factor receptors by overactive PKC may lead to the desensitization of cells to extracellular mitogens and consequently to growth inhibition.

One can speculate that PKC overstimulation may alter oncogene expression or the activity of oncogene products thereby inhibiting cell proliferation. It was shown that TPA strongly inhibited c-H-*ras* oncogene-induced transformation of PKC-overproducing murine fibroblasts, but stimulated c-H-*ras*-induced transformation of control cells with normal levels of PKC [2,17]. Other protooncogens (c-*myc*, c-*fos*) were also reported to be involved in PKC-mediated responses [18–20].

The system used in this work may serve as a convenient model to study the possible link between cell growth inhibition and PKC activation. Pure NCPI will be valuable for future research. The purification process is now in progress in our laboratory.

Acknowledgements: This work was supported by the T.J. Martell Memorial Foundation for Leukemia, Cancer and AIDS Research. TNFα was kindly provided by Dr. Jun Minowuda of Hayashibara Biochemical Laboratories Inc. (Okayama, Japan).

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