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Phorbol ester-induced secretion of human hepatocyte growth factor by human skin fibroblasts and its inhibition by dexamethasone

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Human skin fibroblasts secreted a certain amount of human hepatocyte growth factor (hHGF), as determined by an enzyme-linked immunosorbent assay for hHGF. This hHGF secretion was remarkably stimulated by protein kinase C (PKC)-activating phorbol esters, which was inhibited by the simultaneous addition of dexamethasone. Pretreatment with phorbol 12-myristate 13-acetate (PMA) caused a down-regulation in hHGF secretion. hHGF secreted by the PMA-treated cells showed a potent hepatocyte growth-promoting activity which was neutralized by an anti-hHGF antiserum. These results indicate both that PMA-treated human skin fibroblasts produce biologically active hHGF and the possible involvement of PKC activation in this process.

Human hepatocyte growth factor; Phorbol ester; Protein kinase C; Human skin fibroblast; Dexamethasone; DNA synthesis

1. INTRODUCTION

Hepatocyte growth factor (HGF), which stimulates DNA synthesis on adult rat hepatocytes in primary culture, was purified from the plasma of patients with fulminant hepatic failure [1], rat platetes [2], rabbit serum [3], normal human plasma [3], and conditioned human embryonic lung fibroblast medium [4]. HGF is a heterodimer which consists of a heavy chain of about 60,000 Da, and a light chain of about 35,000 Da, linked together, probably by a single disulfide bond [1–4]. Molecular cloning of human HGF (hHGF) has revealed that it is synthesized as a single polypeptide chain precursor of 728 amino acids with a signal peptide at the N-terminal [5,6]. HGF stimulates the growth of primary cultured rat hepatocytes at less than one-tenth the molar concentrations of transforming growth factor-α and epidermal growth factor, other potent mitogens for hepatocytes [7].

Many lines of evidence support the concept that HGF is a physiological hepatotrophic factor in liver regeneration. Levels of HGF or an HGF-like factor in the serum and liver of mice and rats that were treated with carbon tetrachloride or partially hepatectomized increased markedly prior to liver regeneration [8–10]. These increases were accompanied by an elevation of the mRNA levels of this factor in the liver [11–14]. It was recently shown that cells stimulated to express the HGF gene in hepatotoxin-damaged rat liver were Kupffer and endothelial cells [15]. However, little is known regarding the induction of HGF production by these cells. In this report we show that human skin fibroblasts secreted hHGF and that the secretion was markedly stimulated by addition of protein kinase C (PKC)-activating phorbol esters to the cultures. We also found that this stimulation was inhibited by dexamethasone.

Abbreviations: hHGF, human hepatocyte growth factor; HGF, hepatocyte growth factor; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PKC, protein kinase C; hSF, human scatter factor; MEM, (Eagle's) minimum essential medium; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co., Tokyo; fetal bovine serum was from Gibco, Grand Island, NY; phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 4α-phorbol 12,13-didecanoate (4α-PDD), and dexamethasone were from Sigma Chemical Co., St. Louis, MO; Cellmatrix-1P was from Nitta Gelatine Co., Yao; and methyl-[^{3}H]thymidine (0.74 TBq/mmol) was from Du Pont-New England Nuclear, Boston, MA. Enzyme-linked immunosorbent assay (ELISA) kits for hHGF [16] and rabbit anti-hHGF antiserum [7] were generously supplied by Otsuka Assay Laboratories, Otsuka Pharmaceutical Co. Tokushima. hHGF was purified from the plasma of patients with fulminant hepatic failure, as described previously [1].

2.2. *Culture of human skin fibroblasts and treatment of cells*

Human abdominal skin fibroblasts, obtained from a normal baby (female, 4 months old) [17], and kindly provided by the Department of Dermatology, Shinshu University School of Medicine, Matsumoto,

were used at population doubling levels of 18–30. The cells were grown as monolayers in MEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. After growth to confluency in 24-well plastic dishes (Nunc), the medium (1 ml) was replaced with a fresh one to which the test compounds were added. Culture media were collected after incubation for various times and were immediately frozen at −30°C. Cell monolayers were then washed 4 times with phosphate-buffered saline (PBS) and solubilized in 1 ml of Lowry solution C [18,19], and cellular protein was determined by the method of Lowry et al. [18]. In some cases, after being washed with PBS, cell layers were scraped in ice-cold PBS containing 1 M NaCl and 0.039% Triton X-100. An aliquot of the cell suspension was removed for protein assay. Bovine serum albumin (BSA) was then added at a concentration of 0.25%, following which the cell suspension was sonicated and centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was stored at −30°C for hHGF ELISA. hHGF levels in the culture media and cell extracts were expressed as ng hHGF per mg of cellular protein.

2.3. *ELISA of hHGF*

A sandwich hHGF ELISA was performed at room temperature, as described previously [16]. Calculation of the amount of hHGF used as a standard was based on the result of its amino acid analysis and molecular weights determined by SDS-PAGE [1].

2.4. *Assay of hHGF activity*

The activity of hHGF was determined by measuring its stimulatory effects on DNA synthesis in adult rat hepatocytes in primary culture, as described previously [20]. Hepatocytes, isolated from adult male Wistar rats (about 200 g) by a collagenase perfusion method, were plated in 24-well plastic dishes (Nunc), pre-coated with collagen (Cellmatrix-1P), at a density of 2.5×10^4 cells/0.2 ml/cm^2 (0.38 ml/well), and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The plating medium was Williams medium E, supplemented with 5% fetal bovine serum, 10 nM dexamethasone, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The medium was replaced with fresh medium containing growth factors 4 h after plating. DNA synthesis, determined by labeling cultured hepatocytes with [^{3}H]thymidine (74 kBq/ml, 37 GBq/mmol) for 18 h at 37°C between 29 and 47 h after plating, was expressed as incorporated [^{3}H]thymidine per μg of cellular protein.

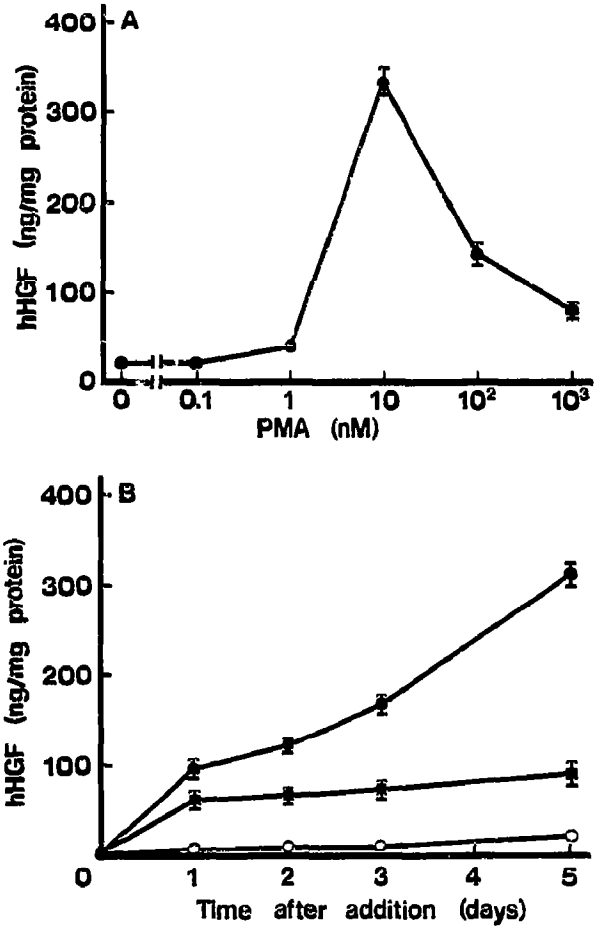

Fig. 1. Dose-response (A) and time-course (B) for PMA-induced hHGF secretion by human skin fibroblasts. Confluent cells were incubated without or with the indicated concentrations of PMA (A) for 5 days and (B) without (○) or with 10 nM PMA (●) or with 100 nM PMA (■) for the indicated days. hHGF levels in the culture media were determined by ELISA. Values are means ± S.D. for triplicate cultures.

3. RESULTS

When confluent human skin fibroblasts were cultured for 5 days, the conditioned medium contained a measurable amount of hHGF as determined by hHGF ELISA, as shown in Fig. 1A. Secretion of hHGF from the cells was markedly stimulated by the addition of protein kinase C-activating phorbol ester, PMA (Fig. 1A). The effect of PMA was maximal at 10 nM, showing about 11-fold stimulation. The amounts of hHGF in extracts of cell layers untreated and treated with 10 nM PMA for 5 days were 4 and 15 ng/mg cellular protein, respectively, which were less than one-seventh and one-twentieth of those in their own culture media. The cells treated with 10 nM PMA secreted hHGF continuously during 5 days of culture, but in the presence of 100 nM PMA maximal amounts were reached after 24 h of incubation (Fig. 1B). Both PDBu and mezerein, other PKC activators, also stimulated hHGF secretion, but 4 α-PDD, known to be inactive for PKC, was without effect, as shown in Fig. 2. Prolonged pretreatment of cultured cells with PMA has been reported to down-regulate PKC by a depletion of this enzyme [21]. As shown in Fig. 3, pretreatment of human skin fibroblasts with 100 or 500 nM PMA for 24 h resulted in a marked decrease in hHGF secretion.

Next, we examined this hHGF to determine whether it was biologically active. As shown in Fig. 4, the hHGF, partially purified from the culture medium of PMA-treated cells by heparin–Sepharose affinity chromatography, had potent hepatocyte growth-stimulating activity. It showed a dose-response similar to that of hHGF purified from the plasma of patients with fulminant hepatic failure, and the activity of both hHGF was neutralized by an anti-hHGF antiserum.

We tested the effects of some other biologically active compounds on hHGF secretion from human skin fibroblasts during 24 h incubation. These compounds were dibutyryladenosine 3′,5′-cyclic monophosphate (1–100 μM), dibutyrylguanosine 3′,5′-cyclic monophosphate (1–100 μM), dibutyrylcytidine 3′,5′-cyclic monophosphate (1–100 μM), ionomycin (30–300 nM),

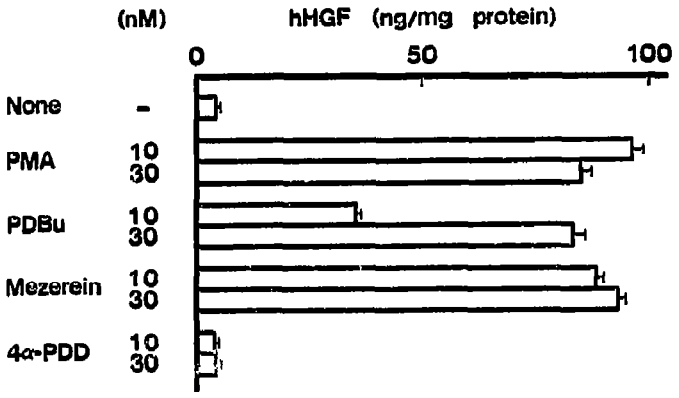

Fig. 2. Effects of various protein kinase C activators on hHGF secretion from human skin fibroblasts. Confluent cells were incubated without or with the indicated protein kinase C activators for 24 h. hHGF levels in the culture media were determined by ELISA. Values are means ± S.D. for triplicate cultures.

A23187 (10–100 nM), dexamethasone (0.01 nM–10 μM), and insulin (0.01–1 μM). None of them significantly stimulated hHGF secretion by the cells (data not shown). We also examined the effects of these compounds in PMA-stimulated hHGF secretion. Most compounds had no appreciable effect (data not shown), but dexamethasone remarkably inhibited the stimulated hHGF secretion, as shown in Fig. 5. This inhibitory effect, observed at concentrations as low as 10 nM, was maximal (about 60% inhibition) at 1 μM.

4. DISCUSSION

The present study demonstrated that the secretion of hHGF by human skin fibroblasts was markedly stimulated by the tumor-promoting phorbol ester, PMA. The following lines of evidence were supportive of a role for PKC activation in the regulation of hHGF secretion. First, the effective concentration of PMA (10 nM) was similar to that required for the activation of PKC [22]. Second, other PKC activators were also effective, whereas inactive 4α-PDD exerted no effect. Third, pretreatment of cells with PMA, which has been reported

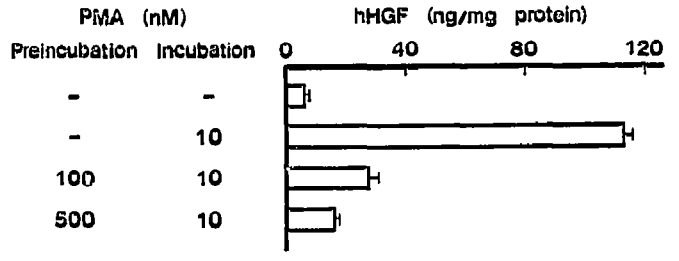

Fig. 3. Effects of pretreatment of human skin fibroblasts with PMA on phorbol ester-induced hHGF secretion. Confluent cells were pretreated without or with 100 or 500 nM PMA for 24 h. The cells were then washed and incubated with 10 nM PMA for an additional 24 h. hHGF levels in the culture media were determined by ELISA. Values are means ± S.D. for triplicate cultures.

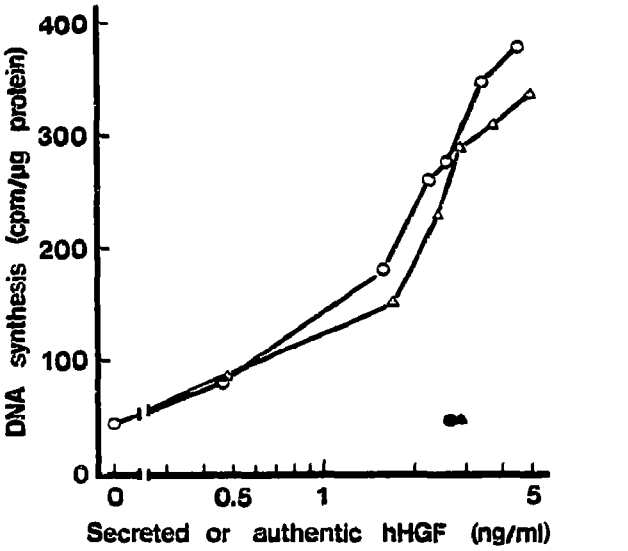

Fig. 4. The hepatocyte growth-stimulating activity of hHGF secreted from PMA-treated human skin fibroblasts, and its inhibition by anti-hHGF antiserum. hHGF in the 5-day-culture media of the fibroblasts treated with PMA was partially purified by heparin–Sepharose chromatography, as described previously [8]. The eluate, with 1.75 M NaCl in PBS containing 0.013% Triton X-100, was dialyzed against PBS after BSA was added at a concentration of 2.5 mg/ml. Rat hepatocytes were incubated with the indicated concentrations of this hHGF (○, ●) or of hHGF purified from the plasma of patients with fulminant hepatic failure (△, ▲). Filled symbols show DNA synthesis in hepatocytes cultured with each hHGF pre-incubated with an anti-hHGF antiserum for 72 h at 4°C. The final concentration of the antiserum in the hepatocyte culture was 0.067%. Concentrations of hHGF in both preparations were determined by ELISA. Values are means for duplicate cultures.

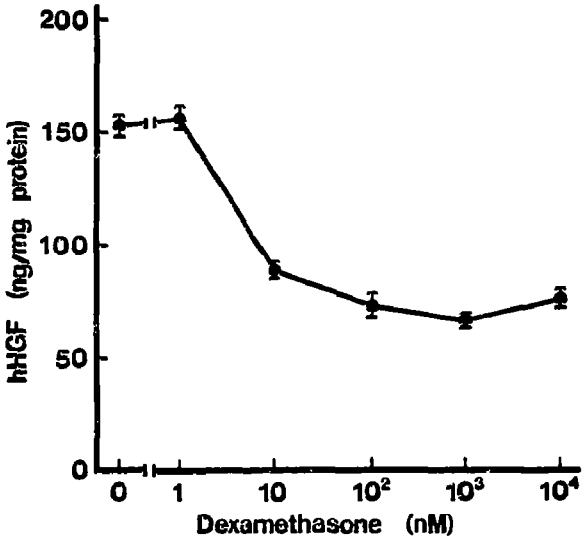

Fig. 5. Inhibition by dexamethasone of PMA-induced hHGF secretion from human skin fibroblasts. Confluent cells were incubated with or without the indicated concentrations of dexamethasone in the presence of 10 nM PMA for 24 h. hHGF levels in the culture media were determined by ELISA. Values are means ± S.D. for triplicate cultures.

to down-regulate PKC activity via depletion of this enzyme, caused a marked decrease in the secretion of hHGF. While this study was in progress, Nishino et al. reported that a promyelocytic leukemia cell line, HL-60, stimulated with PMA produced hHGF [23], indicating that the stimulatory effect of PMA on hHGF secretion is not limited to its effect on human skin fibroblasts. It has recently been reported that hHGF and human scatter factor (hSF), which stimulates the motility of epithelial cells, are identical proteins encoded by a single gene [24–26]. Large amounts of hSF are produced by human embryonic lung fibroblasts such as MRC5 and WI38 [27,28]. We found that the level of hHGF/hSF in the conditioned medium of human skin fibroblasts treated with PMA for 24 h was comparable to that of untreated MRC5 cells (our unpublished results). It would be of interest to examine whether PKC is also involved in active hHGF/hSF secretion by MRC5 fibroblasts.

Dexamethasone inhibited the PMA-stimulated secretion of hHGF. This fact is noteworthy, since hHGF may play an important role in liver regeneration and since regenerating rat liver under the influence of glucocorticoids shows pronounced inhibition of mitosis and DNA synthesis [29]. Although dexamethasone itself has been reported, in some cases, to inhibit DNA synthesis in primary hepatocyte cultures [30], it is possible that the inhibition of hHGF secretion by this steroid contributes to its suppressive effect on liver regeneration.

Normal human skin fibroblasts cultures may be suitable for the identification of the physiological stimulators of hHGF production. The inhibitors of hHGF production can also be detected by the use of such cultures treated with PMA.

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