

Tumor Promoters Induce Basic Fibroblast Growth Factor Gene Expression in Human Dermal Fibroblasts¹

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Abstract

Tumor-promoting phorbol esters have been shown previously to either induce or repress the expression of numerous cellular genes, and this property is likely to be important for the *in vitro* and *in vivo* biological effects of these compounds. In this report, we demonstrate that phorbol 12-myristate 13-acetate induces the accumulation of basic fibroblast growth factor mRNA and protein in human dermal fibroblasts. In contrast, acidic fibroblast growth factor expression was unaffected by this compound. The enhancement of basic fibroblast growth factor gene expression by phorbol 12-myristate 13-acetate was blocked by the isoquinolinesulfonamide derivative H7, a potent inhibitor of protein kinase C. Two additional tumor promoters that bind to and activate protein kinase C, phorbol 12,13-didecanoate and mezerein, also increased basic fibroblast growth factor mRNA levels. Basic fibroblast growth factor is a mitogen for many cell types and can stimulate angiogenesis; thus, some tumor promoter-induced cellular responses may be mediated by this polypeptide.

Introduction

PMA³ is the most potent and intensively studied of the tumor-promoting phorbol esters. This compound modulates the growth and differentiation of various cell types cultured *in vitro* (1) and induces inflammation, cellular hyperplasia, and angiogenesis *in vivo* (1, 2). Most of the biological effects of PMA are thought to occur as a result of its ability to bind and activate the Ca²⁺/phospholipid-dependent protein kinase C family of proteins (3, 4) and to regulate the expression of numerous cellular genes (5). We reported recently that PMA treatment increased both aFGF and bFGF mRNA levels in human smooth muscle cells but did not investigate this response in any detail (6). In this report, we demonstrate that PMA-treated human dermal fibroblasts express increased levels of bFGF, but not aFGF, and that this effect is likely mediated through activation of protein kinase C. The tumor promoters 4 β -PDD and mezerein can also induce bFGF mRNA expression. aFGF and bFGF are structurally related heparin-binding proteins with similar biological activities (7). They are mitogenic for various mesoderm- and neuroectoderm-derived cells and are angiogenic factors (7, 8). The ability of tumor promoters to elevate aFGF and/or bFGF expression *in vivo* may be important for their biological action.

Materials and Methods

Cell Culture. Human skin fibroblast strain GM2037 was purchased from the Human Genetic Mutant Cell Repository, Camden, NJ. Standard growth medium was Dulbecco's modified Eagle's medium (JRH Biosciences) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone Laboratories) and 1 \times antibiotic/antimycotic (GIBCO Laboratories). Fibroblasts were routinely expanded by trypsin treatment and subcultured at a 1:4 split ratio. They were used at similar passage levels for all experiments. To induce quiescence, fibroblasts were incubated for 72 h in normal growth medium containing a reduced serum concentration (0.5%).

RNA Gel Blot Analysis. Serum-starved fibroblasts were either left untreated or treated for the indicated time with the indicated concentration of PMA, 4 β -PDD, 4 α -PDD, mezerein, H7, or HA1004. The phorbol esters and mezerein were purchased from Sigma Chemical Co. and the protein kinase inhibitors were purchased from Seikagaku America. Cells were harvested, total RNA was prepared as described (6), and 10 μ g of each sample were denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted onto Zetabind nylon membranes (Cuno, Inc.) and cross-linked by UV irradiation using a Stratalinker (Stratagene). Hybridization probes were labeled to high specific activity with [³²P]dCTP (3000 Ci/mmol; Amersham Corp.) using a random primer labeling kit (Boehringer Mannheim). The restriction fragments used and the source of the DNA probes were: (a) human aFGF, 2.2-kilobase *Eco*RI fragment of pDH15, purchased from American Type Culture Collection, Rockville, MD; (b) bovine bFGF, 1.4-kilobase *Eco*RI fragment of pJJ11-1, gift of Dr. J. Abraham, California Biotechnology Inc., Mountain View, CA; (c) human GAPDH, 0.8-kilobase *Pst*I/*Xba*I fragment of pHcGAP, purchased from American Type Culture Collection. Membranes were first hybridized with the aFGF or bFGF probes, washed, exposed to film (XAR-5; Eastman Kodak Co.), and then rehybridized with the GAPDH probe. Hybridization and membrane washing conditions were as described (6). Autoradiographic signals were quantitated using a laser densitometer (Pharmacia LKB). aFGF or bFGF mRNA signals were normalized to the signal intensity obtained for GAPDH mRNA to correct for slight differences in the amount of total RNA per gel lane.

Immunoblotting. Serum-starved fibroblasts were either left untreated or treated for the indicated time with PMA (10 ng/ml). Cells were washed twice with cold phosphate-buffered saline, scraped with a rubber policeman, pelleted by centrifugation, and lysed in 66 mM Tris-HCl (pH 6.8)-2% SDS-10 mM EDTA. The extracts were sonicated and insoluble material was pelleted by centrifugation. Protein concentrations were determined using the BCA protein assay (Pierce Chemical Co.). Forty μ g of each sample were combined with an equivalent volume of 0.125 M Tris-HCl (pH 6.8)-4% SDS-20% glycerol-10% 2-mercaptoethanol, denatured at 95°C for 4 min, and subjected to electrophoresis on a 15% polyacrylamide-SDS slab gel. Protein size standards (Bio-Rad Laboratories) and equivalent amounts (100 ng) of recombinant human aFGF (gift of Dr. W. Burgess, American Red Cross) and bFGF (Bachem, Inc.) were also included on the gel. Proteins were transferred electrophoretically to nitrocellulose filters (Bio-Rad). Filters were blocked and then incubated for 1 h at 22°C with rabbit anti-human bFGF polyclonal antiserum (gift of Dr. D. Rifkin, New York University Medical Center, New York, NY). The filter was washed extensively and incubated with a goat anti-rabbit horseradish peroxidase-conjugated

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³ The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDD, phorbol 12,13-didecanoate; SDS, sodium dodecyl sulfate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride.

secondary antibody (Promega Corp.). Bound secondary antibody was visualized by an enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Corp.). Signal intensity was quantitated using a laser densitometer (Pharmacia LKB).

Results and Discussion

PMA Increases bFGF but not aFGF mRNA Expression in Human Fibroblasts. We first determined whether human dermal fibroblasts, like human saphenous vein smooth muscle cells (6), expressed increased levels of the aFGF and bFGF transcripts in response to PMA treatment. Fibroblasts were serum starved for 72 h and then either left untreated or treated with PMA; the treatment conditions (dose and duration of PMA exposure) were identical to those previously used for smooth muscle cells. RNA was prepared and both aFGF and bFGF mRNA levels were assayed by RNA gel blot hybridization. Acidic FGF and bFGF transcripts of the appropriate size (6) could be detected in serum-starved fibroblasts; PMA treatment had no effect on aFGF mRNA levels but increased bFGF mRNA levels ~4-fold (Fig. 1). Time course experiments indicated that neither shorter nor longer periods of PMA treatment had a significant effect on aFGF gene expression (data not shown).

Two previous studies have indicated that tumor-promoting phorbol esters can also regulate bFGF mRNA levels in other cell types. Murphy *et al.* (9) reported that phorbol 12,13-dibutyrate could induce bFGF mRNA expression in the human astrocytoma-derived cell line U87-MG, but they did not analyze aFGF mRNA levels. Bikfalvi *et al.* (10) reported that PMA had no effect on aFGF or bFGF mRNA levels in human omental microvascular endothelial cells but could induce bFGF mRNA expression in human umbilical vein endothelial cells. Taken together, these results indicate that the effect of tumor-promot-

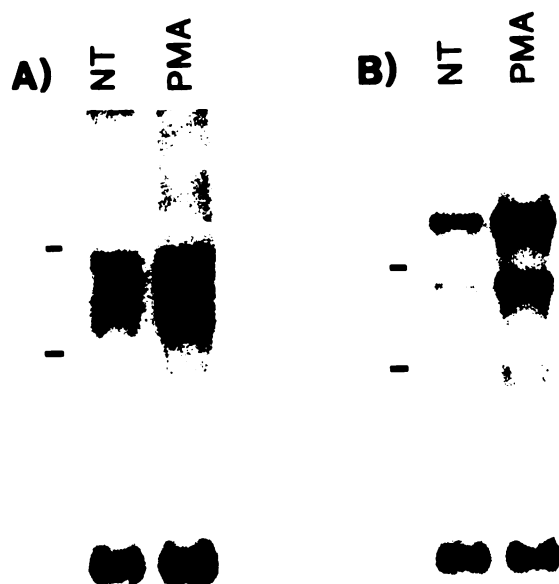


Fig. 1. Expression of aFGF and bFGF mRNA in PMA-treated human dermal fibroblasts. (A) Serum-starved fibroblasts either were left untreated (NT) or were treated for 6 h with PMA (100 ng/ml). RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis using ^{32}P -labeled aFGF (top) or GAPDH (bottom) DNA as the hybridization probes. In this and subsequent RNA gel blot figures, the upper and lower bars on the left side of each blot represent the positions of 28S and 18S rRNA, respectively. Also, only the region of the autoradiogram that contained GAPDH mRNA hybridization is shown. (B) RNA gel blot analysis using ^{32}P -labeled bFGF (top) or GAPDH (bottom) DNA as the probes.

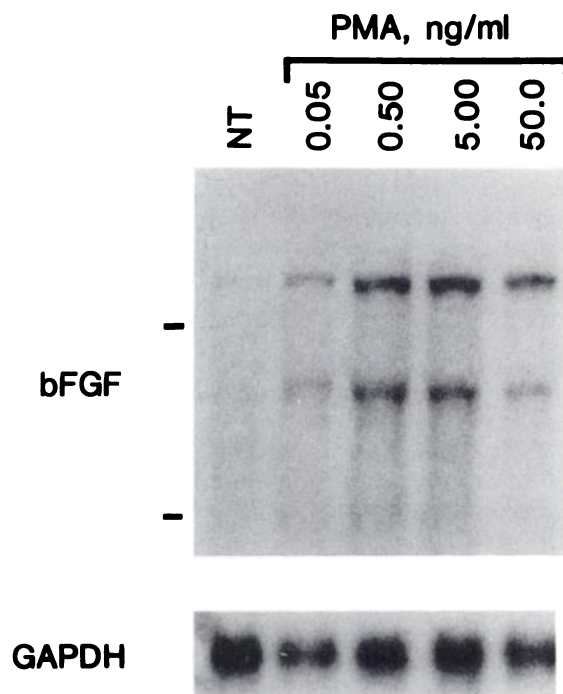


Fig. 2. Expression of bFGF mRNA in PMA-treated human dermal fibroblasts. Dose response. Serum-starved fibroblasts either were left untreated (NT) or were treated for 4 h with the indicated concentration of PMA. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis. In this and subsequent RNA gel blot figures, the complementary DNA probes used are indicated on the left.

ing phorbol esters on aFGF and bFGF gene expression varies depending on the cell type examined.

PMA Treatment Transiently Elevates bFGF mRNA Expression. To determine whether PMA induced bFGF mRNA levels in a dose-dependent manner, fibroblasts were serum starved for 72 h and then treated for 4 h with various concentrations of PMA. RNA was prepared and bFGF mRNA expression was analyzed by RNA gel blot hybridization. Increased bFGF mRNA levels were evident using a log range of PMA concentrations, with maximal induction (~10-fold) detected using a final concentration of 5 ng/ml or ~8 nM (Fig. 2).

The kinetics of bFGF mRNA induction was then examined by treating serum-starved fibroblasts with PMA for different lengths of time. bFGF mRNA levels were transiently induced; increased expression was evident at 2 h after PMA addition, maximal expression occurred at 4 h, and the basal level of expression was again apparent by 16 h (Fig. 3). The PMA-induced increase in bFGF mRNA levels may, at least in part, reflect enhanced bFGF gene transcription, since this response does not occur if the RNA synthesis inhibitor actinomycin D is included during PMA treatment (data not shown).

PMA-treated Fibroblasts Express Increased Levels of bFGF. We determined whether PMA treatment of human fibroblasts resulted in elevated bFGF production by Western blot analysis. Serum-starved fibroblasts were either left untreated or treated with PMA for different lengths of time. Cell lysates were prepared and an equivalent amount of protein was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-bFGF polyclonal antibodies. Three major forms of bFGF with estimated molecular masses of 27, 24, and 18 kDa were detected in both untreated and PMA-treated fibroblasts (Fig. 4). These immunoreactive proteins were absent when either nonimmune rabbit serum or anti-bFGF antiserum prein-

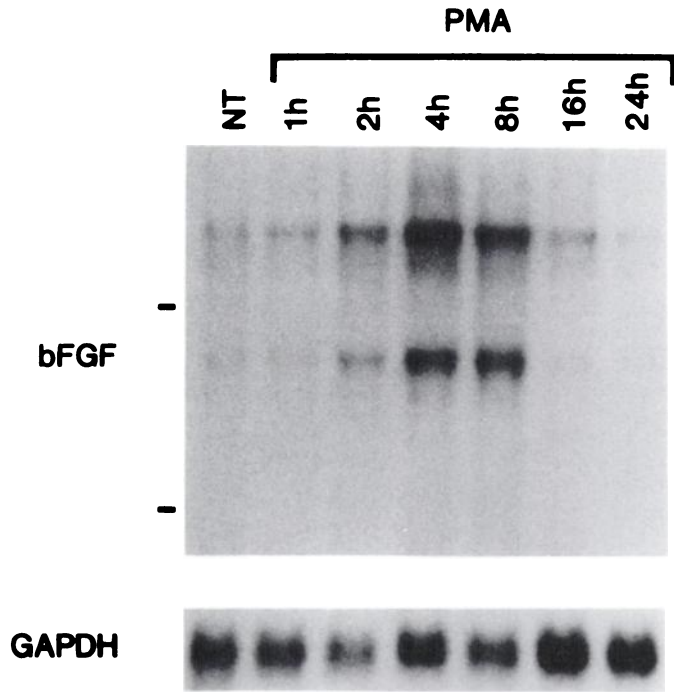


Fig. 3. Expression of bFGF mRNA in PMA-treated human dermal fibroblasts. Time course. Serum-starved fibroblasts either were left untreated (NT) or were treated with PMA (10 ng/ml) for the indicated times. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis.

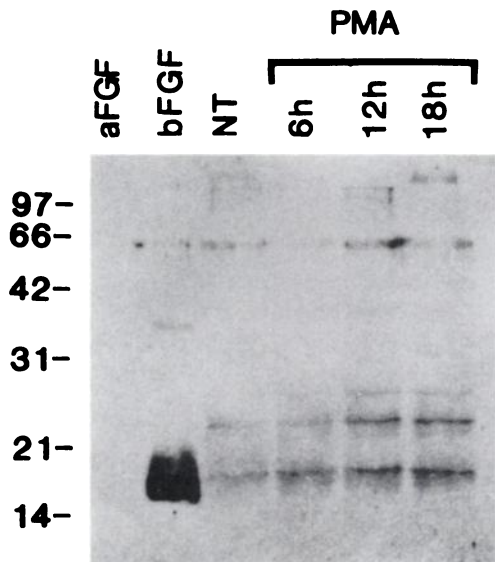


Fig. 4. Expression of bFGF protein in PMA-treated human dermal fibroblasts. Serum-starved fibroblasts either were left untreated (NT) or were treated with PMA (10 ng/ml) for the indicated times. Cell extracts were prepared, and an equivalent amount of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated with anti-bFGF antibodies and bFGF visualized using the enhanced chemiluminescence detection system. Ordinate molecular masses (in kDa) of protein size standards.

incubated with recombinant bFGF were used as the primary antibody reagent (data not shown). As estimated by densitometry, ~4-fold more bFGF was present after 12 or 18 h of PMA treatment.

Multiple forms of bFGF with apparent molecular masses ranging from 18 to 25 kDa have been detected previously in numerous tissues and cell lines. Molecular mass forms of 22.0, 22.5, and 24.2 kDa can arise via translational initiation at CUG

codons located 5' to the AUG codon used for the 18-kDa species (11, 12). The origin and structure of the 27 kDa form of bFGF observed in our Western blot experiments are unknown. A bFGF polypeptide of a similar molecular mass is also present in human mammary epithelial cells (13).

4β-PDD and Mezerein, but not 4α-PDD, Also Induce bFGF mRNA Expression. To evaluate the specificity of the PMA effect on bFGF gene expression, fibroblasts were serum starved and then either left untreated or treated for 4 h with an equivalent concentration of PMA, 4β-PDD, 4α-PDD, or mezerein. RNA was prepared and bFGF mRNA levels were analyzed by RNA gel blot hybridization. The phorbol diesters PMA and 4β-PDD, classified as complete tumor promoters (1) and potent activators of protein kinase C (14), increased bFGF mRNA expression ~4-fold (Fig. 5). The 4β-PDD structural analogue 4α-PDD, which lacks tumor-promoting activity (1) and does not activate protein kinase C (14), had no effect on bFGF mRNA levels. However, mezerein treatment did increase bFGF mRNA levels. Mezerein is a non-phorbol ester diterpene and a potent second-stage tumor promoter (15, 16). Like tumor-promoting phorbol esters, it can bind to and activate protein kinase C (15, 17). These results indicate that at least three different tumor-promoting compounds can increase bFGF mRNA expression levels.

PMA May Induce bFGF mRNA Expression via Activation of Protein Kinase C. A common biochemical property of the tumor promoters that increase bFGF mRNA expression is the ability to activate protein kinase C. The potential role of protein kinase C in PMA-induced bFGF mRNA expression was assessed using the protein kinase inhibitors H7 and HA1004. Although H7 inhibits both cyclic nucleotide-dependent protein kinases and protein kinase C to a similar extent, HA1004 is relatively specific for cyclic nucleotide-dependent protein kinases (18). Serum-starved fibroblasts either were left untreated or treated for 4 h with PMA, H7, HA1004, PMA and H7, or PMA and

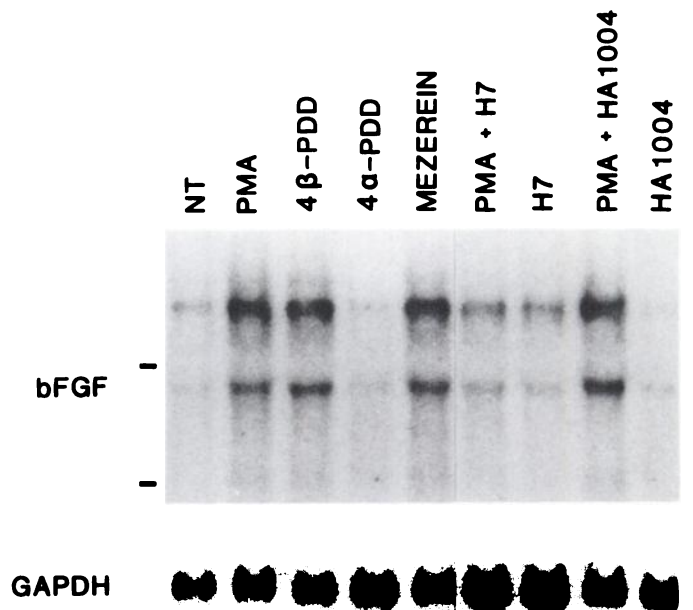


Fig. 5. Effect of different phorbol esters, mezerein, and protein kinase inhibitors on bFGF mRNA expression in human dermal fibroblasts. Serum-starved fibroblasts either were left untreated (NT) or were treated for 4 h with the indicated phorbol ester (10 ng/ml), mezerein (10 ng/ml), or the indicated protein kinase inhibitor (50 μM). For the combination experiments, the inhibitors were added 15 min prior to PMA treatment. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis.

HA1004. RNA was prepared and bFGF mRNA levels were analyzed by RNA gel blot hybridization. H7 strongly inhibited PMA-induced bFGF mRNA expression but the same concentration of HA1004 had no effect (Fig. 5). Treatment with H7 or HA1004 alone did not alter the basal level of bFGF mRNA expression. These results are consistent with the possibility that protein kinase C activation is necessary for PMA regulation of bFGF gene expression.

In conclusion, the results described above demonstrate that tumor-promoting phorbol (PMA, 4 β -PDD) or non-phorbol (mezerein) esters can increase bFGF mRNA levels in human dermal fibroblasts cultured *in vitro*. It is likely that tumor promoter-induced expression of bFGF transcripts is mediated through activation of protein kinase C and results in elevated bFGF production; however, data in support of these proposals have been obtained only using one tumor promoter (PMA). Basic FGF is a potent mitogen for many cell types and an angiogenic factor (7, 8). In contrast to many other polypeptide growth factors, it is synthesized without a signal sequence and thus may not be released from cells via the classical secretory pathway. Nevertheless, there is evidence that bFGF may act in both an autocrine and paracrine manner (19, 20); therefore, it is possible that increased production of bFGF *in vivo* contributes to the molecular mechanism of tumor promotion.

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