Downregulation of c-fms Gene Expression in Human Monocytes Treated With Phorbol Esters and Colony-Stimulating Factor 1

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The colony-stimulating factor-1 (CSF-1) regulates survival, growth, and differentiation of monocytes by binding to a single class of high-affinity receptors. The CSF-1 receptor is identical to the product of the c-fms protooncogene. The present studies monitored the effects of TPA and CSF-1 on c-fms gene expression in human monocytes. The results demonstrate that TPA downmodulates the constitutive expression of c-fms mRNA to low but detectable levels. Treatment of human monocytes with TPA was similarly associated with decreases in levels of the 138- and 125-Kd c-fms-encoded proteins. However, the kinetics of c-fms protein downmodulation indicated independent effects of TPA on c-fms expression at the RNA and protein levels.

Furthermore, c-fms protein levels subsequently recovered despite persistently low levels of c-fms mRNA. Although previous studies demonstrated that c-fms protein is downregulated in the presence of CSF-1, the present results indicate that CSF-1 also downregulates levels of c-fms mRNA. Moreover, the results indicate that CSF-1 increases protein kinase C activity in the membrane fraction. Together, these findings suggest that c-fms gene expression is differentially regulated at both the RNA and protein levels after activation of protein kinase C in human monocytes treated with TPA and CSF-1.

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mmol/L NaCl, 20 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) containing 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 10 μg/mL leupeptin, and 100 μmol/L sodium orthovanadate. The cells were lysed by gentle mixing for 15 minutes at 4°C and centrifuged at 15,000 g, and the clarified supernatant fluid was removed and precleared for one hour with protein A (50 mg/mL). The precleared lysate was then incubated with normal rabbit serum or a rabbit antiserum (no. 3667) to v-fms protein for one hour. Protein A-Sepharose beads were then added and incubated for two hours more at 4°C with gentle mixing. The beads were then pelleted and washed three times in RIP buffer by centrifugation. The immunoprecipitated complexes were dissociated and analyzed by SDS/8% polyacrylamide gel electrophoresis (PAGE) and fluorography.

**Immunocomplex kinase assays.** Immunoprecipitates from unlabelled cell lysates were collected on Protein A-Sepharose beads and washed three times with RIP buffer and three times with kinase buffer (20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L MnCl₂). The washed immunocomplexes on beads (50 μL) were then incubated in the kinase buffer with 20 μCi [γ-³²P]ATP (Amersham Corp) for 15 minutes at 37°C. The reaction was terminated by addition of RIP buffer, and the beads were then washed twice with RIP and once with deionized water. The proteins were separated by electrophoresis in an SDS/8% polyacrylamide gel. The gel was incubated in 2 mol/L NaOH for 90 minutes at 55°C with gentle agitation and washed in 6% (vol/vol) acetic acid/25% (vol/vol) ethanol for 90 minutes before autoradiography.

**Uptake of ³²I/CSF-1.** Recombinant CSF-1 was radioiodinated by the iodogen method to a specific activity of 1.2 x 10⁶ cpm/μg protein. Assays were performed in triplicate on suspensions of purified monocytes in 24-well cluster plates. The monocytes were incubated with [³²P]CSF-1 in RPMI 1640 medium containing 10% fetal bovine serum (FBS) for one hour at 37°C unless otherwise specified. The cells were then washed three times with ice-cold RPMI 1640 medium, solubilized in 2% SDS, and monitored for radioactivity. Specific uptake was determined by the difference between total uptake and nonspecific uptake with a 100-fold excess of unlabelled CSF-1 added. Nonspecific uptake did not exceed 10% of total uptake.

**Protein kinase C activation.** Monocytes were suspended in serum-free RPMI 1640 medium and incubated with CSF-1 or TPA at 37°C. Cells (10⁵) were collected, homogenized by passage through a 25-gauge needle, and immediately reconstituted in buffer A (20 mmol/L Tris-HCl, pH 7.5, 100 μg/mL aprotinin, 0.25 mmol/L leupeptin, and 1 mmol/L PMSF). The cytosolic fraction was obtained after centrifugation, and the particulate fraction was solubilized in buffer A containing 1% Triton X-100 for 15 minutes on ice. Protein kinase C was purified by diethylamino ethanol (DEAE)-cellulose column chromatography and elution with 80 mmol/L NaCl. Enzyme activity was assayed in 10 mmol/L MgCl₂, 1 mmol/L CaCl₂, 10⁶ cpm [γ-³²P]ATP (330 CPM/pmol, Amersham), 20 μg histone H1, 8 μg/mL phosphatidylserine, and 11.7 nmol/L TPA. After incubation at 30°C for ten minutes, protein kinase C activity was determined by subtracting the amount of ³²P incorporation into histone H1 without added phospholipids and calcium from that with these agents. Levels of protein kinase C activity without calcium and phospholipid were <15% of total activity.

**RESULTS**

**Effects of TPA on c-fms mRNA levels.** Previous studies have demonstrated that TPA-induced monocyctic differentiation of HL-60 leukemia cells is associated with downregulation of c-myc gene expression and induction of c-fos and c-fms transcripts.³ Human PB monocytes have low to undetectable levels of c-myc and c-fos mRNA,¹⁰,¹¹ whereas the c-fms gene is constitutively expressed in these cells.³ In contrast to the transient induction of c-myc and c-fos transcripts in monocytes treated with TPA (data not shown), c-fms mRNA levels were downregulated after six hours of TPA exposure and remained at low to undetectable levels for 48 hours (Fig 1). Treatment of monocytes with TPA had little if any effect on c-src transcripts. These findings thus indicated that c-fms gene expression is regulated at the RNA level in human monocytes activated by TPA treatment, presumably through activation of protein kinase C.¹⁸

**Effects of TPA on c-fms protein levels.** Treatment of mouse macrophages with TPA is associated with a rapid downregulation of CSF-1 receptors.⁹,¹⁸ However, no information is available regarding the kinetics of c-fms protein synthesis in human monocytes to provide a basis for comparing c-fms expression at the RNA and protein levels.

The c-fms-encoded proteins were detected by a rabbit antiserum (no. 3667) prepared against a recombinant v-fms polypeptide produced in bacteria.³ Monocytes were pulsed for varying intervals of up to 30 minutes with [³⁵S]methionine and then chased in complete medium containing unlabeled methionine. Cells were harvested at various intervals, lysed, and then immunoprecipitated with the rabbit anti-v-fms serum (Fig 2). A 125-Kd protein was precipitated by ten minutes of labeling (Fig 2). Moreover, a 138-Kd protein was first detectable at 30 minutes during the pulse.

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**Fig 1.** Effects of TPA on c-fms and c-src mRNA levels in human monocytes. Monocytes were treated with 33 mmol/L TPA for the indicated times. Total cellular RNA (20 μg) was monitored by Northern analysis for hybridization to the [³²P]-labeled c-fms DNA probe. After autoradiography, the nitrocellulose filter was stripped by washing in 0.1 x SSC/0.1% SDS at 90°C for ten minutes and rehybridized to the [³²P]-labeled c-src DNA probe.
Fig 2. Pulse-chase labeling and immunoprecipitation of c-fms proteins. Monocytes were incubated with 250 μCi/mL [35S]methionine in methionine-free medium for varying intervals of up to 30 minutes, washed, and then chased in complete medium. The cells were harvested at the indicated times, lysed, and reacted with rabbit antiserum (no. 3667) prepared against v-fms protein. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

The 138-Kd protein remained detectable throughout the chase period, whereas the level of the 125-Kd protein decreased (Fig 2). These findings were consistent with conversion of the 125-Kd cytoplasmic precursor by 30 minutes to the mature membrane-bound 138-Kd c-fms glycoprotein (GP).

To examine the effect of TPA on c-fms protein expression, immunoprecipitation studies were performed on TPA-treated monocytes labeled during the last two hours of drug treatment with [35S]methionine. There were no detectable 138- or 125-Kd proteins in lysates of monocytes labeled with [35S]methionine for two hours and reacted with normal rabbit serum (Fig 3). In contrast, both c-fms GPs were detectable in lysates of control monocytes precipitated with the rabbit antiserum. Treatment of these monocytes with TPA transiently decreased both the 138- and 125-Kd c-fms proteins within two hours of exposure (Fig 3). Moreover, the decrease in the 138-Kd species was more pronounced than that for the 125-Kd c-fms GP. As determined by scanning densitometry, the amount of GP138 and GP125 in TPA-treated cells as compared with untreated monocytes was 9% and 38% at two hours, 4% and 67% at four hours, and 10% and 73% at 18 hours, respectively. Furthermore, similar results were obtained when cell lysates were immunoprecipitated using the 2E8 mouse monoclonal antibody (MoAb) generated against the recombinant v-fms protein (data not shown). Finally, some variability was noted in the interval for reappearance of the 138-Kd product that depended on the different preparations of monocytes. However, GP138 returned to control levels by 24 to 36 hours of continuous TPA exposure.

Effects of TPA on c-fms–associated kinase activity. Immune complex protein kinase assays were performed on lysates of untreated monocytes after immunoprecipitation with either rabbit antiserum or normal rabbit serum. Phosphorylation of GP138 was detectably greater than that observed for GP125 when precipitates were analyzed with the antiserum (Fig 4). In contrast, no phosphorylation was detectable after treatment of lysates from resting monocytes with the normal rabbit serum (Fig 4). Treatment of these monocytes with TPA resulted in a rapid and transient decrease in GP138 kinase activity, whereas this agent had little effect on phosphorylation of GP125 (Fig 4). GP138 kinase activity recovered to control levels by 18 hours in the experiment shown in Fig 4. However, as in recovery of c-fms protein, there was some variability from 18 to 36 hours for return of kinase activity to control levels in different preparations of monocytes. Nonetheless, recovery of c-fms proteins occurred despite persistent downregulation of c-fms mRNA levels.

Similar studies were performed with bryostatin, a macrocyclic lactone that also activates protein kinase C. Bryostatin similarly decreased GP138 kinase activity at four hours of exposure (Fig 4). Furthermore, phosphorylation of GP138 returned partially to pretreatment levels by 18 hours of TPA treatment.

Fig 3. Effects of TPA on c-fms protein levels. Control cells were labeled with [35S]methionine for two hours. TPA-treated cells were labeled for the last two hours of drug treatment by adding [35S]methionine to the culture medium. Cells were harvested at the indicated times of TPA exposure, and lysates containing equal numbers of TCA-precipitable counts were immunoprecipitated with the no. 3667 rabbit antiserum or normal rabbit serum (NS). The immunoprecipitates were analyzed by SDS-PAGE and fluorography.
bryostatin treatment (Fig 4). These findings suggested that a variety of agents which activate protein kinase C result in loss of kinase activity of the c-fms GPs, presumably from internalization and degradation of the receptor.5,19

Effects of TPA on uptake of [125I]CSF-1. Downmodulation and recovery of c-fms protein levels by TPA prompted studies on ligand binding and uptake. [125I]CSF-1 uptake was rapidly decreased by TPA treatment and reached 40% of control uptake after two hours (Fig 5). In contrast, longer incubations with TPA resulted in a slow and nearly complete recovery of uptake (Fig 5). Together, these findings were in concert with the rapid downmodulation of c-fms proteins by TPA and their eventual recovery despite the continued presence of this agent.

Effects of CSF-1 on c-fms expression. We previously demonstrated that CSF-1 mRNA levels are at low to undetectable levels in resting human monocytes and that these transcripts are induced after TPA treatment.21,22 CSF-1 mRNA levels were increased by three hours of TPA exposure21,22 and remained elevated at 24 hours (Fig 6A). Furthermore, this induction of CSF-1 mRNA levels was associated with secretion of CSF-1 into the tissue culture supernatant.21,22 Thus, recovery of c-fms proteins after 24-hour continuous TPA exposure occurred in the presence of elevated levels of CSF-1 and presumably the downregulation of protein kinase C.

Although CSF-1 downregulates CSF-1 receptors in the absence of prolonged phorbol ester treatment, the effect of this factor on c-fms expression at the RNA level is unknown. Therefore, human monocytes were exposed to CSF-1 and
A. MONOCYTES

TPA 24h

28S-

CSF-1

18S-

B. MONOCYTES

CSF-1

2h 6h 24h

28S-

c-fms

18S-

actin

Fig 6. CSF-1 and c-fms mRNA levels in induced monocytes. Monocytes were treated with 33 nmol/L TPA (A) or 1,000 U/mL CSF-1 (B) for the indicated times. Total cellular RNA was monitored by Northern analysis for hybridization to the 32P-labeled DNA probes. (B) The nitrocellulose filter was hybridized to the c-fms DNA probe, washed in 0.1 x SSC/0.1% SDS at 90°C for ten minutes and rehybridized to the 32P-labeled actin DNA probe. When normalized against the changes in actin expression by laser densitometric scanning of both blots, the increase in c-fms RNA expression at 24 hours as compared with untreated monocytes was only 10%.

monitored for levels of c-fms transcripts. c-fms mRNA levels were decreased by 35% after two hours and by 62% after six hours of CSF-1 treatment (Fig 6B). Furthermore, the levels of c-fms mRNA recovered by 24 hours of CSF-1 exposure. These findings indicated that CSF-1, like TPA, regulates c-fms expression at both the RNA and protein levels.

Effects of CSF-1 on protein kinase C activation. The downregulation of c-fms expression by both CSF-1 and TPA raised the possibility that CSF-1 might mediate these effects at least in part through activation of protein kinase C. Therefore, we monitored the activity of protein kinase C in CSF-1-treated monocytes. CSF-1 treatment was associated with an increase in membrane-bound protein kinase C and a decline in cytosolic enzyme activity (Fig 7). This increase in membrane-bound protein kinase C was maximum at 15 minutes of CSF-1 exposure (Fig 7). A similar level of protein kinase C activation was observed in TPA-treated monocytes (Fig 7). These findings indicate that CSF-1 treatment of monocytes is associated with activation of protein kinase C.

DISCUSSION

Treatment of human monocytes with phorbol esters, such as TPA, induces further differentiation of these cells along the macrophage pathway. For example, TPA phosphorylates the CR1 complement receptor and thereby enables these cells to mediate phagocytosis. TPA treatment of resting monocytes also increases production of plasminogen activators which act in the inflammation process. In addition, TPA induces production of CSF-1 and tumor necrosis factor, and enhances the ability of human monocytes to mediate tumoricidal activity. Thus, TPA-treated monocytes may be useful as a model for studying events associated with differentiation and activation along the monocyte-macrophage lineage.

The present studies show that, in addition to induction of c-myc and c-fos transcripts, treatment of human monocytes with TPA is associated with downregulation of c-fms mRNA. This result is in concert with the previous finding that activation of monocytes to macrophages is accompanied by a marked decrease in c-fms mRNA levels. The decline in c-fms expression at the mRNA level was also associated with a rapid downmodulation of CSF-1 receptors. This downmodulation of c-fms protein was determined by immunoprecipitation, immune complex kinase assays, and [125I]CSF-1 uptake. In each of these assays, the downmodulation of CSF-1 receptors was maximum within two hours of TPA treatment. In contrast, the decline in c-fms mRNA levels was more gradual and these transcripts remained detectable at three hours of TPA exposure. These findings suggested that c-fms expression at the RNA and protein levels may be regulated independently in TPA-treated human monocytes.

Although other studies have shown that TPA rapidly downmodulates CSF-1 receptors in mouse macrophages, previous research regarding the effects of TPA on c-fms gene expression in human monocytes has not been published. The antiserum used in the present study immunoprecipitated both the GP125 intracellular precursor and the mature GP138 human cell surface receptor. Pulse-chase protein labeling experiments in untreated human monocytes demonstrated conversion of newly synthesized GP125 to the mature form by 30 to 60 minutes. Furthermore, turnover of both species was increased by TPA exposure, although the results indicated that TPA induced a more rapid turnover of the
membrane GP138 receptor as compared with the cytoplasmic GP125. The autophosphorylation of both GP138 and GP125 was also rapidly decreased by TPA treatment. This finding was in concert with the increased turnover of c-fms protein and probably reflects the decrease in receptor level. Furthermore, treatment of monocytes with other agents that activate protein kinase C resulted in findings similar to those obtained with TPA (eg, bryostatin, a macrocyclic lactone that activates protein kinase C,26 also dramatically decreased autophosphorylation of the c-fms proteins). Indeed, other agents known to activate protein kinase C such as phorbol 12,13-didecanoate (PDD), phorbol 12,13-dibutyrate (PDBU), 4β-phorbol 12,13-dibenoate (PDBZ), and 1-oleoyl-2-acetyl-glycerol (OAG) also inhibited this kinase activity. In contrast, 4α-phorbol 12,13-didecanoate (4αPDD), which does not activate protein kinase C, had no detectable effect on phosphorylation of either GP138 or GP125 (data not shown). Together, these findings suggested that activation of protein kinase C may be associated with rapid downmodulation of c-fms protein in human monocytes. In vitro studies have indicated that c-fms proteins are phosphorylated by protein kinase C in a calcium- and phosphatidylinerine-dependent manner.27 Whether protein kinase C can phosphorylate serine residues of the c-fms protein and lead to subsequent internalization of the receptor remains to be determined.

Studies with mouse macrophages have demonstrated that downmodulation of CSF-1 receptors by TPA is transient despite continuous drug treatment.9 Similar findings were obtained in the present studies, although recovery in mouse cells occurred by five hours,9 whereas that in human monocytes required ~24 hours. Recovery of c-fms protein in human monocytes was also detectable by autophosphorylation and [125I]CSF-1 uptake, suggesting that these receptors were capable of CSF-1 binding and induction of tyrosine kinase activity. De novo synthesis of c-fms protein at 24 hours occurred despite downregulation of c-fms transcripts to low but detectable levels. These results provide further support for the differential regulation of c-fms gene expression at the RNA and protein levels.

Downregulation of the CSF-1 receptor in TPA-treated monocytes can also be explained, at least in part, by the transient induction of CSF-1 expression in these cells. We demonstrated that CSF-1 transcripts are detectable in monocytes within three hours of TPA treatment.31,22 Furthermore, CSF-1 protein production as monitored by a CSF-1–specific radioimmunoassay (RIA) was increased by as early as three hours in supernatants of TPA-treated monocytes. Thus, downregulation of c-fms protein is in concert with production of CSF-1. Moreover, CSF-1 transcripts remained detectable after treatment of the monocytes with TPA for 24 hours. This expression of CSF-1 mRNA at 24 hours was also associated with elevated levels of CSF-1 protein in culture supernatants. Together, these findings indicated that recovery of c-fms protein occurred in the presence of CSF-1 and that downregulation of this receptor in the presence of ligand is not detectable after prolonged TPA exposure. In this regard, persistent activation of protein kinase C results in downregulation of this protein34 and thereby the potential to phosphorylate the CSF-1 receptor on serine.

Finally, the present results demonstrate that activation of human monocytes is associated with decreases in the level of c-fms transcripts. This finding and previous studies8,19,29 indicate that CSF-1 regulates c-fms gene expression at both the RNA and protein levels. Furthermore, the production of CSF-1 by TPA-treated monocytes may contribute to downmodulation of c-fms transcripts. Although the mechanisms responsible for this effect remain unclear, we have shown that CSF-1 treatment of human monocytes is associated with an increase in protein kinase C activity. Further studies are underway to determine the signaling pathway responsible for this effect. Nonetheless, CSF-1, like TPA, appears to regulate c-fms gene expression through activation of this enzyme.

REFERENCES


Downregulation of c-fms gene expression in human monocytes treated with phorbol esters and colony-stimulating factor 1

E Sariban, K Imamura, M Sherman, V Rothwell, P Pantazis and D Kufe