Prolonged Activation of the Mitogen-activated Protein Kinase Pathway Is Required for Macrophage-like Differentiation of a Human Myeloid Leukemic Cell Line[1]

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Abstract

The role of the mitogen-activated protein kinase (MAPK) signal transduction pathway in the proliferation of mammalian cells has been well established. However, there are relatively few reports concerning cell differentiation being mediated by MAPK. The effect of phorbol 12-myristate 13-acetate (PMA) on cell differentiation and signal transduction in a human myeloid leukemia cell line, TF-1a, was investigated. When TF-1a cells were treated with 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M PMA for 24 h, they underwent 98, 93, 91, and 51% macrophage-like differentiation, respectively. PMA treatment rapidly (10 min) induced phosphorylation of MAPK kinase (MEK and p44/42 MAPK), which persisted for at least 24 h. p44/42 MAPK immunoprecipitates from lysates of PMA-treated cells had increased ability to phosphorylate the transcription factor Elk-1. This is important because phosphorylated Elk-1 can be considered an "end-product" of the MAPK pathway. In contrast, treatment of TF-1a cells with granulocyte/macrophage-colony stimulating factor induced only transient activation of MEK and p44/42 MAPK (10–20 min) and an increase (~50%) in cell proliferation, without any change in cellular differentiation. These results suggest that macrophage-like differentiation may be dependent on prolonged activation of the MAPK pathway. Additional support for this conclusion was obtained from experiments showing that treatment of TF-1a cells with antisense oligonucleotides for MEK1 coding sequences prior to adding PMA inhibited macrophage-like differentiation. Furthermore, transient transfection with an inactive, dominant-negative MEK mutant also inhibited PMA-induced differentiation, whereas transient transfection with a plasmid coding for constitutively activated MEK led to macrophage-like differentiation in the absence of PMA.

Introduction

Activation of the MAPK[3] pathway after ligand binding to various receptors has been correlated with numerous cellular responses, including proliferation, differentiation, and regulation of specific metabolic pathways in different cell types. It is now well established that MAPK is a key regulator of cell proliferation (1–3). Studies from fibroblasts and hematopoietic cells suggest that the activation of Ras, Raf, MEK, and MAPK appears to be a linear pathway that is stimulated by growth factor receptors and leads to cell proliferation (4–7). The 44 kDa MAPK (ERK1) and 42 kDa MAPK (ERK2) are phosphorylated and activated by highly specific MEK1 and MEK2 (8–12). It has been argued that it is the duration of MAPK activation that determines whether a stimulus induces proliferation or differentiation (2, 13). Thus far, MAPK-dependent differentiation has been demonstrated in only two cell types. Studies of rat PC 12 neuroblastoma cells (14–16) suggest that cell differentiation requires prolonged activation of MAPK (lasting hours to days), in contrast to transient activation of MAPK occurring in response to proliferative signals. Megakaryocytic differentiation in the K562 cell line, obtained from a patient with an acute transformation of chronic myeloid leukemia, is also associated with activation of MAPK (17). In K562 cells, active MAPK remained elevated for 2 h and returned to near-basal level by 24 h in response to PMA. However, megakaryocytic differentiation in the human erythroid/megakaryocytic cell line, HEL, appears to be dependent on PKC activation (18). Since PKC has been reported to direct phosphorylation of Raf (19), which is upstream of MEK, it is possible that PKC-induced differentiation may be through the activation of the MAPK pathway. However, direct evidence of cell differentiation induced by the PKC-MAPK pathway has not been reported. A recent report shows that activation of PKC is associated with the MEK kinase 1/stress-activated protein kinase pathway (20). BCL-X_L has also been proposed to be involved in human myeloid cell differentiation (21). Thus, the role of MAPK in the control of cell differentiation, particularly in hematopoietic

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; WT, wild type; BrdUrd, bromodeoxyuridine.

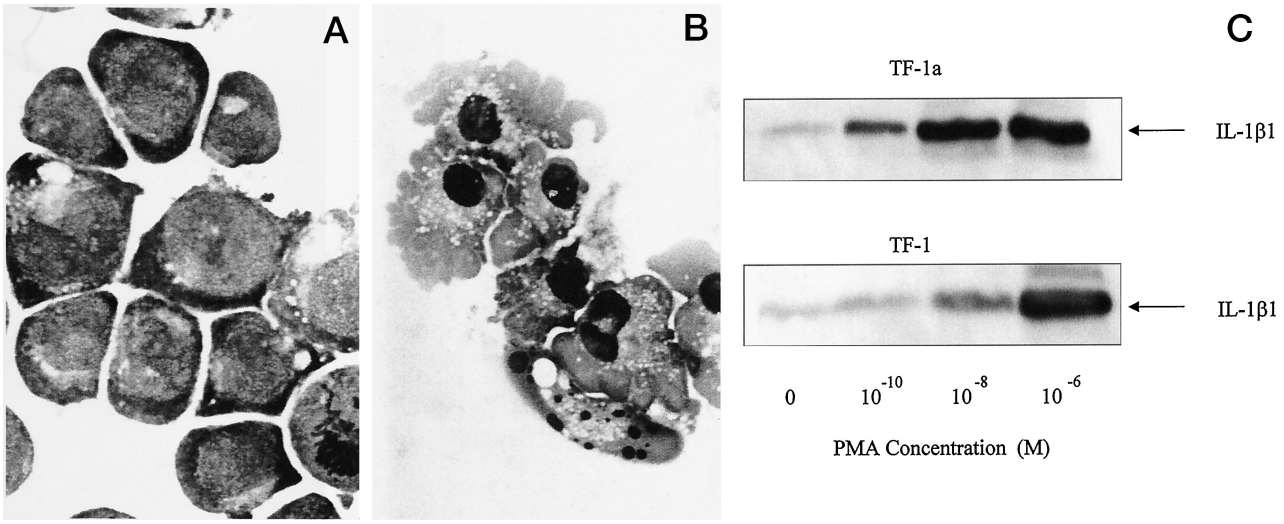

Fig. 1. Induction of macrophage-like differentiation in TF-1a cells. *A*, TF-1a cells are cytologically identical to TF-1 cells. They consist of a relatively homogeneous population of medium-size cells with the appearance of blasts. They contain moderate amounts of dark basophilic, agranular cytoplasm with frequent small cytoplasmic vacuoles and a smooth cytoplasmic border. The nuclei are oval with fine chromatin and 1–3 macronucleoli. *B*, the addition of PMA to TF-1a cells results in an increase in the amount of cytoplasm, a decrease in the nuclear size with chromatin clumping and loss of nucleoli, the appearance of fine granules and vacuoles in the cytoplasm, and cell spreading. *C*, untreated and PMA-treated cells for 24 h were collected by centrifugation and lysed in lysis buffer. Aliquots of lysates were subjected to SDS-PAGE and analyzed by Western blot with an antibody specific for IL-1β.

cells, is not well established. Further evidence is needed to reveal whether prolonged activation of MAPK is required for cell differentiation. In the study reported here, we investigated the induction of macrophage-like differentiation and its possible linkage with prolonged activation of the MAPK pathway in TF-1a myeloid leukemia cells (22). TF-1a is a factor-independent cell line derived from the human factor-dependent erythroid leukemia TF-1 cell line (23). The TF-1 cell line was isolated from a patient with erythroleukemia, which is dependent on GM-CSF or IL-3 for its growth *in vitro.* Erythropoietin also sustains the short-term growth of TF-1 cells but does not induce erythroid differentiation. TF-1 cells can be induced to differentiate into two different pathways, dependent on the type of an inducer. Hemin and delta-aminolevulinic acid induces erythroid differentiation with hemoglobin synthesis in TF-1 cells, whereas PMA brings about dramatic macrophage-like differentiation of the cells (23). The TF-1a cells retain the ability to increase their proliferation in response to GM-CSF and IL-3 (22). Compared with its parent TF-1 cells, this subline can survive longer in serum-free medium and has the ability to resist apoptosis as it exits from the cell cycle and undergoes differentiation. These characteristics of TF-1a cells enabled us to study the kinetics of MAPK activation over a relatively long period of time. To determine the role of the MAPK pathway in macrophage-like differentiation of TF-1a cells, we investigated the ability of MEK1 antisense oligonucleotides and dominant-negative MEK1 to block PMA-induced differentiation and the ability of a constitutively form of MEK1 to induce macrophage-like differentiation of TF-1a cells. These results suggest that prolonged activation of the MAPK pathway is necessary and possibly sufficient for macrophage-like differentiation of TF-1a cells.

Results

Macrophage-like Differentiation of TF-1a Cells. TF-1a and parental TF-1 cells appear to be cytologically identical, consisting of a relatively homogeneous population of medium-size cells with the appearance of blasts. They contain moderate amounts of dark basophilic, agranular cytoplasm with frequent small cytoplasmic vacuoles. They have a smooth cytoplasmic border. The nuclei are oval with fine chromatin and 1–3 macronucleoli. Many binucleated and occasional large multinucleated forms are present (Fig. 1*A*). The addition of PMA to the cells results in typical macrophage-like changes, characterized as a decrease in nuclear size and an increase in the degree of nuclear chromatin condensation. The majority of the cells have moderately abundant light basophilic, agranular to finely granular cytoplasm with irregular cytoplasmic borders (Fig. 1*B*). CD14 remained undetectable. These results are in agreement with reports published previously from the study on TF-1 cells (23). Morphological differentiation is dose dependent. At least 50% of TF-1a cells had macrophage-like alterations in response to 10^{-6} to 10^{-9} M PMA. Compared with TF-1 cells, TF-1a showed a higher sensitivity to PMA treatment (Table 1).

Macrophages are known to produce IL-1 (24–27). To assess whether the induction of macrophage-like differentiation of TF-1a cells is accompanied by an increase of IL-1β production, Western blotting with anti-IL-1β antibody was performed. As shown in Fig. 1*C*, proliferating TF-1a cells expressed very limited IL-1β, and the addition of PMA markedly increased the IL-1β levels. This effect is dose dependent, with maximal expression of IL-1β being detected at a concentration of 10^{-6} M PMA. A similar increase in the level of IL-1β was also observed in TF-1 cells in response to PMA treatment.

Table 1 TF-1a cell differentiation induced by PMA

TF-1a cells (2×10^6 in 2 ml) were treated *in vitro* with increasing concentrations of PMA for 24 h. The cultures were evaluated after staining with Wright-Giemsa for macrophage differentiation (increased cytoplasm, nuclear condensation with nucleolar loss, and development of cytoplasmic granules). A total of 100 cells in duplicate for each group were enumerated under 10×100 magnification, and the proportion of macrophages was reported. The results shown are the means of two independent experiments. Cell viability measured by trypan blue staining was >90% for TF-1a and 85% for TF-1 cells.

PMA concentration (M)	% macrophage morphology	
	TF-1a	TF-1
0	0	0
10^{-9}	51	15
10^{-8}	91	55
10^{-7}	93	71
10^{-6}	98	76

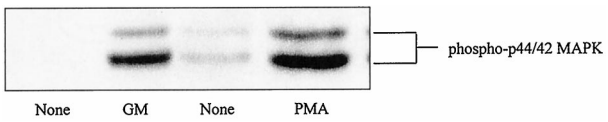

Fig. 2. PMA rapidly induces phosphorylation of MAPK. TF-1a cells starved in serum-free medium for 48 h were treated with GM-CSF (10 ng/ml) or PMA (10^{-7} M) for 5 min at 37°C, after which the cells were collected by centrifugation, washed once in PBS, and lysed in 1× sample buffer. Aliquots of lysates containing the same cell numbers were subjected to Western blot analysis and probed with an antibody specific for phosphorylated p44/42 MAPK. Identical results have been obtained in five independent experiments.

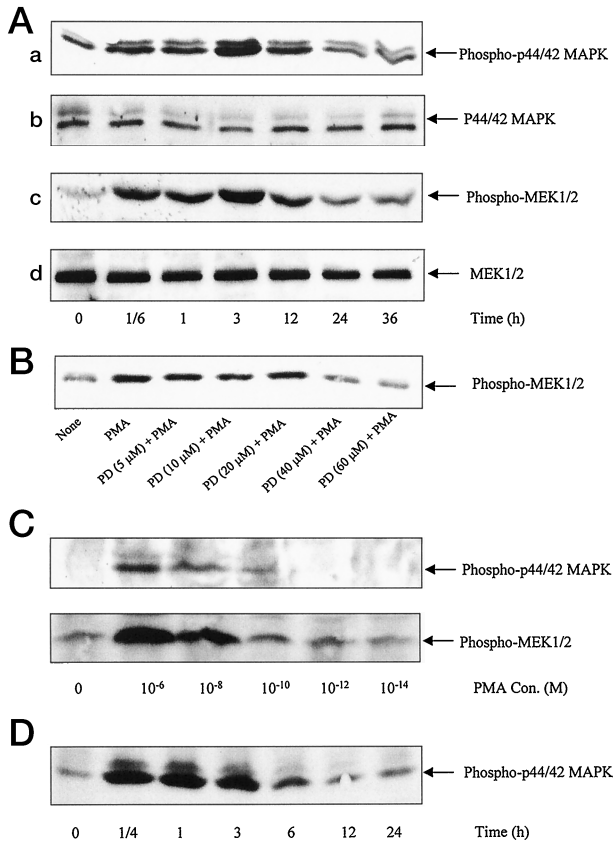

Fig. 3. PMA induces prolonged phosphorylation and nuclear translocation of MAPK. *A*, serum-starved TF-1a cells (48 h) were treated with or without PMA (10^{-7} M) for 0–36 h, after which the cells were collected and lysed. Aliquots of the lysates were analyzed by Western blot and probed with an anti-MAPK or MEK (*b* and *d*) or antiphosphorylated MAPK or MEK antibody (*a* and *c*). *B*, serum-starved TF-1a cells were pretreated with pD098059 at various concentrations for 1 h. Subsequently, PMA (10^{-6} M) was added to the cells for another 1 h incubation; after which, cells were collected, and phosphorylation of MEK was detected by Western blotting, using antiphosphorylated MEK1/2 antibody. *C*, serum-starved TF-1a cells untreated or treated with PMA (10^{-14} to 10^{-6} M) for 1 h were collected and analyzed by Western blotting as described in *A*. *D*, nuclear extracts were made from TF-1a cells untreated or treated with PMA for the times indicated. Aliquots of the extracts were loaded on SDS-PAGE, and the expression of MAPK was determined by Western blot analysis, using an antiphosphorylated MAPK antibody. Identical results have been obtained in three independent experiments.

Prolonged Activation of MAPK Pathway by PMA *versus* Transient Activation of the Signal by GM-CSF. To determine whether the macrophage-like differentiation is correlated with MAPK pathway activation, the expression of phosphorylated MEK and MAPK in response to PMA was first investigated. The cells were also exposed to GM-CSF, which has been reported to be a potent stimulator of p44/42 MAPK phosphorylation (7). Western blot analysis was performed, using an antiphosphorylated MAPK antibody. Fig. 2 illustrates that both GM-CSF (10 ng/ml) and PMA (10^{-7} M) rapidly induced phosphorylation of p44/42 MAPK after exposure to GM-CSF or PMA for 5 min. Since the signal duration has been linked to the decision to undergo either proliferation or differentiation, the time course of the phosphorylation of p44/42 MAPK and MEK was examined. As shown in Fig. 3*A*, phosphorylation of p44/42 MAPK induced by PMA was rapid and persistent. Peak activation occurs at 3 h after PMA treatment, after which the signal slightly decreases but remains at a high level up to 36 h, as compared with control cells. The kinetics of MEK phosphorylation nearly parallel those seen with p44/42 MAPK. (Fig. 3*A*). PMA had no effect on levels of total MAPK and MEK protein, because the levels of these two proteins detected by anti-MAPK and MEK antibodies remained constant at all time points tested up to 36 h after initiating PMA treatment (Fig. 3*A*). To determine the specificity of PMA-induced phosphorylation of MEK, PD098059, an inhibitor of MEK activity (28), was added to cells 1 h before the addition of PMA. After another 1 h of culture in the presence of PMA at 37°C, the cells were collected, lysed, and subjected to Western blotting for detection of MEK phosphorylation. PMA induced phosphorylation of MEK because preincubation of cells with PD098059 at a concentration of 40 μM or more completely suppressed the activation of MEK (Fig. 3*B*). The PMA-induced activation of the MAPK pathway is dose dependent, with maximum activation being observed at a concentration of 10^{-6} M PMA. Low concentrations of PMA ($<10^{-10}$ M) did not induce phosphorylation of p44/42 MAPK or MEK (Fig. 3*C*). Western blot experiments using the nuclear extracts made from TF-1a cells showed that PMA rapidly induced p44/42 MAPK (Fig. 3*D*) but not MEK (data not shown) nuclear translocation (see next section). PMA had no effect on the proliferation of TF-1a

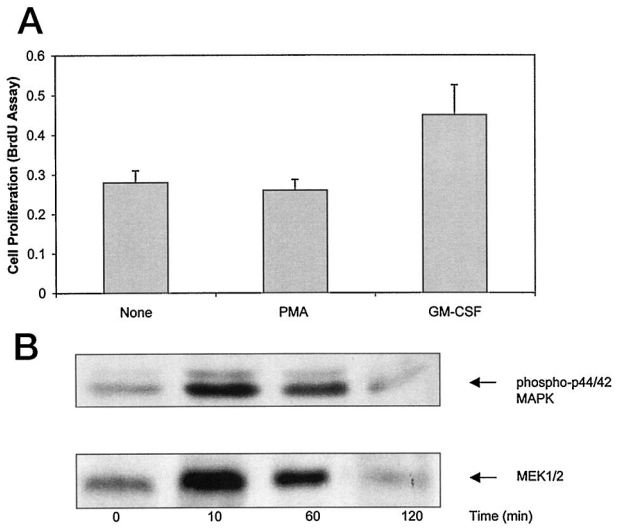

Fig. 4. GM-CSF stimulates TF-1a cell proliferation and induces transient phosphorylation of MAPK and MEK. *A*, growing TF-1a cells were incubated in a 96-well plate in a final volume of 100 μl/well in the presence or absence of GM-CSF (5 ng/ml) or PMA (10^{-7} M). After 2 days of incubation at 37°C, BrdUrd labeling agent was added to cells for another 12 h. Subsequently, the culture medium was removed, and anti-BrdUrd antibody was added to the cells. The immune complexes then were detected by a subsequent substrate reaction, using an ELISA plate reader at 450 nM as described in "Materials and Methods." Values are means of three independent experiments; *bars*, SD. The statistical significance of differences between group means was determined using Student's *t* test. *B*, serum-starved TF-1a cells were stimulated with GM-CSF for the times indicated, after which Western blotting was performed using antiphosphorylated MAPK and MEK antibodies as described in "Materials and Methods." Identical results have been obtained in three separate experiments.

cells, as measured by BrdUrd incorporation (Fig. 4) or MTT (data not shown) assays. In contrast, GM-CSF only induced a transient phosphorylation of MAPK and MEK. The peak signal induced by GM-CSF was at 10 min, which then returned to basal level within 2 h (Fig. 4*B*). GM-CSF is a major growth factor capable of stimulating the proliferation of several factor-dependent leukemic cell lines, such as TF-1 (23) and megakaryoblastic Mo7e cell lines (29). GM-CSF can also increase the proliferation rate of several factor-independent cell lines (22, 30). The addition of GM-CSF increased proliferation of TF-1a cells by ~60% ($P < 0.01$; Fig. 4*A*) but failed to induce macrophage-like differentiation in the cells (data not shown).

Nuclear Translocation of MAPK Detected by Western Blotting and Immunostaining. Since MAPK nuclear translocation in fibroblasts is essential for passing the restriction point and initiating proliferation (31), the expression of phosphorylated p44/42 MAPK in the nucleus was examined by Western blot using nuclear extracts. Our primary aim was to find whether the phosphorylated p44/42 MAPK induced by PMA was translocated to the cell nuclei for a transient or a prolonged time. For this purpose, the nuclear extracts from the cells treated with or without PMA for different periods of time were examined by Western blotting, using antiphosphorylated MAPK antibody. From Fig. 3*D*, it is clear that phosphorylated p44/42 MAPK was present at a low (basal) level in the nuclei of TF-1a cells grown in the absence of PMA, whereas the level of phosphorylated p44/42 MAPK rapidly increased (within 15 min) in the nuclei of the cells treated with PMA. Again, the translocation was persistent and remained up to 6 h, after which the signal decreased and returned to basal level by 24 h. To directly observe nuclear translocation of MAPK, an indirect immunofluorescence assay was carried out. TF-1a cells were treated with 10^{-7} M PMA for various times, after which the cells were incubated with antibody specific for phosphorylated MAPK and FITC-labeled secondary antibody, and translocation of MAPK was visualized on the images captured by an Axiovezt 100 M Zeiss microscope. As shown in Fig. 5*A*, a low level of phosphorylated p44/42 MAPK is present in untreated cells, and this was primarily in the cytoplasm. This appears to represent a basal level (22). After 10 min of PMA treatment, the MAPK started to migrate into nuclei (Fig. 5*B*), and the level of phosphorylated MAPK in the nuclei was markedly increased at 1 and 3 h (Fig. 5, *C* and *D*). By 6 h, the nuclear staining was significantly decreased (Fig. 5*E*), and most of this staining reappeared in the cytoplasm 24 h after treatment (Fig. 5*F*). These kinetics were consistent with the results obtained from Western blotting analysis of nuclear extracts described above.

Activated MAPK Induces Prolonged Phosphorylation of Elk-1. Next, it was asked whether the prolonged phosphorylated MAPK was still active functionally. For this purpose, a nonradioactive MAPK kinase assay was performed *in vitro.* With this method, an antibody specific for phosphorylated p44/42 MAPK (Thr202/Tyr204) was used to immunoprecipitate phosphorylated p44/42 MAPK from cells treated with PMA. The resulting immunoprecipitate was then incubated with an Elk-1 fusion protein for 30 min at 30°C in the presence of ATP and kinase buffer. Subsequently, phosphorylation of Elk-1 at Ser383 was measured by Western blotting using an antibody specific for Ser383 phosphorylation-specific Elk-1. The immunoprecipitates obtained with the antiphosphorylated MAPK antibody from the cells treated with PMA for several time periods up to 24 h contained significant kinase activity for Elk-1 phosphorylation (Fig. 6, *top panel*). The levels of phosphorylation of Elk-1 after treatment with PMA for 10 min to 24 h were approximately the same. The phosphorylation was dose dependent within the range of 10^{-9} to 10^{-6} M PMA (Fig. 6, *bottom panel*).

Antisense MEK Oligonucleotides Suppress the MAPK Pathway Activation and Macrophage-like Differentiation in TF-1a Cells. Although we have demonstrated that the macrophage-like differentiation was closely correlated with prolonged activation of the MAPK pathway, this does not necessarily prove that MAP kinase directly caused differentiation. Since prolonged incubation (24 h) with PD098059 caused cytotoxicity (possibly because of the solvent of PD098059, DMSO), antisense MEK1 oligonucleotides were used to determine whether MAPK is a key regulator of macrophage-like differentiation. Previous studies have shown that oligonucleotides can enter the nucleus when added to the culture (32, 33), and their uptake was increased in human leukemic cells as compared with normal hematopoietic cells (33). Therefore, an adaptation of this technique was used here. As shown in Table 2, PMA induced macro-

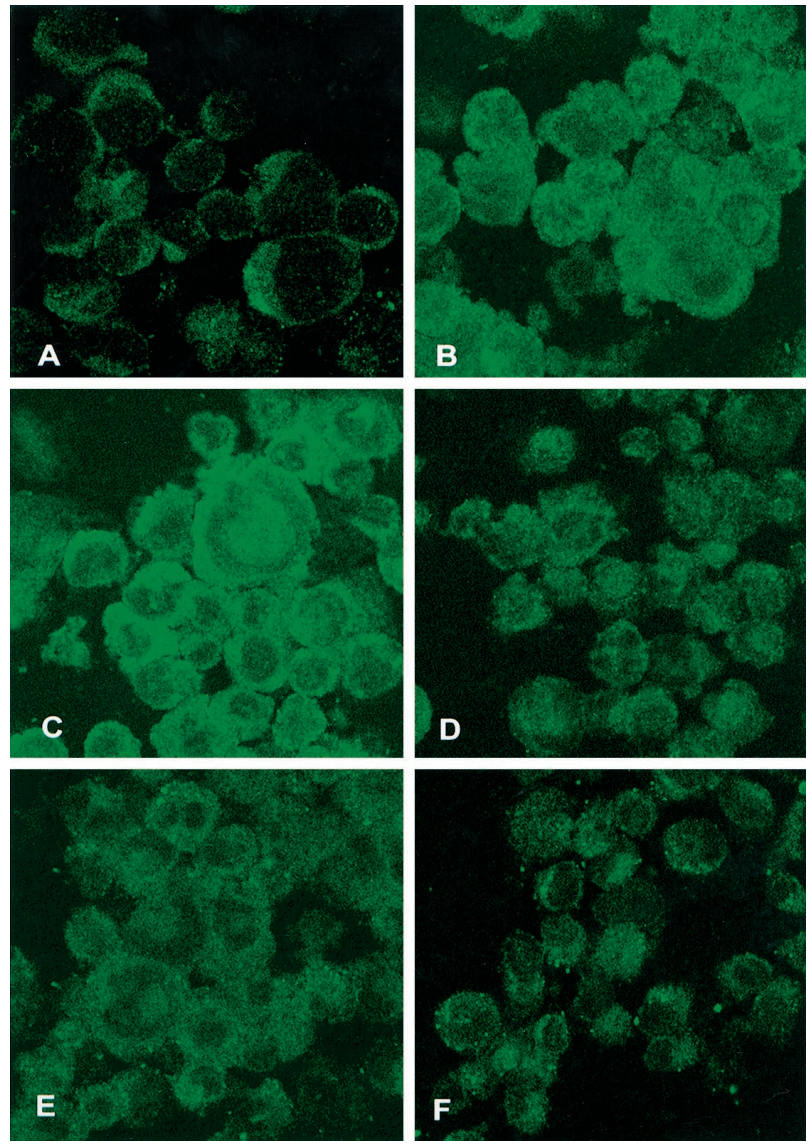

Fig. 5. Nuclear localization of phosphorylated MAPK detected by immunostaining. TF-1a cells were treated with 10^{-7} M PMA for 0 min (*A*), 10 min (*B*), 1 h (*C*), 3 h (*D*), 6 h (*E*), and 24 h (*F*), respectively, after which the cells were collected and then fixed in 4% paraformaldehyde. Subsequently, the cells were incubated with antiphosphorylated MAPK antibody for 1 h and FITC-labeled rabbit IgG for 25 min at room temperature. MAPK immunofluorescence then was visualized on images captured by a Axiovert 100 M microscope using the LSM 510 programme, as described in "Materials and Methods." Similar results have been obtained in two independent experiments.

phage-like differentiation in ~98% of cells by 24 h; however, the degree of differentiation decreased dramatically to 16% of cells after the addition of the MEK1 antisense oligonucleotides. The sense or nonsense oligonucleotides had no significant effect on blocking cell differentiation. Of note, TF-1a cells treated with antisense oligonucleotides exhibited nuclear budding, which was not present in the original control cells (Fig. 7). The inhibition of differentiation by MEK-1 antisense oligonucleotides appeared to correlate with the inhibition of the MAPK pathway (Fig. 8), because they significantly suppressed both MEK and p44/42 MAPK phosphorylation (~60–70% at 12 and 24 h, respectively), whereas the sense oligonucleotides did not inhibit the phosphorylation of these substrates. To confirm that the antisense oligonucleotide-induced blocking effect was not from nonspecific cytotoxicity, cell viability was examined by trypan blue staining. The cells treated with sense, antisense, or no oligonucleotides had similar viability (>90%) after 12 and 24 h of incubation.

Alterations in PMA-induced Macrophage-like Differentiation of TF-1a Cells by Transient Transfection of Dominant-Negative and Constitutively Activated MEK1 cDNAs. In an attempt to confirm the results from approaches using the antisense oligonucleotides, transient transfections of TF-1a cells with WT MEK, dominant-negative MEK (8E), or constitutively activated MEK mutant (R4F) were performed. As another negative control, the vector alone (pCEP4) also was expressed in these cells. The effect of mutant MEK expression on cell differentiation was compared with control cells in the presence or absence of PMA. PMA (5×10^{-8} M) caused macrophage-like differentiation by 65% of the cells without transfection and 64% of the cells transfected with vector pCEP4. Overexpression of WT MEK1 did not alter the morphology of the transfected TF-1a cells,

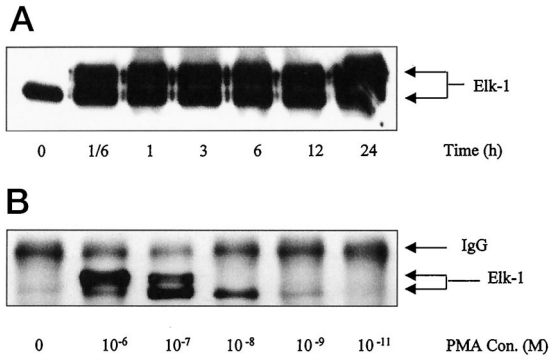

Fig. 6. Constitutive activation of MAPK induces prolonged phosphorylation of Elk-1. TF-1a cell lysates were precipitated with antiphosphorylated MAPK antibody. The resulting immunoprecipitates were then incubated with an Elk-1 fusion protein in the presence of ATP and kinase buffer. Subsequently, phosphorylation of Elk-1 at Ser383 was analyzed by Western blotting using an antiphosphorylated Elk-1 antibody. *A*, cells treated with 10^{-7} M PMA for the times indicated. *B*, cells treated with various concentrations of PMA for 1 h at 37°C. Identical results have been obtained in three separate experiments.

Table 2 Antisense oligonucleotides suppress macrophage differentiation of TF-1a cells

Addition of antisense oligonucleotides (AS), but neither sense (SS) nor nonsense (NS) oligonucleotides, inhibited differentiation of TF-1a cells (2×10^6) upon the addition of PMA. Of note, cells in the AS + PMA group exhibited nuclear budding, which was not present in the original control cells. A total of 100 cells in duplicate for each group were enumerated under 10 × 100 magnification, and the proportion of macrophages was reported. The results shown are means of two independent experiments. All cell viability tested was >90% measured by trypan blue staining.

Treatment	% macrophage morphology
0	0
PMA	98
AS + PMA	16
NS + PMA	96
SS + PMA	95

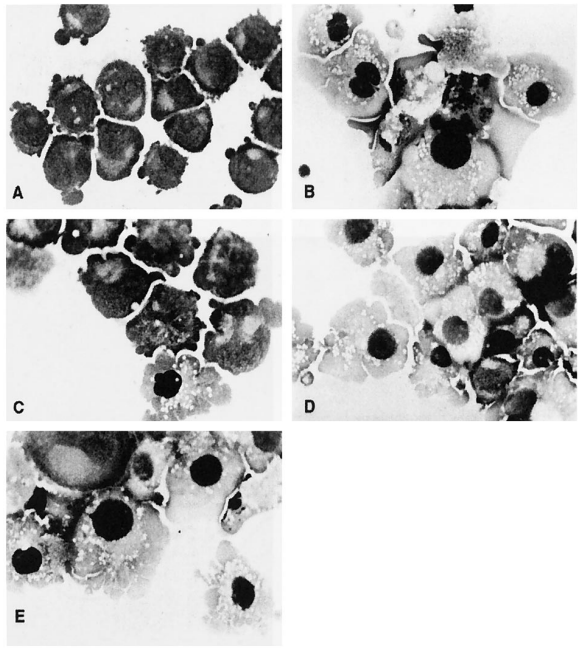

Fig. 7. Antisense MEK oligonucleotides inhibit TF-1a macrophage-like differentiation. TF-1a cells in log phase were treated with oligonucleotides (5 μM) for 2 h at 37°C. Subsequently, the cells were incubated in the presence or absence of PMA (10^{-7} M) for another 24 h, after which the cells were harvested by centrifugation and stained with Wright-Giemsa for the examination of macrophage-like differentiation. *A*, cells in culture without PMA treatment. *B*, cells in culture with PMA (10^{-7} M) treatment (refer to Fig. 1 legend for the description of normal morphology and that of differentiation). *C*, cells + PMA + MEK antisense oligonucleotides. *D*, cells + PMA + nonsense oligonucleotides. *E*, cells + PMA + MEK sense oligonucleotides. The addition of MEK antisense oligonucleotides inhibited differentiation of TF-1a cells. Macrophage-like differentiation of TF-1a cells induced by PMA was not significantly affected by either nonsense oligonucleotides or MEK sense oligonucleotides. Identical results have been obtained in two separate experiments.

and the addition of PMA still induced macrophage-like differentiation in these cells (Fig. 9). These results demonstrated that neither the plasmid alone or WT MEK interfered with the effect of PMA on cell differentiation. However, transfection with a dominant-negative MEK (8E) greatly diminished the efficiency of PMA-induced differentiation, with only ~32% macrophage-like differentiation (a decrease of ~50%; $P < 0.05$ compared with the cells treated with PMA). In contrast, the constitutively active mutant (MEK1-R4F) induced macrophage-like differentiation from a basal level of 1–2% to a MEK1-R4F-stimulated level of ~35% in the absence of PMA ($P < 0.01$; compared with that of PCEP4+, PMA−). Constitutively active MEK at least partially mimics the morphological response of the cells to PMA.

Discussion

Despite the recent advances in understanding signaling pathways, the role of the MAPK pathway in mediating cell differentiation has not been well established, and many gaps remain to be filled. The fact that MAPK becomes activated after mitogenic or nonmitogenic stimulation does not prove that it is necessary for proliferation or differentiation. In this study, data obtained suggest that prolonged MAPK is required for macrophage-like differentiation of human myeloid leukemic TF-1a cells. The results reported here demonstrate that PMA induced macrophage-like differentiation of TF-1a cells, characterized by a decrease in nuclear size, an increase in the amount of nuclear chromatin condensation, absence of nucleoli, increased cytoplasm, and up-regulation of IL-1β. PMA rapidly induced phosphorylation of MEK and MAPK. These phosphorylations persisted for at least 24 h, which suggests that the MAPK pathway might play an important role in the control of differentiation. To test this hypothesis, a number of approaches were used to delineate the possible contribution of the MAPK pathway to TF-1a cell macrophage-like differentiation. We examined whether MAPK was translocated into the nucleus after the addition of PMA, because this reagent is not able to induce $p44^{mapk}$ nuclear translocation in fibroblasts (31). By examining the activity of p44/42 MAPK in nuclear extracts and the presence of p44/42 MAPK in nuclei by immunofluorescence, we demonstrated that MAPK is primarily cytoplasmic before stimulation. After PMA treatment, MAPK rapidly translocated into the nucleus. This translocation persisted, at least for 6 h,

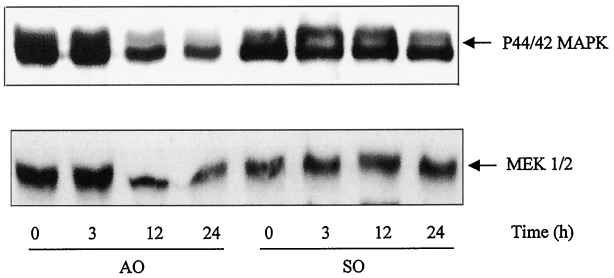

Fig. 8. Antisense MEK oligonucleotides suppress phosphorylation of MAPK and MEK. TF-1a cells growing in RPMI 1640 with 2% FBS cells were pretreated with antisense or sense MEK oligonucleotides for 2 h at 37°C before the addition of PMA (10^{-7} M), after which the cells were incubated for the times indicated. Subsequently, the cells were collected by centrifugation and lysed in sample buffer, followed by Western blotting using antiphosphorylated MAPK and MEK antibodies. Identical results have been obtained in three separate experiments.

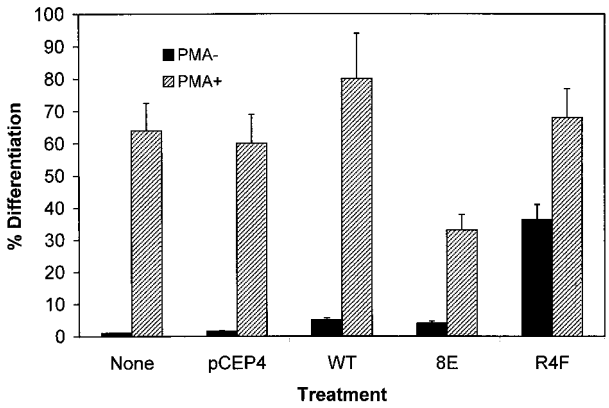

Fig. 9. Effect of mutant MEKs on macrophage-like differentiation of transfected TF-1a cells. TF-1a cells in log phase were treated with a DEAE-Dextran mixture containing 5 μg of plasmid DNAs (MEK1, 8E, R4F, and pCEP4) for 15 min at 37°C as described in "Materials and Methods." After the reaction, the DEAE-Dextran solution was removed by centrifugation, and the cells were resuspended in fresh culture medium. Subsequently, PMA (5×10^{-8} M) was added and incubated for 36 h, after which the cells were collected by centrifugation and stained with Wright-Giemsa for the examination of macrophage-like differentiation. The results shown are means of two independent experiments; *bars*, SD. The statistical significance of differences between group means was determined using Student's *t* test. Cell viabilities after transfection and PMA treatment were in a range of 83–92% as determined by trypan blue staining.

after which the signal declined to the basal level. This finding is consistent with previous studies in K562 cells (17). Because phosphorylated MAPK-specific antibody was used to visualize translocated MAPK, the translocated MAPK observed was phosphorylated and, therefore, activated. This raises the possibility that at least some of nuclear transcription factors such as c-Jun (34, 35), c-myc (36), p62 (37), and Elk-1 (38) that are known to be activated by phosphorylation might be activated by MAPK. The duration of activation of MAPK that was observed in these studies should be sufficient for MAPK to activate a nuclear substrate. To determine this, Elk-1 was selected as a test substrate for MAPK function. Elk-1 is a member of the Ets family of transcription factors that has been reported to be an important physiological substrate of ERK, mediating serum-induced expression of immediate-early genes and resulting neuronal differentiation of PC12 cells (39). Our results showed that immunoprecipitates of p44/42 MAPK from all time periods after treatment with PMA phosphorylated Elk-1, and that the levels of phosphorylation of Elk-1 from 10 min to 24 h were approximately the same.

The direct role of the prolonged activation of the MAPK pathway in TF-1a cell differentiation was demonstrated by using MEK antisense oligonucleotides. The levels of the MEK and p44/42 MAPK were found to be similarly suppressed by the MEK antisense oligonucleotides (Fig. 8), which is consistent with the role of the MEK as a regulator working upstream of p44/42 MAPK. At a concentration of 7.5 μM of the antisense oligonucleotides, we obtained a ~60–70% reduction in the level of p44/42 MAPK and MEK, respectively. Under these conditions, PMA-induced macrophage-like differentiation of TF-1a cells was greatly diminished (~80%) by the antisense oligonucleotides (Table 2). Neither the sense nor nonsense oligonucleotides had any significant effect on PMA-induced differentiation.

If prolonged activation of the MAPK pathway is essential for macrophage-like differentiation, as discussed above, then it would be expected that constitutively active mutants of MEK would induce macrophage differentiation in the absence of PMA and that the presence of a dominant-negative MEK would inhibit the ability of PMA to induce macrophage-like differentiation of TF-1a cells. Therefore, transient transfection with these plasmid cDNAs was carried out. As controls, the vector pCEP4, as well as the WT MEK, were also expressed in these cells. Indeed, the MEK1-dominant negative mutant (8E) was found to inhibit the ability of PMA to induce differentiation. In contrast, the constitutively active MEK mutant induced differentiation in the absence of PMA, although to a lesser degree than that induced by PMA alone. These experiments provide strong evidence that prolonged activation of the MAPK signaling pathway is essential for PMA-induced differentiation of TF-1a cells, and they suggest that prolonged MAPK activation may even be sufficient for inducing the macrophage-like differentiation.

It has been argued that it is the duration of MAPK activation that determines whether proliferation or differentiation will happen after stimulation (2, 14). Studies with PC12 cells suggest that prolonged activation of ERK leads to nuclear translocation, which has been proposed to explain the correlation between the kinetics of ERK activation and that of differentiation *versus* proliferation responses. In K562 cells (17), the time of expression of phosphorylated MAPK is somewhat shorter than in PC12 cells, with a peak at 2 h after addition of an inducer and return to basal levels by 24 h. In TF-1a cells, phosphorylation of MAPK and MEK was more prolonged, lasting up to 36 h after initiating PMA treatment. In contrast, GM-CSF, a well-known proliferation stimulator for human hematopoietic cells including TF-1 and TF-1a cells, only caused transient phosphorylation of MEK and MAPK. These findings suggest that prolonged activation of MAPK may be important for induction of differentiation. However, there are differences in the duration of phosphorylation/activation of MAPK in the nucleus *versus* the findings

in whole-cell lysates. The shorter time of expression of activated MAPK in the nucleus suggests that not all activated MAPK in the cytoplasm is translocated to the nucleus. It is important to note that the ability of MAPK to activate Elk-1 is stable over at least the first 24 h after PMA treatment. It is possible that some activated MAPK molecules may return to the cytoplasm after they finish their kinase job in the nucleus (as seen from 24 h of fluorescent staining in the nucleus) and maintain an active status, following which these molecules may easily re-enter the nucleus as the level of MAPK in the nucleus decreases. This presumed "shuttle" may be important for maintaining the differentiation process. Thus far, the mechanism of nuclear translocation of phosphorylated MAPK is obscure because MAPK does not seem to possess any canonical nuclear localization signal in its sequence.

In summary, it was shown here that PMA induces prolonged activation of the MAPK pathway, nuclear translocation of phosphorylated MAPK, and macrophage-like differentiation of TF-1a myeloid leukemic cells. By using antisense MEK oligonucleotides and constitutively active and dominant-negative MEK mutants, it was demonstrated that the prolonged activation of MAPK is required for macrophage-like differentiation of TF-1a cells. The data reported here also demonstrate that the time of expression of activated MAPK in the nucleus is different from that in the cytoplasm. Similar observations have been found in KG-1 human myeloid leukemia cell line (Ref. 40; data not shown).

Materials and Methods

Reagents. Recombinant human GM-CSF was purchased from Immunex (Seattle, WA), recombinant human IL-3 from R&D Systems (Minneapolis, MN), and PMA from Sigma Chemical Co. (St. Louis, MO). PD098059, polyclonal antiphosphorylated MEK and MAPK antibody kits, and the MAP Kinase Assay kit were purchased from New England BioLabs (Beverly, MA). An anti-MAPK antibody was a gift of Dr. J. Wu, H. Lee Moffitt Research Institute, Tampa, FL. Anti-IL-1β antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The kits (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, BrdUrd) for proliferation assays were purchased from Boehringer Mannheim (Indianapolis, IN). Several plasmid cDNAs were used in this study. WT MEK1, catalytically inactive MEK1 (8E) and constitutively active MEK1 (R4F) were kindly provided by Dr. Natalie G Ahn (17). The kit for transient transfection was purchased from Pharmacia Biotech (Piscataway, NJ). NP40, BSA, and goat antirabbit FITC-labeled IgG were obtained from Sigma.

Maintenance of TF-1a Cells. TF-1a (CD34+, CD38−; Ref. 22) is a factor-independent subline isolated from the human factor-dependent myeloid leukemic cell line, TF-1 (CD34+, CD38+; Ref. 22). TF-1a cells were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in humidified air containing 5% CO_2. All the media and sera were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Cell Cytology and Induction of Differentiation. Cells were cultured in the medium described above in the presence or absence of GM-CSF. During log-phase growth, PMA was added to the cells and incubated for 24–48 h. Cytospins were prepared by spinning aliquots of harvested cells at 500 rpm for 4 min, after which the cells were stained with Wright-Giemsa stain, and the level of cell differentiation was determined by morphological examination under a light microscope at 10 × 100 magnification.

Assays of Cell Proliferation. Cell proliferation was examined by directly counting cells with a hemocytometer and by indirect colorimetric immunoassay (BrdUrd incorporation). The BrdUrd assay is based on the incorporation of the pyrimidine analog BrdUrd instead of thymidine into the DNA of proliferating cells. Briefly, cells were incubated in 96-well microplates in a final volume of 100 μl/well in the presence or absence of PMA and GM-CSF (5 ng/ml). After 48 h incubation, BrdUrd was added to the cells for another 12 h. Subsequently, the culture medium was removed, and anti-BrdUrd antibody was added to the cells. The immune complexes were then detected by a subsequent substrate reaction and measured using an ELISA reader at a wavelength of 450 nm as recommended by the manufacturer.

Preparation of Cell Lysates and Nuclear Extracts. Exponentially growing TF-1a cells were washed free of serum and growth factors and incubated in serum-free RPMI 1640 for 24 h at 37°C in a humidified atmosphere of air containing 5% CO_2. Before stimulation, TF-1a cells were pelleted and resuspended in serum-free medium without growth factors. These cells were then exposed to PMA at 37°C for 0–36 h, after which the cells were washed once with cold PBS and lysed in 1 × SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue). Aliquots of each lysate containing the same cell number were subjected to Western blotting and were probed with anti-p44/42 MAPK and antiphosphorylated MAPK antibodies. To prepare nuclear extracts, TF-1a cells (1×10^7) were washed twice with PBS and once with the PBS containing 1 mM sodium orthovanadate (Na_3VO_4) and 5 mM NaF. Subsequently, the cells were washed with 2 ml of 1× hypotonic buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, and 1 mM EGTA] and lysed in 1× hypotonic buffer supplemented with 0.2% NP40 (41). Thereafter, the nuclear pellets were collected by centrifugation at 15,000 × *g* for 10 min and resuspended in 50–100 μl of 1× high salt buffer (420 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, and 20% glycerol), after which they were incubated at 4°C for 30 min with constant rotation. Subsequently, the nuclear extracts (the supernatants) were collected by centrifugation and were stored at −80°C. Protein concentrations of the nuclear extracts were determined by colorimetric assay using DC protein assay kit (Bio-Rad, Hercules, CA), following the manufacturer's instruction.

Western Blotting Analysis. An aliquot of each lysate was heated at 100°C for 4 min before SDS-PAGE. Proteins were separated on a 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). The proteins in the membrane were then immunoblotted with anti-IL-1β, antiphosphorylated p44/42 MAPK, or MEK antibody overnight at 4°C. The first antibody-containing solution was then removed, and the blot was washed three times in TBST buffer [20 mM Tris, 137 mM NaCl (pH 7.6), and 0.1% Tween 20]. To detect the antibody reaction, the blot was incubated with horseradish peroxidase-conjugated antirabbit secondary antibody at room temperature for 1 h, and the product was detected by chemiluminescence (NewEngland BioLabs, Beverly, MA), as recommended by the manufacturer.

Immunofluorescent Analysis of Fixed Cells. TF-1a cells untreated or treated with PMA were plated in six-well plates for 0–24 h at 37°C in humidified air containing 5% CO_2, after which the cells were collected by centrifugation and resuspended in PBS. About 5×10^4 cells were cytospun on slides with a Shandon cytocentrifuge (Pittsburg, PA) for 3 min at 500 rpm and were immediately fixed with 4% paraformaldehyde at 4°C for 20 min. The cross-linking was stopped with 1% glycine in PBS, and the cells were permeabilized for 2 h by several changes of PBS containing 1% glycine and 0.5% Triton X-100. Rabbit polyclonal antibody against phosphorylated MAPK was diluted 1:250 with PBS containing 0.1% NP40 and 1% BSA and incubated with the cells for 1 h at room temperature. After several washes with PBS for 2 h, the cells on the slides were incubated with 1:125 diluted goat antirabbit FITC-labeled IgG in 0.1% NP40, 1% BSA in PBS for 25 min at room temperature. Subsequently, the slides were washed, dried, and covered with coverslips in Vectashield mounting media of antifade/4′,6-diamidino-2-phenylindole (1:1) (Vector Laboratories, Inc., Burlingame, CA). Immunofluorescent confocal laser scanning microscopy was performed with an Axiovert 100 M microscope (Zeiss, Germany), and images were captured with the LSM 510 programme (Zeiss, Germany).

Functional MAPK Activity Assay. Untreated or PMA-treated serum-free TF-1a cells were chilled on ice, washed with cold PBS, and lysed in cold lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin). Cell lysates were centrifuged at 13,000 × *g* for 10 min at 4°C, and the supernatant fluid fractions were transferred to fresh tubes. Protein concentrations of the lysates were determined by colorimetric assay using the DC protein assay kit, as described in "Preparation of Cell Lysates and

Nuclear Extracts." Lysates containing 200 μg of total protein in 500 μl were immunoprecipitated by incubating at 4°C overnight with antiphosphorylated p44/42 MAPK monoclonal antibody. Subsequently, protein A-Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ) was added. The immunoprecipitates were washed three times with cell lysis buffer and twice with kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanade, and 10 mM $MgCl_2$). The kinase activity assay was performed by incubating the immunoprecipitates in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of Elk1 fusion protein (NewEngland Biolab). After 30 min at 30°C, the reaction was stopped by adding 30 μl of 3× SDS sample buffer and loaded onto 10% SDS-PAGE. Phosphorylation of Elk-1 was analyzed by Western blotting using an antiphosphorylated Elk-1 antibody (NewEngland Biolab), following the manufacturer's protocol.

Oligonucleotide Uptake. Phosphorothioate antisense (5′-GCTTCT-TCTTGGGCATCT-3′, corresponding to the start codon of MEK1), sense (5′-AGATGCCCAAGAAGAAGC-3′), and nonsense (5′-AAATTCGTG-GACGTTCGC-3′) oligonucleotides were purchased from Bio-Synthesis, Inc. (Lewisville, TX). The oligonucleotides were purified by polyacrylamide gel electrophoresis. Briefly, cells were incubated with the oligonucleotides (5–7.5 μM) described above for 2 h at 37°C, 5% CO_2. Subsequently, the cells were incubated in the presence or absence of PMA (10^{-7} M) for another 24 h, after which they either were harvested by centrifugation and stained with Wright-Giemsa for the examination of macrophage-like differentiation or lysed in 1× sample buffer for Western blot analysis.

Transient Transfection. TF-1a cells in log phase were washed once with Tris-HCl (pH 7.5) and resuspended in Tris-HCl at the concentration of 2×10^6/ml. A DEAE-Dextran-DNA mixture containing 5 μg of plasmid DNA was prepared following the manufacturer's instructions, and 200 μl of the mixture were added, drop by drop, to the 1 ml of cell suspension described above. The cells were then incubated for 15 min at 37°C and rocked every 5 min to spread the solution evenly across the cells. Then cells were collected by centrifugation, washed once in Tris-HCl (pH 7.5), and resuspended in complete culture medium. The next day, PMA was added, and the mixture was incubated for another 24 or 48 h. Subsequently, the cells were harvested and stained with Wright-Giemsa for the examination of differentiation as described previously. Transfection efficiencies were measured indirectly by expression of green fluorescent protein (Clonetech, Palo Alto, CA) in paralleled transfection assays for each experiment.

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