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Signaling Mechanism of PMA-Induced Differentiation of K562 Cells

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We have studied the signaling pathways responsible for the monocytic and/or megakaryocytic differentiation of K562 cells. The results demonstrated that although the mitogen-activated protein kinase (MAPK) was activated during the phorbol myristate acetate (PMA)-induced monocytic and/or megakaryocytic differentiation of K562 cells, the overexpression of Ha-*ras* which can activate the MAPK did not induce the monocytic and/or megakaryocytic differentiation of K562 cells. Instead PMA-induced megakaryocytic differentiation of K562 cells was inhibited by the pretreatment of pyrrolidine dithiocarbamate, a specific nuclear factor κB (NF-κB) inhibitor. Taken together, these results suggest that the activation of NF-κB rather than that of MAPK might be involved in the PMA-induced megakaryocytic differentiation of K562 cells.

The K562 cell line was established from the pleural effusion of a patient with chronic myelogenous leukemia in blast crisis (1). It had been known that K562 cells have the capacity to express characteristics of erythrocytic, monocytic, and megakaryocytic differentiation when exposed to various agents (2). As shown by several investigators, the K562 cells can be induced to differentiate into cells with monocytic and/or megakaryoblastic characteristics by tumor-promoting phorbol esters, e.g., phorbol myristate acetate (PMA), a protein kinase C (PKC) activator (3, 4–7). Recently it has been demonstrated that PKCα is involved in the megakaryocytic differentiation of K562 cells (8). In the HL60 human myeloid leukemia cells, PKC-β is necessary and sufficient for PMA-induced macrophage differentiation (9, 10), and the Raf-1 and mitogen-activated protein kinase (MAPK) are activated during the monocytic differentiation of HL60 cells induced by the treatment of PMA (11). PKC can phosphorylate directly and activate the cytoplasmic Raf-1 serine/threonine kinase (12–14) which can activate MAPK via MAPKK (MAPK kinase) (15–17).

Although PMA is a well-characterized activator of PKC (18), less is known about the signaling pathways responsible for monocytic and/or megakaryocytic differentiation of K562 cells. In the present studies, the signaling mechanism of PMA-induced differentiation of K562 cells was determined to see whether the activation of MAPK can induce the monocytic and/or megakaryocytic differentiation of K562 cells.

MATERIALS AND METHODS

Cell culture. K562 cells were grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultured cells were passed twice each week, seeding at a density of about 2×10^5/ml.

Transfection. Croaker's method (19) was slightly modified to transfect pMMTVrasH and pZIPrasH(20) (kindly donated by G.M. Cooper, Harvard Medical School) into K562 cells. 20 μg of supercoiled plasmid DNA was added to K562 cells (2×10^6) and electroporation was carried out using Electroporator (ECM600, Biotechnology & Experimental Research Inc.) at 2,000 V/cm and 25 μF condition. Electroporated cells were cultured in G418 (450 μg/ml) containing medium. After 2 weeks, individual G418 resistant colonies were isolated and expanded in the presence of G418 to generate stable transformants.

Northern blot. Northern blot analysis was done as described (21). For blot hybridization, an equal amount of RNA was electrophoresed in 1% agarose-formaldehyde gels, transferred to nylon membrane. Membrane was probed with a random-

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primed BamH1 fragment of pZIPrasH (kindly provided by G. M. Cooper). and autoradiographed. Transcript sizes were estimated by comparison with the migration of rRNAs.

Immune complex kinase assays for MAPK activity. Cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl pH 8, 1% Triton X-100, 10% glycerol, 131 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin and 10 μg/ml aprotinin) for 10 min with occasional vortexing. Lysates were cleared of nuclei and detergent insoluble material by centrifuging for 10 min at 14,000 rpm. 100 μg of cell lysate were immunoprecipitated with the anti-MAPK antibody (Zymed Laboratories, Inc.) and protein A-Sepharose beads (Sigma Chemical Co.) for overnight. Immune precipitates were collected by centrifuging for 10 sec at 14,000 rpm and were washed three times with 1 ml of kinase buffer (30 mM Tris pH 8, 10 mM $MgCl_2$) before being resuspended in 30 μl of kinase assay cocktail containing kinase buffer, 7 μg of myelin basic protein (MBP, Sigma Chemical Co.), 2 μM cold ATP and 1 μCi of [γ-^{32}P]ATP (Amersham Co.) per sample. Incubation was carried out for 30 min at 30°C and were terminated by the addition of 2 × SDS-PAGE sample buffer, followed by boiling for 5 min at 95°C. Samples were resolved on a 12% SDS-PAGE. The gel was dried and autoradiographed. The bands were excised from the dried gel, and the incorporated radioactivity was determined by Cerenkov counting.

Non-specific esterase staining. The cells were collected, washed three times with serum-free RPMI and cytocentrifuged (up to 10^5 cells per slide). Non-specific esterase staining was done with staining kit (Muto Pure Chemicals, LTD, Japan) according to manufacturer's manual. Non-specific esterase-stained Cytospin (Shandon Southern Instrument Inc.) preparations were examined microscopically for intracellular dark red granules, which is positive in monocyte and megakaryocyte (22).

Flow cytometric analysis. Quantitative fluorescence analysis was performed with the Fluorescence-Activated Cell Sorter (Becton-Dickinson, Mountain View, Calif.). For each label, 2 to 5 × 10^5 cells were analyzed for light scattering and for fluorescence intensity.

RESULTS

Activation of MAPK by the treatment of PMA. To examine whether the MAPK activity can be modulated during the monocytic and/or megakaryocytic differentiation of K562 cells, K562 cells were treated with the various concentration of PMA, and MAPK activity was determined by the immune complex kinase assay. The MAPK activity was increased dose-dependently with the maximal increase (about 4.2 fold) at 32 nM of PMA (Fig. 1), indicating that the increased activity of MAPK was associated with the monocytic and/or megakaryocytic differentiation of K562 cells induced by PMA.

*Effect of overexpressed Ha-*ras*on the differentiation of K562 cells and MAPK activity.* If the activation of MAPK is associated with the monocytic and/or megakaryocytic differentiation of K562 cells, overexpression of Ha-*ras* that lead to the activation of MAPK (23) should induce the monocytic and/or megakaryocytic differentiation of K562 cells. As shown in Fig. 2A, Ha-*ras* was overexpressed by the treatment of dexamethasone in K562 cells that had been transfected by a pMMTVrasH plasmid containing normal Ha-*ras.* To determine whether the monocytic and/or megakaryocytic differentiation of the pMMTVrasH-transfected K562 cells can be inducible by the overexpression of Ha-*ras,* cells were treated with 1 μM of dexamethasone to induce the expression of Ha-*ras.* Surprisingly, the pMMTVrasH-transfected K562 cells were not differentiated by the overexpression of Ha-*ras* (Fig. 2B). Dexamethasone (1 μM) did not inhibit the differentiation of K562 cells by PMA (10 nM). Also we could not find out any evidence of differentiation during the transient expression of pZIPrasH construct (data not shown).

Because the pMMTVrasH-transfected K562 cells were not differentiated by the induction of

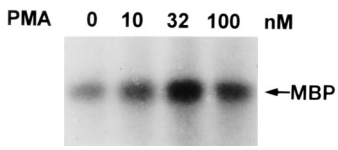

FIG. 1. Activation of MAPK by the treatment of PMA. K562 cells were treated with PMA for 10 min. Cell lysate (100μg) was immunoprecipitated with anti MAPK antibody. Immune complex kinase assay was done to measure kinase activity toward exogenously added MBP. Products from the kinase reaction were separated by 12% SDS/PAGE and autoradiographed.

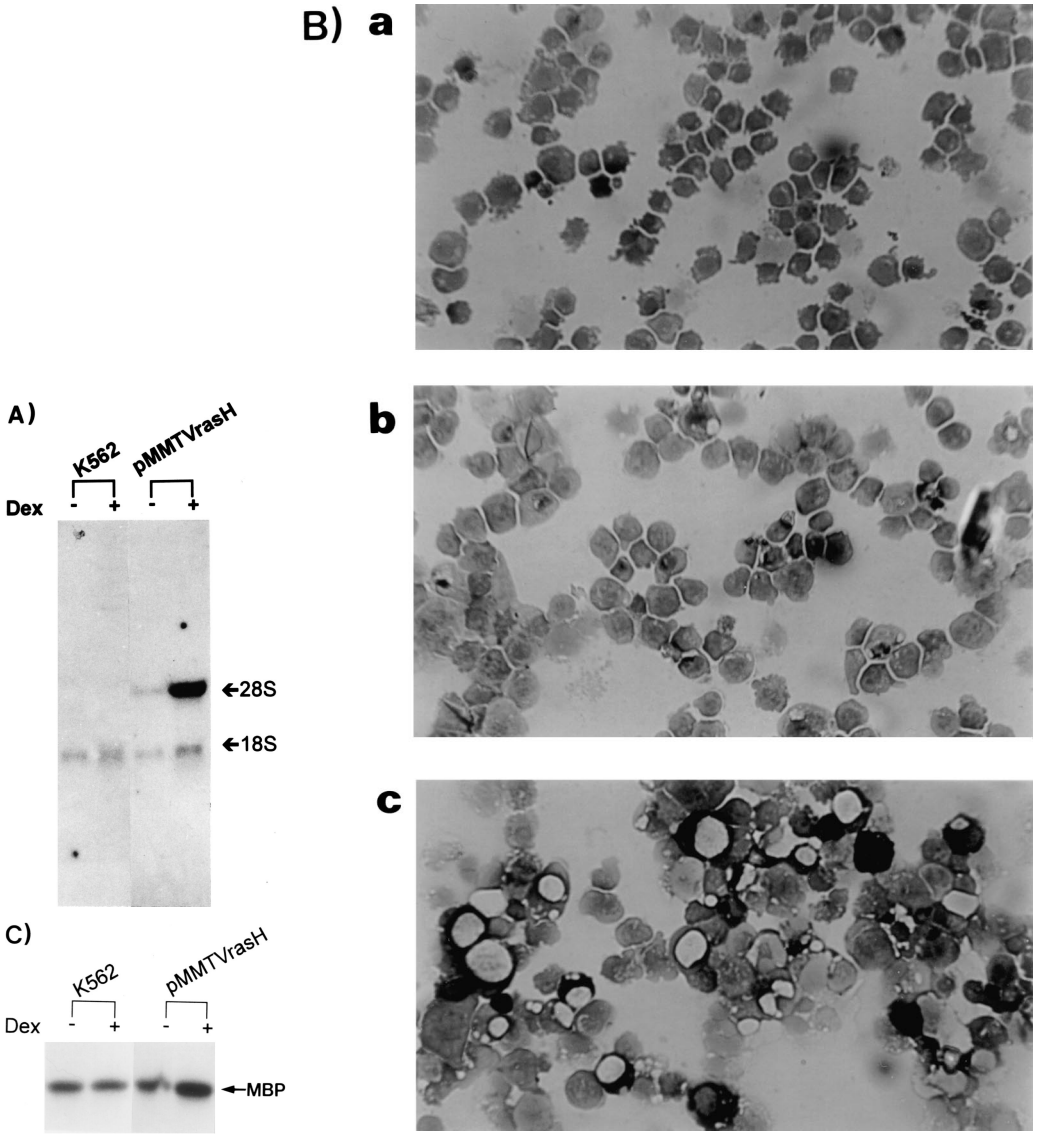

FIG. 2. A) Expression of Ha-*ras* in pMMTVrasH-transfected K562 cells. K562 cells and pMMTVrasH-transfected K562 cells wereanalysed for overexpression of Ha-*ras* by Northern blotting. Dexamethasone (1 μM) was added 24 h prior to sample preparation as indicated (Dex +). B) Effect of overexpressed Ha-*ras* on the differentiation of K562 cells. 2×10^5 cells/ml of pMMTVrasH-transfected K562 cells were treated with (b) or without (a) dexamethasone (1 μM). After 3 days of incubation, Cytospin preparations were stained to examine non-specific esterase. a) pMMTVrasH-transfected K562 cells, b) pMMTVrasH-transfected K562 cells treated with dexamethasone and c) K562 cells treated with dexamethasone and PMA (10 nM). C) MAPK activation by the overexpression of Ha-*ras.* After treatment of dexamethasone (1 μM) for 24 h to overexpress the transfected Ha-*ras,* Immune complex kinase assay was done as described in the legend to Fig. 1.

Ha-*ras*, it should be known that the differentiation of the pMMTVrasH-transfected K562 cells was not induced by the overexpression of Ha-*ras* in spite of the activation of MAPK. As shown in Fig. 2C, MAPK activity was increased about 2.5 fold by the overexpression of Ha-*ras.* This finding suggests that although the MAPK activity is increased during the PMA-induced differentiation of K562 cells (Fig. 1), the MAPK activation *per se* can not induce the monocytic and/or megakaryocytic differentiation of K562 cells. Therefore, other pathways downstream to PKC including the

activation of NF-κB must be involved in the monocytic and/or megakaryocytic differentiation of K562 cells.

Effect of PDTC on the PMA-induced differentiation of K562 cells. In response to various stimuli including PMA, the NF-κB can be activated (24–26) and the activation of NF-κB could be blocked specifically by the treatment of the pyrrolidine dithiocarbamate (PDTC) (27, 28). As shown in Fig. 3A, more than 80% of PMA-treated cells were positive non-specific esterase staining, and it was inhibited to about 40% by the pretreatment of PDTC. In addition, the percentage of strongly positive cells were markedly decreased by the pretreatment of PDTC. The ability of PDTC to block the PMA-induced differentiation of K562 cells was confirmed by flow cytometric analysis (Fig. 3B). Monoclonal antibodies against CD14 and CD 61 which are specific to monocytic and megakaryocytic lineage, respectively, were used. After induction of differentiation with PMA, expression of CD61 was markedly increased from 1% to about 45%. The increased expression of CD61 was inhibited to about 24% by the pretreatment of PDTC. The expression level of CD14 was not modulated significantly by the treatment of PMA and pretreatment of PDTC (data not shown). These results indicate that the activation of NF-κB might be involved in the PMA-induced megakaryocytic differentiation of K562 cells.

DISCUSSION

Protein kinase C has been the subject of intense interest since it was first discovered that it is the

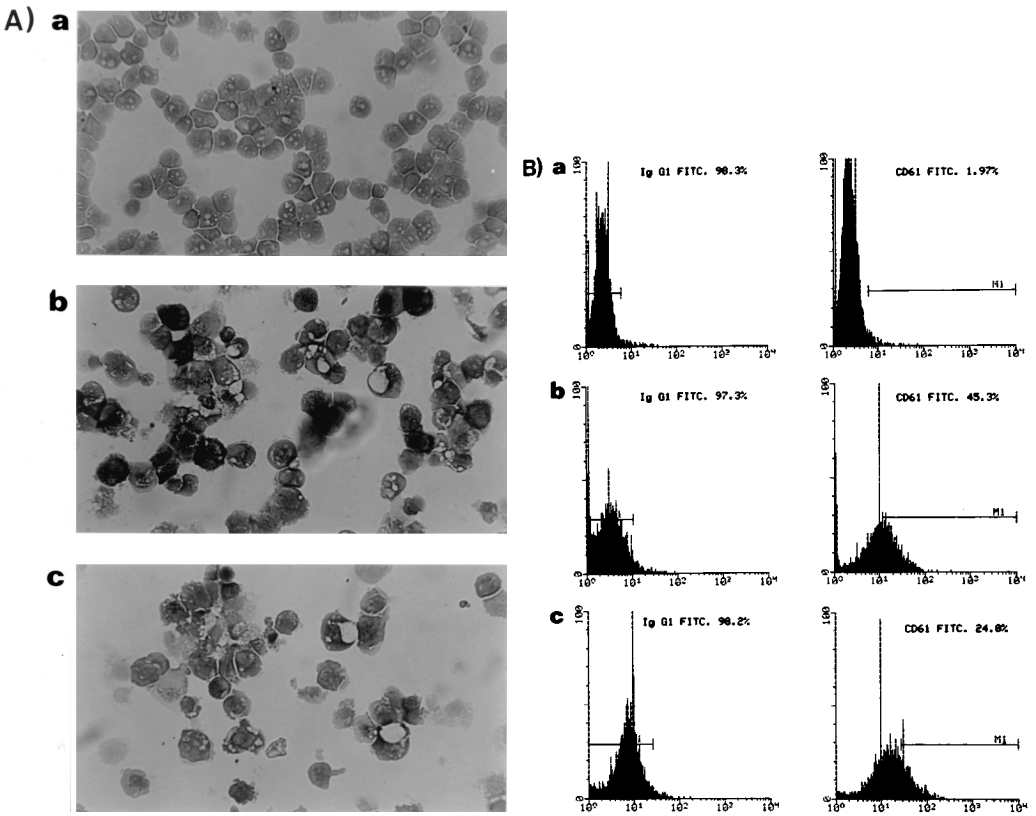

FIG. 3. A) Effect on PDTC on the PMA-induced differentiation of K562 cells. 2×10^5 cells/ml of K562 cells were pretreated for 1 h with PDTC (1 μM) and followed by the treatment of PMA (10 nM). After 3 days of incubation, Cytospin preparations were stained to examine non-specific esterase. a) untreated cells, b) PMA-treated cells, and c) PDTC and PMA-treated cells. B) Flow cytometric analysis of K562 cells. Cells were treated as above and analysed with CD61 monoclonal antibody. a) untreated cells, b) PMA-treated cells, and c) PDTC and PMA-treated cells. Left panels show the non-specific binding of IgG_1, and right panels show the specific binding of CD61 monoclonal antibody.

major intracellular receptor for phorbol ester tumor promoters such as PMA (18). In many cell types, including fibroblasts (29) and T lymphocytes (30), PMA stimulates cellular proliferation, suggesting a role for PKC activation in this process. Paradoxically, PMA has also been shown to induce terminal differentiation in several human tumor cell lines including human chronic myelogenous leukemic cells (K562) (31, 32) and human promyelocytic leukemic cells (HL60) (33–35). In the HL60 human myeloid leukemia cells, the Raf-1 and MAPK were activated during the monocytic differentiation of HL60 cells induced by the treatment of PMA (11). However, the relationship between MAPK activation and the induction of differentiation has been unclear. Consequently, we asked whether PMA-induced signal can stimulate MAPK activity in K562 cells, and MAPK activation by overexpression of Ha-*ras* can induce the megakaryocytic differentiation of K562 cells.

In this study, the MAPK activity was increased dose-dependently by the treatment of PMA. This result is consistent with the other results using HL60 and U937 cells in which the MAPK was activated by the treatment of PMA (11, 23) and indicate that K562 cell could be differentiated into monocytic and/or megakaryocytic lineage by the similar mechanism with HL60 and U937 cells. However, it is not clear whether the activation of MAPK is required for the differentiation induction of K562 cells.

Because Ras can activate MAPK through Raf-1 and MAPKK, overexpression of Ha-*ras* could induce the monocytic and/or megakaryocytic differentiation of K562 cells. However, pMMTVrasH-transfected cells were not differentiated by the overexpression of Ha-*ras.* It is unlikely that this phenomenon is due to clonal variation, because all isolated clones have shown same results. Thorn et al. (36) also had not observed the differentiation in N-*ras* transfected K562 cells. From this result it is suggested that signal transduction pathways other than Raf-1/MAPK cascade should be involved in the monocytic and/or megakaryocytic differentiation of K562 cells.

Activation of protein kinase C by phorbol esters lead to activation of NF-κB (25). Therefore, PDTC, a specific inhibitor of NF-κB, was thus used to examine whether the activation of NF-κB is involved in the monocytic and/or megakaryocytic differentiation of K562 cells. The PMA-induced differentiation of K562 cells was inhibited by the pretreatment of PDTC which does not interfere with the PMA-induced membrane association and kinase activity of PKC (37), and other DNA binding activities and the induction of AP1 by PMA (27). The increased expression level by PMA of CD61 was inhibited by the pretreatment of PDTC. Recently it has been described that PMA induces a macrophage-like morphology with enhanced expression of proteins associated with megakaryocytes in K562 cells (38). We have found similar phenomenon during the PMA-induced differentiation of K562 cells. Therefore these finding indicate that although the MAPK activity was increased during the PMA-induced differentiation of K562 cells, NF-κB activation rather than MAPK activation might be responsible for the PMA-induced megakaryocytic differentiation of K562 cells. Further work will be required to elucidate the specificity and generality of NF-κB's role in the differentiation of leukemic cells.

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