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MAPK-dependent expression of $p21^{WAF}$ and $p27^{kip1}$ in PMA-induced differentiation of HL60 cells

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Abstract Treatment of HL60 cells with phorbol 12-myristate 13-acetate (PMA) results in growth arrest and differentiation towards the macrophage lineage. PMA-induced changes are easily monitored by morphological changes while cells in suspension start adhering onto substrate. PMA induces rapid activation of the extracellular signal-regulated kinases (ERKs). Activation of the ERK pathway is essential to PMA-induced differentiation of HL60 cells. PMA also induces the expression of the cyclin-dependent kinase inhibitors $p21^{WAF}$ and $p27^{kip1}$, which is modulated by the use of an inhibitor of the ERK cascade. This implies that a link exists between ERK activation and $p21^{WAF}$ and $p27^{kip1}$ induction in the process of terminal differentiation.

Key words: Differentiation; Mitogen-activated protein kinase; $p21^{WAF}$; $p27^{kip1}$

1. Introduction

Activation of mitogen-activated protein kinase (MAPK) family members is involved in a wide range of cellular functions. MAPK activity is elevated in response to proliferative factors [1,2], as well as in response to differentiating factors such as nerve growth factor (NGF) [3]. In PC12 cells, prolonged activation of extracellular signal-regulated kinase (ERK)-1 and -2 correlates with the NGF-induced differentiation [4]. NGF suppresses the activity of cyclin-dependent kinases (CDKs) in several cell types and this inhibition of CDK activity appears to be critical in the ability of NGF to induce differentiation [5,6]. The cell cycle inhibitors p21 (WAF) and p27 (kip1) play a critical role in the control of the cell cycle by interacting with multiple cellular targets. $p21^{WAF}$ has been implicated in mediating growth arrest in response to a variety of conditions including terminal differentiation [7]. Induction of $p21^{WAF}$ in response to DNA damage occurs primarily through a transcriptional mechanism that requires interaction of tumor suppressor protein p53 with a p53 binding site in the $p21^{WAF}$ promoter [8]. However, induction of $p21^{WAF}$ in other situations, such as during cellular differentiation, does not appear to require p53 [9,10]. The mechanism that regulates $p21^{WAF}$ expression in these p53-independent circumstances is not clear.

In this study, we have addressed the role of MAPK signaling pathway in regulating $p21^{WAF}$ and $p27^{kip1}$ expression, and their involvement in phorbol 12-myristate 13-acetate (PMA)-induced terminal differentiation of HL60 cells. Data are presented that directly link activation of the MAPK pathway to the induction of $p21^{WAF}$ and $p27^{kip1}$ and cellular differentiation in response to PMA.

2. Materials and methods

Reagents and chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise indicated.

2.1. Cell culture and induction of differentiation

HL60 promyelocytic leukemia cells were obtained form the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco BRL). For MAPK assay, cells in logarithmic growth were serum-starved in RPMI medium containing 0.5% FBS for 24 h. To induce differentiation, the cells in logarithmic growth were seeded at 2×10^5/ml and treated with 25 nmol/l PMA dissolved in dimethyl sulfoxide (DMSO) (final concentration of DMSO in culture medium was 0.02%). Control cultures were treated similarly with the solvent DMSO. In this condition, the initial adherence of the cells to the bottom of the plastic plate was seen at 24 h. To test the role of MAPK activation in differentiation, HL60 cells were pre-incubated for 30 min with 50 μM MAPK kinase (MEK) inhibitor PD098059 (Calbiochem, San Diego, CA, USA).

2.2. Preparation of cell extracts

Cells were washed with ice cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 50 mM NaF, 2 mM sodium orthovanadate and 10 mM sodium pyrophosphate) for 30 min on ice. The lysates were then centrifuged at $14\,000\times g$ for 10 min and the supernatant was either used or stored at −80°C.

2.3. Antibodies

For Western blotting analysis, we used the following antibodies: anti-active ERK and anti-active p38 antibodies were from Promega, Madison, WI, USA. The $p21^{WAF}$ (C-19) polyclonal antibody and ERK-2 (C-14) polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and $p27^{kip1}$ monoclonal antibody from Transduction Laboratories (Lexington, KY, USA). P38 polyclonal antibody was obtained from New England Biolabs, Beverly, MA, USA.

2.4. Immunoblotting

Equal amounts of protein (40–60 μg) from HL60 cell extracts were separated by polyacrylamide gel electrophoresis, and then transferred to nitrocellulose. The membrane was blocked for 1 h with 5% BSA in Tris-buffered saline (TBS), pH 7.8, and then incubated with antibodies overnight at 4°C. The membrane was washed with TBS containing 0.1% Tween 20. Immunoreactive proteins were detected by using horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Height, IL, USA) and an enhanced chemiluminescence detection system (Amersham).

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2.5. Assay of cellular proliferation

Cells were seeded at 5×10^4/ml in 60×15 mm dishes and grown for 4 days in normal growth medium or medium supplemented as described. Cells were counted every day microscopically using a hemocytometer. All experiments were counted in triplicate.

2.6. Adhesion assay

HL60 cells were seeded at 5×10^4/ml in normal growth medium or medium supplemented as described. After 48 h, medium was removed. Residual non-adherent cells were removed by washing with 3 ml of PBS twice. Adherent cells were fixed in 3% paraformaldehyde in PBS for 10 min at room temperature and stained with 0.5% crystal violet in 20% methanol for 30 min. The stain was eluted with 0.1 M sodium citrate, pH 4.2, in 50% ethanol and the eluate optical density measured spectrophotometrically at 550 nm.

3. Results

Treatment of HL60 cells with PMA induces functional and morphological changes that are characteristic of terminally differentiated macrophages. To understand the early events that lead to PMA-induced macrophage differentiation in HL60 cells, we studied the activation of three major pathways involving MAPK family members ERK/MAPK, JNK/SAPK and p38 by analyzing their phosphorylation state using phosphospecific antibodies. A time-course analysis of endogenous phosphorylated ERKs shows that PMA induces a rapid activation of ERK-1 and -2 within 5 min of PMA treatment and then this activation goes down at 1 h (Fig. 1A). After stripping of the membrane, incubation with anti-ERK-2 antibody shows that equal amounts of ERK-2 are present in each lane. Under similar conditions, the phosphorylation state of JNK and p38 remained unchanged (data not shown).

To test whether or not PMA-induced activation of ERK-2 is due to MEK, we used a specific MEK inhibitor, PD098059. As shown in Fig. 1B, 50 μM PD098059 completely blocked ERK phosphorylation, indicating that this activation is

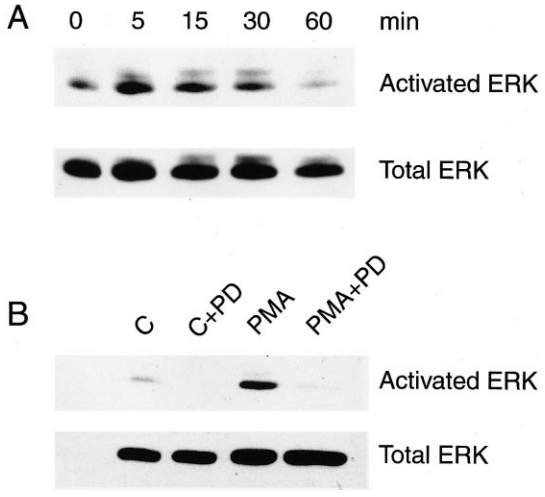

Fig. 1. ERK activation in HL60 cells exposed to PMA. (A) Time-dependent activation of ERK. After serum starvation, cells were exposed to PMA for the indicated times. Lysates were analyzed by Western blot using either anti-active ERK antibody (upper panel) or anti-total ERK-2 antibody (lower panel). (B) Lysates from HL60 cells were prepared 15 min after exposure to PMA or vehicle (C). Cells were pre-incubated with PD098059 (PD) where shown. Western blot analysis was carried out by using either anti-active ERK antibody (upper panel) or anti-total ERK-2 antibody (lower panel). Anti-total ERK-2 antibody cross-reacts with ERK-1.

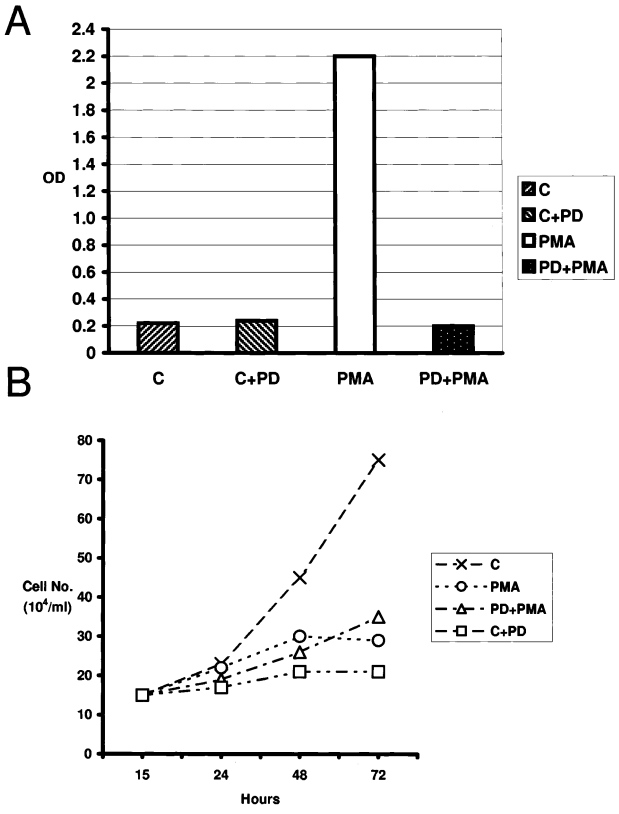

Fig. 2. (A) Adhesion assay of HL60 cells in the presence or absence of PMA plus or minus PD098059 (PD). Crystal violet eluate optical density at 586 nm was measured as described in Section 2. (B) Growth curve of HL60 cells in the presence or absence of PMA plus or minus PD098059 (PD). Cells were seeded and counted as described in Section 2.

MEK-dependent. PD098059 also inhibits the basal level of phosphorylation of ERK in control cells. The lower panel of Fig. 1B shows that equal amounts of ERK-2 are present in each lane.

To investigate whether or not the ERK pathway plays any role in the process of PMA-induced morphological changes and differentiation, we used the same MEK inhibitor. Preincubation with PD098059 in the culture medium before the addition of PMA completely blocked the morphological changes associated with differentiation (data not shown). To substantiate this observation, an adhesion assay with crystal violet showed that the eluate obtained from PMA-treated cells had increased optical density (Fig. 2A), indicating cell adhesion to the dish. In contrast, the eluate obtained from cells pretreated with PD098059 and then treated with PMA had an optical density close to the control cells, indicating that no adherent cells were present (Fig. 2A). In addition, we found that PMA treatment resulted in growth inhibition of HL60 cells that was evident at 48 h (Fig. 2B). To test whether modulation of MAPK activity is involved in the growth arrest, HL60 cells were incubated with PD098059. PD098059 itself inhibited the serum-dependent growth rate of control cells, indicating that MAPK activation is necessary for normal growth rate of HL60 cells.

During the process of terminal differentiation induced by PMA, HL60 cells appear to completely leave the cell cycle. To investigate the possible mechanism of cell cycle arrest, we

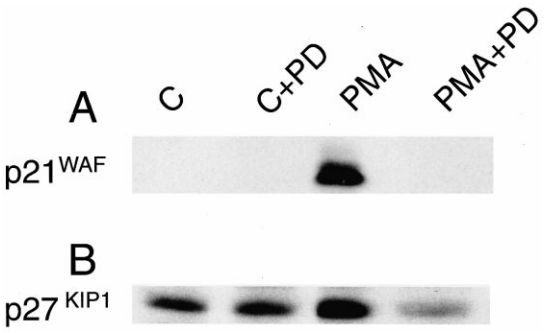

Fig. 3. PMA-induced expression of $p21^{WAF}$ and $p27^{kip1}$. Lysates were obtained from HL60 cells treated for 24 h with either DMSO (C) or PMA, in the presence or absence of PD098059 (PD). (A) Western blot analysis by using anti-$p21^{WAF}$ antibody. (B) Western blot analysis by using anti-$p27^{kip1}$ antibody.

studied the expression levels of $p21^{WAF}$ and $p27^{kip1}$ that both bind to and inactivate a variety of cyclin/CDK complexes and thereby regulate the cell cycle. Exponentially growing HL60 cells express no detectable amount of $p21^{WAF}$ (Fig. 3A) but have detectable levels of $p27^{kip1}$ (Fig. 3B). Induction of $p21^{WAF}$ and an increased expression of $p27^{kip1}$ are detected after 24 h of PMA treatment. This correlates well with the time when these cells have a lower growth rate and start to show the changes in adherence behavior. The high levels of expression of both the CDK inhibitors are sustained up to 48 h of PMA treatment (data not shown). To test whether or not the ERK pathway is also involved in the regulation of $p21^{WAF}$ and $p27^{kip1}$, cells were exposed to PD098059 prior to the addition of PMA. It was observed that PD098059 completely blocked the induction of both $p21^{WAF}$ (Fig. 3A) and $p27^{kip1}$ (Fig. 3B). This result clearly shows that the ERK pathway is involved in PMA-induced $p21^{WAF}$ and $p27^{kip1}$ expression in HL60 cells.

4. Discussion

Our data provide evidence that PMA-induced activation of ERK in HL60 cells leads to growth arrest and terminal differentiation. The MEK inhibitor PD098059 blocks this effect. PMA also induces $p21^{WAF}$ and $p27^{kip1}$, which correlates well with morphological changes of the cells and growth arrest. HL60 cells lack p53 that is one of the activators of $p21^{WAF}$ transcription. Our data suggest that increased ERK activity leads to the induction of both $p21^{WAF}$ and $p27^{kip1}$. In fact, PD98059 blocks both ERK activation and $p21^{WAF}$ and $p27^{kip1}$ induction. At the same time, PD98059 also blocks the morphological changes induced by PMA. This clearly indicates the involvement of the ERK pathway in $p21^{WAF}$ and $p27^{kip1}$ induction that may lead to growth arrest and differentiation.

Although a role of the ERK pathway in growth arrest or cellular differentiation is not unprecedented [3,11,12], our results are the first of its kind that establish a link between ERK activation and $p21^{WAF}$ and $p27^{kip1}$ induction in the process of cellular differentiation. The mechanism by which PMA-induced activation of ERK leads to induction of $p21^{WAF}$ and $p27^{kip1}$ and terminal differentiation in HL60 cells is not clear. A recent report suggests that induction of $p21^{WAF}$ in response to growth factor stimulation is mediated by activation of ERK [13]. It is possible that ERK-dependent $p21^{WAF}$ induction occurs both in response to growth factor stimulation and in a differentiating condition. Therefore, a p53-independent pathway exists that involves ERK and that can induce $p21^{WAF}$ under appropriate stimulus. PMA could stimulate this pathway to induce cell cycle arrest and differentiation in HL60 cells. Further knowledge of the mechanisms contributing to control $p21^{WAF}$ expression could improve our understanding of how mitogenic factors and differentiating agents effect the target cells.

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