Role of MAPKs and NF-κB in diosgenin-induced megakaryocytic differentiation and subsequent apoptosis in HEL cells

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Abstract. Megakaryocytopoiesis is characterized by progressive polyploidization and acquisition of megakaryocytic markers. MAPK pathways play a key role during megakaryocytic differentiation of megakaryocyte precursors or leukemic cells. Apoptosis is the physiological fate of normal megakaryocyte after differentiation and maturation. The aim of this study was to investigate the signaling pathways involved in diosgenin-induced differentiation and the fate of diosgenin-differentiated HEL cells. The present report shows that diosgenin induced megakaryocytic differentiation of HEL cells through a combined activation of ERK and inhibition of the p38 MAPK pathways. Inhibition of ERK activation by a MEK inhibitor abrogated diosgenin-induced differentiation. Afterwards, differentiated cells showed a marked inhibition of expression of survival factors NF-κB, Akt and Bcl-xL and activation of caspase-3 together with PARP cleavage leading to apoptotic death of diosgenin-differentiated cells.

Introduction

Megakaryocytopoiesis is a unique process requiring proliferation of hematopoietic stem cells and differentiation of megakaryocyte progenitors. Maturation of megakaryocytes is characterized by progressive polyploidization and acquisition of megakaryocytic markers such as the CD41-CD61 integrin (1,2).

The role of the mitogen-activated protein kinase (MAPK) pathways during hematopoietic cell differentiation is now established but remains only partially understood. The MAPK family includes the extracellular signal-related kinases (ERKs), the p38 MAPK and the c-Jun amino-terminal kinase (JNK) (3). A growing number of studies have demonstrated the key role of the ERK pathway during megakaryocytic differentiation (4-6). Recently, the p38 MAPK pathway has been shown to play a crucial role in erythroid differentiation (7). JNK also seemed to be involved in erythroid differentiation and particularly in erythropoietin signaling (8).

Previous studies reported that several hematopoietic cytokines, such as interleukin-3 or erythropoietin could activate Akt (9,10). Furthermore, thrombopoietin signaling has been demonstrated to involve Akt phosphorylation in primary megakaryocytes, megakaryocytes and platelets (11).

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that has been shown to promote cell survival by initiating the transcription of genes involved in cell proliferation or encoding anti-apoptotic proteins (12). It has been demonstrated that NF-κB activation participated in megakaryocytic differentiation induced by PMA and was also involved in thrombopoietin receptor signaling (13,14).

Diosgenin [(25R)-5-spirosten-3ß-ol] is a steroidal saponin, which has been reported to have various biological effects *in vivo* (15,16). Moreover, in function of the used dose, diosgenin has been shown to exert antiproliferative and pro-apoptotic actions on rheumatoid arthritis synoviocytes (17) or on cancer cells *in vitro* (18-20) and *in vivo* (21) and differentiating effect (22).

The aim of this study was to investigate the signaling pathways involved in diosgenin-induced differentiation of HEL cells. We focused particularly our attention on expression of phosphorylated kinases such as MAPKs and Akt but also on activity of the transcription factor NF-κB.

Materials and methods

Cell line, cell culture and treatment. The HEL cell line was kindly provided by Professor J.P. Cartron (INSERM U76, Paris, France). Cells were seeded at 10^5 cells/ml in tissue culture flasks, grown in RPMI-1640 medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Gibco BRL), 1% sodium pyruvate, 1% HEPES (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humified atmosphere with 5% CO_2 at 37˚C. Cells were allowed to grow for 24 h in culture medium prior to exposure or not to 10 μM diosgenin or 10 nM phorbol myristate acetate (PMA) (Sigma, Saint Quentin Fallavier, France). The same amount of vehicle (<0.1% ethanol) was added to control cells. Cell viability was determined by trypan blue dye exclusion method.

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Evaluation of nuclear ploidy and cell phenotype. For DNA content analysis, 10^6 cells treated or not with 10 μM diosgenin were fixed and permeabilized in 70% ethanol in phosphate-buffered saline (PBS) at -20˚C overnight, washed in PBS, treated with RNase (40 U/μl, Boehringer Mannheim, Meylan, France) for 1 h at room temperature and stained with propidium iodide (PI) (50 μg/ml). Flow cytometric analyses were performed as previously described (19,23).

The membrane phenotype was also analysed by flow cytometry. Staining was performed on 10^6 cells treated or not with diosgenin. Briefly, cells were suspended in PBS containing 1% BSA, monoclonal antibody anti-CD41-(FITC) (Southern Biotech, Cliniscience, Montrouge, France) and monoclonal antibody anti-GpA-(RPE) (Dako, Trappes, France) and then incubated 1 h at room temperature. Cells were then washed in PBS-BSA and resuspended in PBS for FACS analysis.

Protein expression analysis. After diosgenin treatment, cells were washed and lysed in RIPA lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% NP-40, 0.1% SDS, 20 μg/ml aprotinin) containing protease inhibitors (Complete™ Mini, Roche Diagnostics, Meylan, France). Western blot was performed as previously described (18). Briefly, proteins (20-80 μg) were separated by electrophoresis on SDS-polyacrylamide gels, transferred to PVDF membranes (Amersham Pharmacia Biotech, Saclay, France) and probed with respective antibodies against Akt, phospho-Akt (Thr 308), Bcl-xL and PARP all purchased from Santa Cruz Biotechnology (TEBU, Le Perray en Yvelines, France). After incubation with secondary antibodies (Dako), blots were developed using enhanced chemiluminescence reagents (Amersham) and immediately exposed to X-ray film. Membranes were then reblotted with monoclonal antibody anti-ß-actin (Sigma).

Quantification of MAP kinase activation by ELISA. After diosgenin treatment, 10^6 cells were homogenized in lysis buffer containing protease and phosphatase inhibitors in accordance with the manufacturer's protocol (R&D Systems, Lille, France). Before assay, one plate was coated with capture antibody (4 μg/ml) overnight at room temperature. The plate was then washed and blocked for 1-2 h at room temperature. Before use, cell lysates were centrifuged at 2000 g for 5 min, supernatants were diluted 6-fold and phospho-ERK1/ERK2, phospho-JNK or phospho-p38α detection was done according to the manufacturer's instructions (R&D Systems).

Electromobility shift assay (EMSA). EMSA experiments were performed using DIG Gel Shift Kit (Roche Diagnostics). Briefly, nuclear extracts were prepared from cells treated or not with 10 μM diosgenin. Cells were resuspended in lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, protease inhibitors Complete Mini, 0.5% Nonidet P-40). Nuclei were pelleted (2000 g, 10 min at 4˚C) and resuspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 15% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, protease inhibitors Complete Mini). Lysates were clarified by centrifugation (13000 g, 10 min at 4˚C) and supernatants were collected. NF-κB binding reactions were carried out with 10 μg nuclear proteins incubated with digoxigenin (DIG) labeled NF-κB probe (24) according to the manufacturer's protocol. The samples were loaded on a 5% native polyacrylamide gel in Tris-Borate-EDTA buffer. After transfer to nylon membranes and incubation with anti-DIG antibody conjugated with alkaline phosphatase, gel mobility shift was visualized by incubation with CSPD® chemiluminescence reagent and exposed to X-ray film. Quantification of each band was performed by densitometry analysis software in respect of band intensity and band area. Results are expressed relative to controls in arbitrary units.

Caspase-3 activity assay. Caspase-3 activity was assayed using Quantikine® human active caspase-3 (R&D Systems). Following induction by 10 μM diosgenin, cells were incubated with 10 μM biotin-ZVKD-fmk inhibitor for 1 h. Caspase-3 activity was then measured in accordance with the manufacturer's protocol (R&D Systems). Briefly, cells were harvested, washed in PBS and resuspended in extraction buffer containing protease inhibitors. Standards and sample extracts containing covalently linked active caspase-3-ZVKD-biotin were added to a microplate pre-coated with monoclonal antibody specific for caspase-3. Then, streptavidin conjugated to horseradish peroxidase was added to the wells. The amount of active caspase-3 was quantified by colorimetry at 450 nm after addition of the HRP substrate.

Statistical analysis. The median and standard deviation (SD) were calculated using Excel (Microsoft Office, Version 98). Statistical analysis of differences was carried out by analysis of variance (ANOVA) using StatView Version 5.0 (SAS Institute Inc., Cary, NC). A P-value of <0.05 (Fisher's PLSD test) was considered to indicate significance.

Results

Diosgenin-induced megakaryocytic differentiation of HEL cells requires ERK activation. Megakaryocytopoiesis is characterized by progressive polyploidization and acquisition of megakaryocytic markers such as the CD41-CD61 integrin (1,2).

As illustrated in Fig. 1, diosgenin treatment of HEL cells resulted in a marked decrease of the GpA erythroid marker and an increase in CD41 membrane expression. In the first 48 h following exposure of HEL cells to 10 μM diosgenin, GpA expression underwent a 60% decrease and a slight increase in CD41 expression was observed. Afterwards, GpA levels remained stable and CD41 levels rose progressively until 144 h when they reached maximum expression. By studying the expression of CD41 and GpA, we confirmed that with this new markers diosgenin induced megakaryocytic differentiation of HEL cells.

Physiological factors (thrombopoietin) as well as synthetic inducers (phorbol esters) were shown to induce sustained activation of the ERK pathway for megakaryocytic differentiation of either hematopoietic progenitors or cultured leukemia cell lines (4-6,25).

The treatment of HEL cells with 10 μM diosgenin caused an initial activation of ERK kinases within 5 min (5-fold over uninduced cells), which was sustained through 12 h (Fig. 2a). However, between 12 and 48 h stimulation, phospho-ERK levels recovered to base-line levels. After 96 h diosgenin

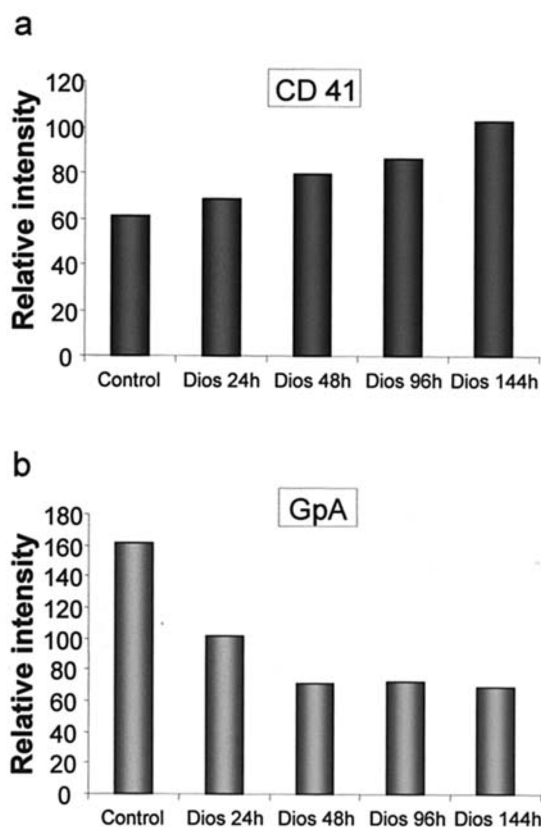

Figure 1. Diosgenin-induced increased expression of megakaryocytic marker CD41 and reduced expression of erythroid marker glycophorin A (GpA). Flow cytometry analysis of immunofluorescence staining of diosgenin treated and untreated cells with anti-CD41-FITC (a) and anti-GpA-RPE (b). One representative of three independent experiments is shown.

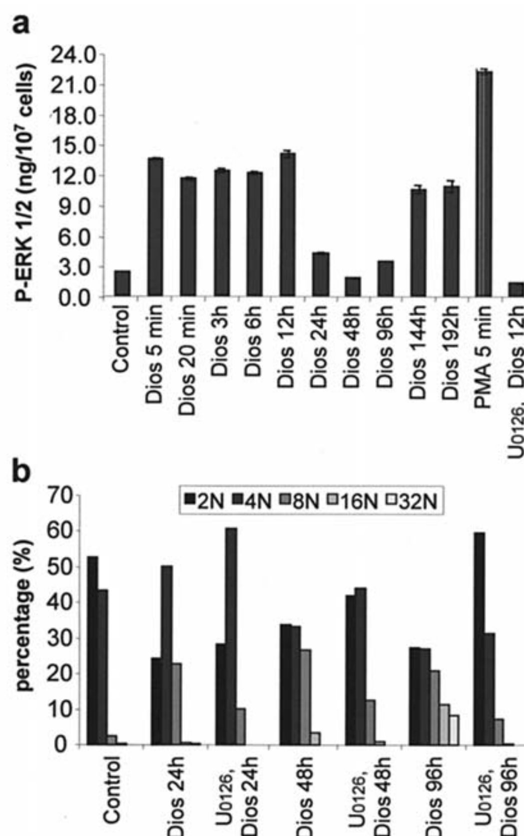

Figure 2. Activation of ERK signaling pathway in diosgenin-induced megakaryocytic differentiation of HEL cells. Quantification of active phospho-ERK 1 and 2 (a). HEL cells were grown in culture media alone or treated with 10 μM diosgenin. PMA treatment (10 nM) of HEL cells served as a positive control of ERK activation and the pretreatment with the MEK inhibitor, U0126, was done to confirm the capacity of U0126 to inhibit ERK activation. Phospho-ERK 1 and 2 were then quantified in cell lysates by 'human phospho ERK1/ERK2' enzyme-linked immunosorbent assay (ELISA). Values are expressed as mean ± SD and one representative of four independent experiments is shown. Effect of U0126 (MEK inhibitor) on HEL cell polyploidization (b). HEL cells were preincubated 3 h with 20 μM U0126 before 10 μM diosgenin treatment or were untreated (control). Cells were then permeabilized, incubated with RNase (40 U/μl) and stained with propidium iodide (50 μg/ml). Nuclear ploidy classes were determined by flow cytometry.

treatment, ERK seemed to be reactivated and again reached high levels. PMA treatment for 5 min served as a positive control of ERK activation.

To further elucidate the role of ERK activation following diosgenin stimulation, we used the MEK inhibitor U0126. Stimulation of ERK1/2, but not JNK and p38 MAPK, was shown to be selectively blocked by U0126 (26). As illustrated in Fig. 2a, pretreatment with 20 μM U0126 3 h before diosgenin treatment completely abrogated ERK stimulation induced by 12 h of diosgenin. U0126 also blocked cellular enlargement (data not shown) and nuclear polyploidization (Fig. 2b). As shown in Fig. 2b, with U0126 pretreatment, although the proportion of 8N ploidy class was increased after 24 and 48 h stimulation, diosgenin failed to increase nuclear ploidy higher than 8N, and cell ploidy remained principally 2N and 4N whereas diosgenin alone increased nuclear ploidy up to 32N. This illustrated that, as for other inducers, sustained ERK activation was involved in diosgenin-induced differentiation.

Diosgenin induced transient reductions in JNK activity and sustained inhibition of p38 MAPK activity. The JNK signaling pathway is involved in the regulation of many cellular events including growth control, transformation and apoptosis (3). Nevertheless, to our knowledge, no study has evoked the possible role of JNK in megakaryocytic differentiation. We therefore evaluated the JNK phosphorylation state in HEL cells stimulated by diosgenin. As shown in Fig. 3a, diosgenin induced an initial reduction in JNK activity within 5 min, which lasted over 6 h. Afterwards, JNK phosphorylation recovered to base-line levels and declined again after 96 h of treatment. These data seemed to indicate a possible involvement of JNK pathway in diosgenin-induced signaling. Sorbitol treatment for 30 min served as a positive control of JNK activation.

Activation of p38 MAPK was shown to be involved in proliferation, cell survival, or differentiation signaling. Previous studies have demonstrated that p38 activity was implicated in erythroid differentiation of primary erythroid cells or induced leukemia cells (7,8,27). On the other hand, the role of p38 in megakaryocytic differentiation remains unclear. Thus, we studied the effect of diosgenin treatment on the p38 phosphorylation state. As illustrated in Fig. 3b, diosgenin induced a rapid and sustained de-activation of p38. In fact, diosgenin decreased phospho-p38 levels as soon as 5 min after induction and this inhibition lasted for all remaining treatment times. These data showed that p38 inhibition was implicated

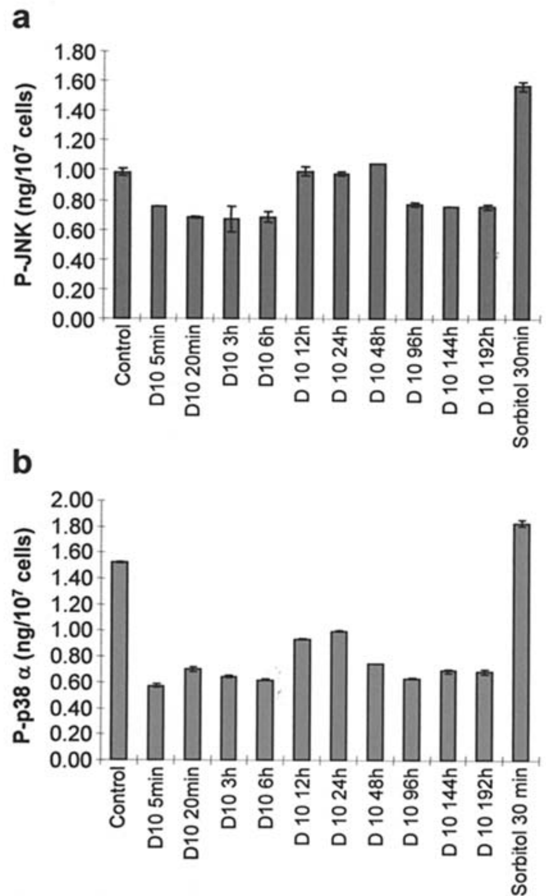

Figure 3. Differentiating dose of diosgenin inhibited active JNK and p38α. HEL cells were grown in culture media alone or treated with 10 μM diosgenin and 300 mM sorbitol served as a positive control of JNK and p38α activation. P-JNK and P-p38α were then quantified in cell lysates by respective 'human phospho-JNK (Pan)' (a) and 'human phospho-p38α (T180/Y182)' (b) ELISAs. Values are expressed as mean ± SD and one representative of three independent experiments is shown.

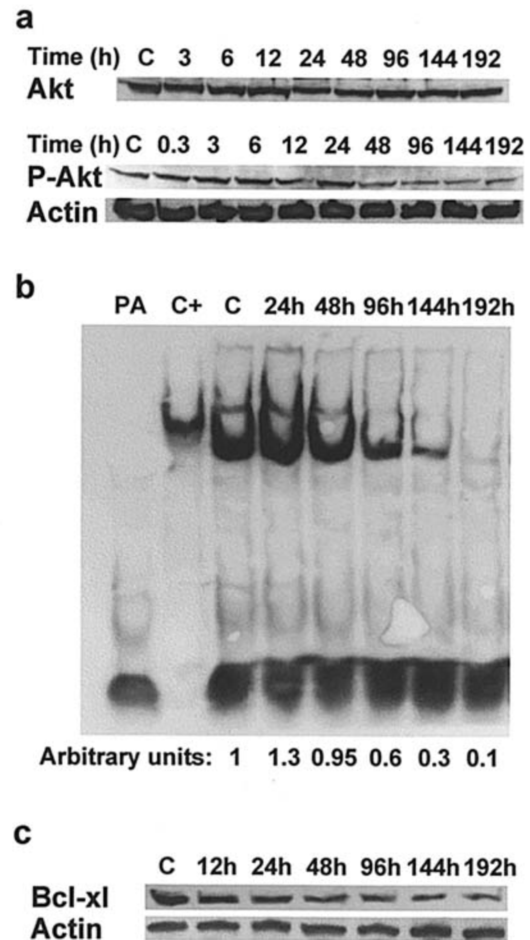

Figure 4. Down-regulation of survival factors after diosgenin-induced HEL cell differentiation. Cells were treated or not with 10 μM diosgenin. Total proteins were extracted and protein expression was evaluated by western blotting. Akt and phospho-Akt [P-Akt (Thr 308)] (a) and Bcl-xl (c) expression was estimated using rabbit polyclonal antibodies (a) and mouse monoclonal antibody (c). Membranes were then reprobed with anti-ß-actin antibody to ensure equal amounts of protein in each lane. NF-κB nuclear translocation was evaluated by Electromobility Shift Assay (EMSA) (b). Cells were treated or not with 10 μM diosgenin and NF-κB transolcation was assessed by 'DIG Gel shift kit' (Roche). Extracted nuclear proteins (10 μg) were incubated with DIG labeled NF-κB probe. Mobility shift was evaluated after migration on a 5% native polyacrylamide gel, electroblotting on to nylon membranes and incubation with anti-DIG polyclonal sheep antibody conjugated to alkaline phosphatase. Mobility shift was then visualized by chemiluminescence. PA means probe alone and C+ is an internal positive control of the kit. Quantification of each band was performed by densitometry analysis software in respect of band intensity and band area. Results are expressed relative to controls (C) in arbitrary units.

in diosgenin signaling during megakaryocytic differentiation. Sorbitol treatment for 30 min served as a positive control of p38 activation.

Inhibition of survival factors Akt, NF-κB and Bcl-xl during diosgenin-induced megakaryocytic differentiation. We examined Akt expression and NF-κB activation patterns in response to diosgenin treatment. We showed that although the expression of Akt remained stable after diosgenin stimulation, Akt activity, reflected by its phosphorylation state, increased during the first 24 h and then decreased compared to control activity (Fig. 4a).

Furthermore, following diosgenin stimulation, no significant variation of NF-κB DNA binding was found until 24 h (data not shown) when a slight activation was observed (Fig. 4b). Then, diosgenin progressivly inhibited NF-κB activation starting at 48-96 h of treatment leading to a complete inhibition at 192 h of treatment (Fig. 4b).

We then examined the level of Bcl-xl expression, another well-identified survival factor (28). Bcl-xl up-regulation has been implicated in the differentiation of hematopoietic cells and particularly in megakaryocytic differentiation (29). In HEL cells treated with diosgenin we observed a progressive decrease in Bcl-xl expression (Fig. 4c).

This observation, which correlated with those of NF-κB translocation and phospho-Akt level, led us to check whether the fall in the expression of these survival factors could be associated with an apoptotic phenomenon. Furthermore, decreased expression of Bcl-xl has previously been observed in ageing cultured megakaryocytes and Bcl-xl was even shown to be absent from senescent megakaryocytes (29).

Diosgenin-differentiated megakaryocytes underwent caspase-3 activation and PARP cleavage. Since Zauli *et al* identified an apoptotic process in senescent megakaryocytes

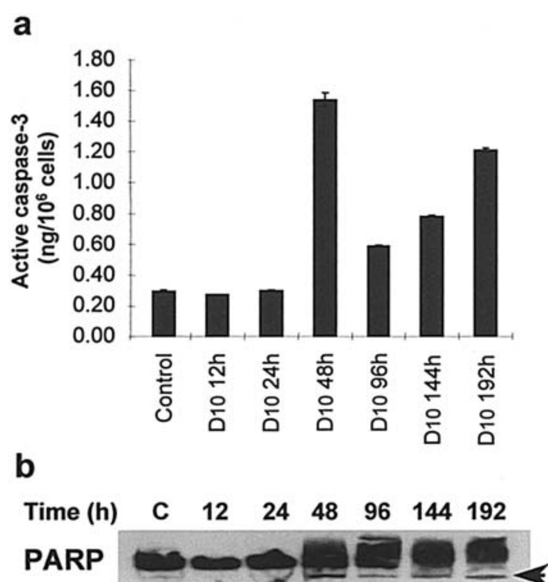

Figure 5. Effect of differentiating dose of diosgenin on caspase-3 activity and related PARP cleavage. Active caspase-3 was quantified using Quantikine human active caspase-3 kit (R&D Systems) (a). HEL cells were grown in culture media alone or treated with 10 μM diosgenin or 10 nM PMA. Cells were then incubated with 10 μM biotin ZVKD-fmk inhibitor. Cell lysates were assessed for caspase-3 activity by colorimetry after incubation with streptavidin conjugated to horseradish peroxidase and addition of a HRP substrate solution. PARP cleavage was analysed by western blotting (b). Total proteins were extracted from cells treated or not with 10 μM diosgenin. PARP cleavage was assessed by observation of a 85 kDa band (arrow) after probing with a mouse anti-human PARP antibody. One representative of five independent experiments is shown.

(30), a growing number of studies involved apoptosis in thrombogenesis. Recently, the process of platelet production has been tightly related to specific caspase activation within megakaryocytes (31,32). Based on these facts, we examined caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage in 10 μM diosgenin differentiated HEL cells. As shown in Fig. 5a, during diosgenin treatment, two distinct bursts of caspase-3 activation were observed. At 48 h stimulation, we observed a fast and dramatic increase in active caspase-3. Then, active caspase-3 levels decreased at 96 h diosgenin treatment but remained higher than controls. Afterwards, caspase-3 activity reached again high levels by the end of the treatment.

In parallel, we observed PARP cleavage in tight relation with caspase-3 activation. The 85 kDa cleaved form appeared after 48 h diosgenin treatment (Fig. 5b). Then, the intensity of the cleaved fragment followed the kinetics of caspase-3 activation (Fig. 5b).

Discussion

Megakaryocytopoiesis is a highly regulated phenomenon that involves a wide spectrum of cytokines and growth factors in physiological conditions. Nevertheless, several chemical agents such as phorbol esters were identified as megakaryocytic differentiation inducers for cultured megakaryocyte progenitors or leukemia cells.

Acute myeloid leukemia (AML) cells generally have the ability to differentiate following various stimulations and the differentiation of AML cells has important therapeutic implications (33). In a previous study, Beneytout *et al* demonstrated only that diosgenin induced megakaryocytic differentiation of HEL cells with increased cell size, nuclear complexity and GpIb expression without studying signaling pathways (22). In the present report we showed that diosgenin induced megakaryocytic differentiation of the HEL cell line through sustained activation of ERK and inhibition of p38 MAPK pathways. This led to nuclear polyploidization and increased expression of the platelet marker CD41 associated with a decrease in the erythroid marker GpA. Afterwards, differentiated cells showed a marked inhibition of survival factors NF-κB, Akt and Bcl-xL expressions and activation of caspase-3 together with PARP cleavage leading to apoptotic death of diosgenin-differentiated cells.

Megakaryocytic maturation comprises changes in cell morphology, nuclear ploidy and expression of specific cell surface markers that will be conserved on platelets. CD41 together with CD61, have been identified as markers of the megakaryocytic lineage (2). HEL cells express both erythroid, such as GpA, and megakaryocytic markers CD41-CD61 (34). Induction of megakaryocytic differentiation of HEL cells with nanomolar doses of PMA was shown to enhance CD41 expression and down-regulated GpA mRNA but apparently not GpA protein expression (35). Our results showed that diosgenin induced an increased expression of CD41 and a strong decrease in GpA expression, therefore increasing the relative abundance of megakaryocytic markers towards erythroid markers.

During megakaryocytic differentiation, the role of the ERK signaling pathway has been widely demonstrated. TPO induced a rapid activation of ERK in normal megakaryocytes as well as in TPO responsive cell lines (6,11,25). Furthermore, phorbol esters induced a sustained activation of ERK in various leukemic cell lines where it was clearly associated with megakaryocytic differentiation (4,5). In the present report, we showed that diosgenin induced sustained activation of the ERK pathway. Furthermore, ERK activation appeared to be essential for diosgenin-induced megakaryocytic differentiation since ERK inactivation inhibited cell size increase and strongly slowed down diosgenin-induced polyploidization. In addition, we demonstrated that diosgenin also induced a concomitant inhibition of the p38 MAPK and to a much lesser extent an inhibition of the JNK pathway. Strikingly, we observed that the inhibition profiles of p38 and JNK were reverse reflections of the ERK activation profile.

Erythroid differentiation was shown to principally require p38 activation and/or ERK inhibition (8,27). These observations suggested that erythroid differentiation involves p38 activation and ERK inhibition and that, conversely, megakaryocytic differentiation requires, as with diosgenin stimulation, ERK activation and p38 inhibition.

It has been demonstrated that the fate of senescent megakaryocytes in physiological conditions or in culture was death by apoptosis (30,36). For these reasons, we were interested in studying the kinetics of expression of survival factors Akt, NF-κB and Bcl-xL as well as factors involved in apoptosis such as caspase-3 activity and PARP cleavage during diosgenin-induced megakaryocytic differentiation of HEL cells. In the present report we demonstrated that diosgenin inhibited

Akt phosphorylation, NF-κB binding activity and Bcl-xL expression. Inhibition of Akt activation was demonstrated to induce apoptosis and Akt inhibition exhibited promising properties particularly in potentiating antileukemic drug actions (37). On the other hand, NF-κB inhibition was shown to potentiate apoptosis induced by cytotoxic drugs (38). Several natural compounds also showed their ability to induce apoptosis through NF-κB inhibition, in cancer cells (39,40), and particularly in leukemia cells (41,42). Similarly, NF-κB inhibition correlated with apoptosis induction in diosgenin-differentiated cells. Diosgenin also inhibited Bcl-xL, an anti-apoptotic protein of the Bcl-2 family (28). Bcl-xL was shown to be absent from apoptotic senescent megakaryocytes (29). We hypothesized that Bcl-xL inhibition also participated in apoptosis of diosgenin-differentiated megakaryocytes.

Together with the inhibition of survival factors, we demonstrated that diosgenin induced strong caspase-3 activation and PARP cleavage in differentiated cells.

Caspase activation was reported to be involved in the process of platelet shedding from mature megakaryocytes (31,32). In our study, we observed two distinct bursts of caspase-3 activation and the second caspase-3 activation correlated with cellular fragmentation (data not shown). De Botton *et al* demonstrated that early caspase-3 activation occured during megakaryocyte maturation (31). Primary activation was located in the cytoplasm, did not lead to cell fragmentation and was demonstrated to be essential for platelet generation as caspase inhibition blocked proplatelet formation. De Botton *et al* also suggested that during late differentiation, the localized activation of caspase-3 switched to a diffuse form, thus leading to apoptosis of senescent megakaryocytes (31). With diosgenin stimulation, we observed the same pattern of caspase-3 activation; early activation that was distinct from apoptosis induction and a second wave of caspase-3 activation that led to cell death.

In conclusion, diosgenin induced the megakaryocytic differentiation of HEL cells through a combined activation of the ERK signaling pathway and the inhibition of the p38 MAPK pathway. Differentiated cells showed a marked inhibition of survival factors Akt, NF-κB, Bcl-xL and activation of caspase-3 together with PARP cleavage leading to apoptosis of diosgenin-differentiated cells.

As a natural compound, diosgenin appeared to be well tolerated *in vivo* (15,16,21). Future studies should evaluate whether diosgenin could be used as a therapeutic agent for erythroleukemia or megakaryoblastic leukemia. The studies on retinoic acid, another pro-differentiating and pro-apoptotic agent (43), could served as a model for studies on diosgenin since retinoic acid is now used in therapy for promyelocytic leukemia.

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