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Journal of Cellular Biochemistry 107:785–796 (2009)

Journal of Cellular Biochemistry

A Proteomic Approach to the Identification of Molecular Targets in Subsequent Apoptosis of HEL Cells After Diosgenin-Induced Megakaryocytic Differentiation

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ABSTRACT

Diosgenin is a plant steroid which is able to induce megakaryocytic differentiation of human erythroleukemia (HEL) cells followed by apoptosis at a later stage. Apoptosis markers and phospho-kinases involved during the subsequent apoptosis of megakaryocytes after diosgenin-induced differentiation in these cells were detected using a proteomic approach. In mature megakaryocytes undergoing apoptosis, we observed increased expression of intrinsic apoptosis markers such as Bax/Bcl-2 ratio and cleaved caspase-9 as well as extrinsic apoptosis markers including cell death receptors and cleaved caspase-8. Furthermore, we demonstrated the link between both apoptotic pathways by Bid cleavage and confirmed the executive phase of apoptosis by caspase-3 cleavage. For the first time, we examined kinase activation and showed that kinases including Src, Tor, Akt, CREB, RSK and Chk2 may be implicated in signalling of subsequent apoptosis of mature megakaryocytes after diosgenin-induced differentiation of HEL cells. J. Cell. Biochem. 107: 785–796, 2009.

KEY WORDS: DIOSGENIN; ERYTHROLEUKEMIA CELLS; DIFFERENTIATION; APOPTOSIS; KINASE; PROTEOME ARRAY

Differentiation therapy is a new approach to cancer treatment that involves agents which can induce cancer cell differentiation. This approach is more specific and less toxic than traditional ones [Leszczyniecka et al., 2001].

Diosgenin [(25*R*)-5-spirosten-3β-ol] is a steroidal saponin that can be found in several plant species, particularly in fenugreek seeds (*Trigonella foenum graecum*) and wild yam roots (*Dioscorea villosa*). Diosgenin has been reported to have various biological effects in vivo [Nervi et al., 1988; Higdon et al., 2001] and in vitro. Diosgenin (40 μM) has recently been shown to exert antiproliferative and pro-apoptotic actions on rheumatoid arthritis synoviocytes [Liagre et al., 2004, 2007] or on cancer cells in vitro [Moalic et al., 2001; Corbiere et al., 2004; Leger et al., 2004a; Raju and Bird, 2007] and in vivo [Raju et al., 2004]. Diosgenin was also shown to induce megakaryocyte differentiation in human erythroleukemia (HEL) cells [Beneytout et al., 1995]. This capacity to induce differentiation leads to mature megakaryocytes after 192 h diosgenin treatment (10 μM selective dose), followed by release of platelet-like bodies [Leger et al., 2006a]. Recently, overexpression of cyclooxygenase-2 and thromboxane synthase has been correlated with induction of megakaryocyte differentiation by diosgenin in HEL cells [Cailleteau et al., 2008].

Megakaryocytopoiesis is a unique process requiring proliferation of hematopoietic stem cells and differentiation of megakaryocyte progenitors. Only among blood precursors, megakaryocytes undergo a process of endomitosis, producing polyploid cells with multiples of the normal chromosome complement (up to 64N) [Gordge, 2005]. After maturation and complex invagination of the plasma membrane, a system of demarkation membrane allows formation of ''proplatelets.'' Predictably, the senescent megakaryocyte nuclei, left after platelet release, are disposed of by apoptosis and phagocytosis. In our model of megakaryocyte differentiation, there is a subsequent apoptosis after diosgenin-induced differentiation of HEL cells into megakaryocytes with an activation of caspase-3 and PARP (poly[ADP-ribose] polymerase) cleavage [Leger et al., 2006a].

Apoptotic cell death is an active and physiological process mediated by various signalling pathways. Apoptosis can be

Abbreviations used: Chk2, checkpoint kinase 2; CREB, cAMP response element-binding; HEL, human erythroleukemia; HSP, heat shock protein; IAP, inhibitor of apoptosis protein; RSK, 90-kDa ribosomal S6 kinase; Src, Rous sarcoma virus; STAT, signal transducer and activators of transcription; t-Bid, truncated Bid; TPO, thrombopoietin; TRAIL, TNF-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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Received 26 November 2008; Accepted 18 March 2009 • DOI 10.1002/jcb.22176 • © 2009 Wiley-Liss, Inc.
Published online 4 May 2009 in Wiley InterScience (www.interscience.wiley.com).

initialised by a variety of extracellular (extrinsic apoptosis) or intracellular (intrinsic apoptosis) stimuli.

The aim of this study was to investigate the signalling pathways using a proteomic approach to identify and characterize markers of subsequent apoptosis of mature megakaryocytes in diosgenin-induced differentiation of HEL cells. We particularly investigated the expression of different apoptotic markers such as Bcl-2 family members, death receptors and activated caspases.

In the first part of this study we analysed the expression of intrinsic apoptosis markers. We focused our attention on traditional markers and on the p53 protein which is known to induce either cell cycle arrest or apoptosis of potentially malignant cells [Ko and Prives, 1996] notably by its ability to activate pro-apoptotic Bcl-2 family members. In the second part of our study we examined extrinsic apoptosis, especially for death receptors : Fas, tumor necrosis factor-α (TNF-α) receptor and TNF-related apoptosis inducing ligand (TRAIL) receptors to identify expression of these receptors during diosgenin-induced megakaryocytic differentiation and subsequent apoptosis in HEL cells.

Finally, it is also known that phospho-kinases can play an important role in the regulation of cell cycle and apoptosis. We selectively investigated the involvement of activated phospho-kinases in our model. Akt is a central phospho-kinase with anti-apoptotic activity which inhibits pro-apoptotic Bcl-2 protein family members. There is an interaction between STAT (signal transducer and activators of transcription) proteins and Tor to activate Akt [Nicholson and Anderson, 2002]. We also know that RSK (protein of 90-kDa ribosomal S6 kinase) can regulate CREB (cAMP response element-binding) protein which in turn regulates Bcl-2 protein expression [Creson et al., 2009]. Checkpoint kinase 2 (Chk2) is a multifunctional enzyme whose functions are central to the induction of cell cycle arrest and apoptosis by DNA damage [Vashistha et al., 2008].

Using a proteomic approach, we were able, for the first time, to characterize apoptosis biomarkers implicated in subsequent apoptosis of mature megakaryocytes after diosgenin-induced differentiation of HEL cells. Furthermore, we describe the involvement of phospho-kinases in apoptosis signalling pathways in these cells.

MATERIALS AND METHODS

CELL LINE, CELL CULTURE, TREATMENT

The HEL cell line was kindly provided by J. P. Cartron (INSERM U76, Paris, France). Cells were seeded at 10^5 cells/ml in 75 cm^2 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 [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Cultures were maintained in a humidified 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 (Sigma–Aldrich, Saint Quentin Fallavier, France).

A stock solution of 10^{-2} M diosgenin was prepared in ethanol and diluted in culture medium to give the appropriate final differentiating concentration (10 μM). The same amount of vehicle (=0.1% ethanol) was added to control cells. Cell viability was determined by the trypan blue dye exclusion method.

PROTEIN EXPRESSION ANALYSIS AFTER TREATMENT

HEL 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% sodium dodecyl sulfate [SDS], and 20 μg/ml aprotinin) containing protease inhibitors (Complete Mini, Roche Diagnostics, Meylan, France). Western blot was performed as described previously [Moalic et al., 2001]. Briefly, proteins (20–60 μg) were separated by electrophoresis on SDS–polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Saclay, France), and probed with respective antibodies against P-Src and full length Bid (Santa Cruz Biotechnology, Santa Cruz, CA; TEBU-BIO, Le Perray en Yvelines, France) and against truncated Bid (Acris Antibodies, Interchim, Montluçon, France). After incubation with secondary antibodies (Dako, Trappes, France and Jackson ImmunoResearch, Interchim, Montluçon, France), blots were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and immediately exposed to X-ray film. Membranes were then reblotted with monoclonal anti-β-actin antibody (Sigma–Aldrich). Western blots were analyzed by densitometry (Kodak 1D3.5 scientific imaging system), and protein expression was normalized to β-actin.

PROTEOME PROFILER™ ARRAY

We used two types of proteome profiler array: The Human Apoptosis Array and The Human Phospho-Kinase Array (R&D Systems). After 10 μM diosgenin exposure for 1, 48, or 192 h, protein expression levels in control or treated HEL cells were evaluated using the two kits according to the manufacturer's instructions. Briefly, capture and control antibodies were spotted in duplicate on nitrocellulose membranes (35 apoptosis-related proteins for the Human Apoptosis Array and 46 kinase phosphorylation sites for the Human Phospho-Kinase Array). Cell extracts were diluted and incubated with the Human Apoptosis Array or the Human Phospho-Kinase Array overnight. The array was washed to remove unbound proteins, followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied, and a signal was produced at each capture spot corresponding to the amount of protein bound. After incubation, membranes were developed using enhanced chemiluminescence reagents and immediately exposed to X-ray film. Blots were analyzed by densitometry (Kodak 1D3.5 scientific imaging system), and protein expression was normalized to a positive control which was represented in each membrane.

CASPASE-8 AND CASPASE-9 ACTIVITY ASSAYS

Caspase-8 and caspase-9 activities were respectively assayed using a caspase-8 and caspase-9 colorimetric assay (R&D Systems). HEL cells, treated with 10 μM diosgenin for 1, 48, or 192 h and control cells, were first lysed to collect their intracellular contents. Protease activity of the cell lysate was then tested by the addition of a caspase-specific peptide that was conjugated to the colour

reporter molecule *p*-nitroanaline (*p*NA). Peptide cleavage by the caspase releases the chromophore *p*NA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. Caspase-8 and -9 enzymatic activities, which are directly proportional to absorbance, were monitored at 405 nm using a spectrophotometer.

STATISTICAL ANALYSIS

Median and standard deviations (SD) were calculated using Excel software (Microsoft Office, version 98). Statistical analysis of differences was carried out by analysis of variance (ANOVA) using StatView (version 5.0, SAS Institute, Cary, NC). A P value less than 0.05 (Fisher's PLSD test) was considered to indicate significance.

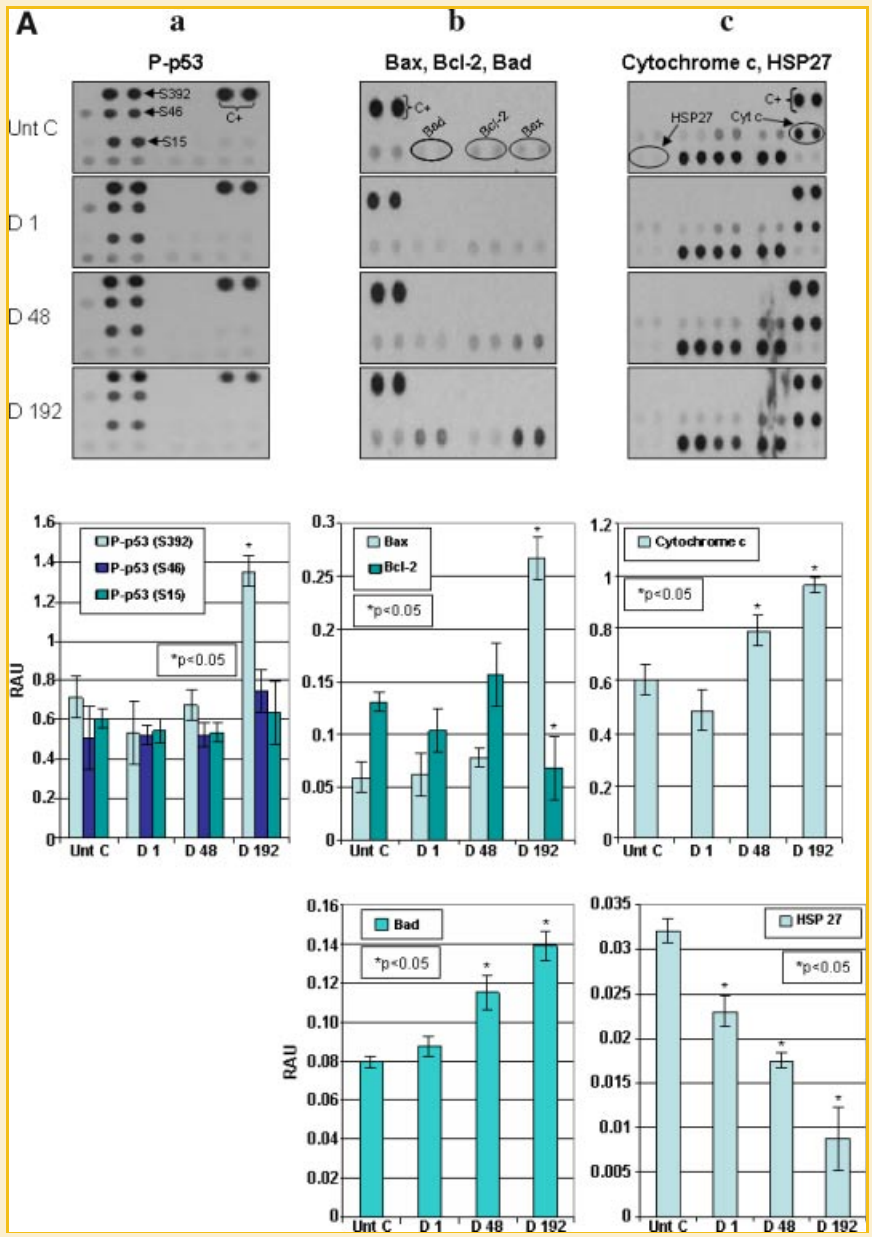

Fig. 1. Effect of differentiating dose of diosgenin (10 μM) on intrinsic apoptosis marker expression during differentiation of HEL cells into megakaryocytes. A: Phospho(P)-p53 (S392, S46 and S15) (a), Bax, Bcl-2 and Bad (b), cytochrome c and HSP27 (c) expression during diosgenin-induced megakaryocyte differentiation of erythroleukemia cells. HEL cells were treated or not (Unt C = Untreated cells) with 10 μM diosgenin (D) for 1, 48 and 192 h. Protein extracts prepared from cells were subjected to Human Apoptosis Array Kit (R&D Systems). Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown. B: Survivin (a), XIAP, cIAP (cIAP-1 and cIAP-2) (b), HTRA2/Omi and SMAC/Diablo (c) expression during diosgenin-induced differentiation of erythroleukemia cells. HEL cells were treated or not (Unt C = Untreated cells) with 10 μM diosgenin (D) for 1, 48 and 192 h. Protein extracts prepared from cells were subjected to Human Apoptosis Array Kit (R&D Systems). Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown.

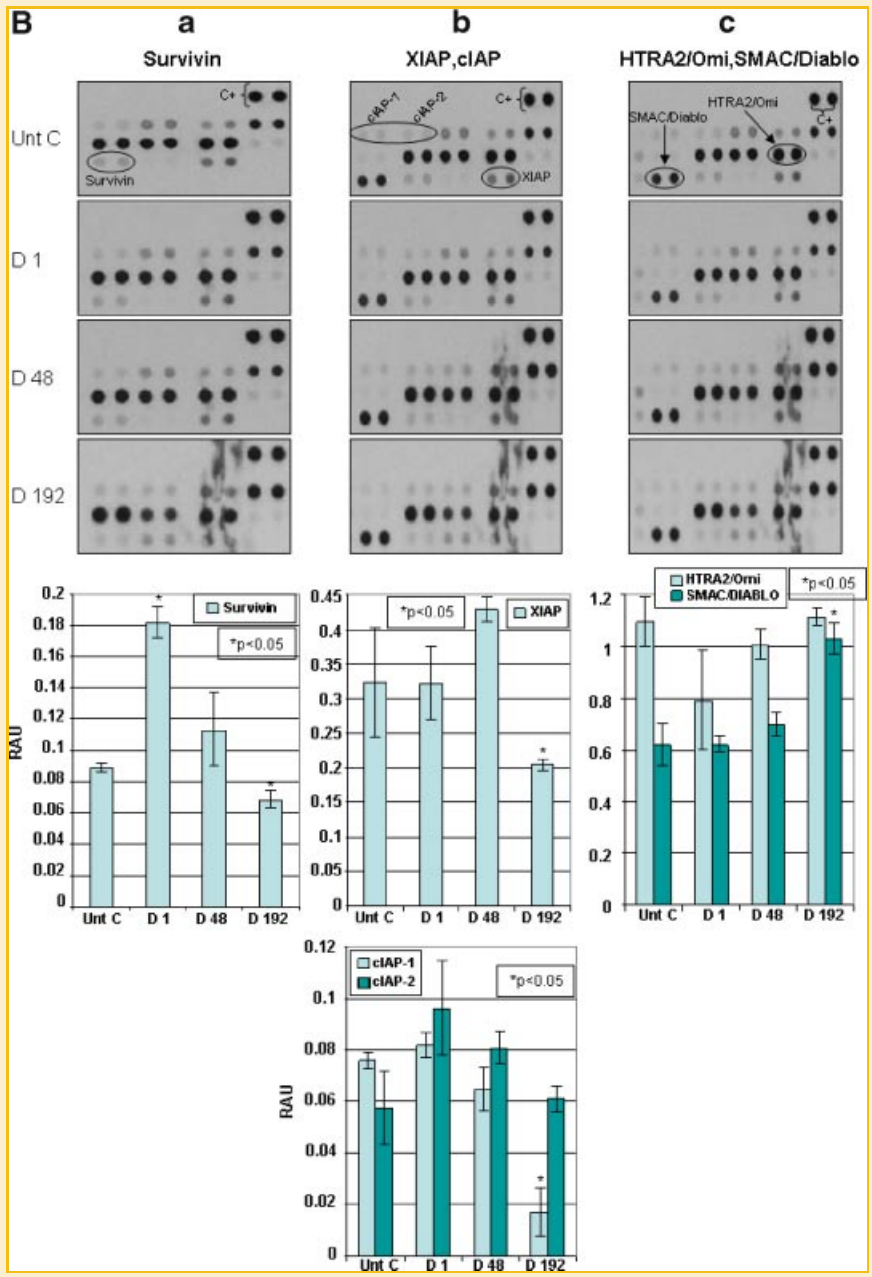

Fig. 1. (*Continued*)

RESULTS

INCREASED INTRINSIC APOPTOSIS MARKER EXPRESSION DURING SUBSEQUENT APOPTOSIS IN DIOSGENIN-INDUCED MEGAKARYOCYTE DIFFERENTIATION OF HEL CELLS

Using a phospho-kinase array, we showed that phospho-p53 (S392) expression increased two fold after 10 μM diosgenin treatment for 192 h compared to untreated cells (Fig. 1A(a)). By using an apoptosis array, we showed that Bax expression increased after 192 h diosgenin treatment, and, at the same time expression of the anti-apoptotic protein Bcl-2 decreased (+4.33- and −2-fold vs. untreated cells, respectively; Fig. 1A(b)). Furthermore, pro-apoptotic protein Bad expression increased after 48 and 192 h diosgenin treatment (+1.4- and +1.7-fold vs. untreated cells, respectively; Fig. 1A(b)). We demonstrated that the cytochrome *c* rate increased after 48 and 192 h diosgenin treatment (+1.3- and +1.6-fold vs. untreated cells, respectively; Fig. 1A(c)). This up-regulation was correlated with down-regulation of HSP27 (heat shock protein 27). HSP27 expression diminished over time with a maximum decrease after 192 h diosgenin treatment (−4.6-fold vs. control cells; Fig. 1A(c)).

In our conditions, we showed an increase in Survivin, a member of the IAPs (inhibitor of apoptosis protein) family, at 1 h diosgenin

treatment (+2.1-fold vs. untreated cells; Fig. 1B(a)), then a decrease after 192 h treatment (−1.3-fold vs. control cells) when HEL cells differentiated into mature megakaryocytes. Furthermore, we observed a specific downregulation of cIAP-1 expression after 192 h diosgenin treatment (−4.4-fold vs. untreated cells), but not for expression of the other cIAP isoform (cIAP-2) which did not change (Fig. 1B(b)). X-linked Inhibitor of Apoptosis Protein (XIAP) was also down regulated after 192 h 10 μM diosgenin treatment (−1.6-fold vs. untreated cells; Fig. 1B(b)). Expression of the pro-apoptotic marker SMAC/Diablo increased after 192 h treatment (1.7-fold vs. untreated cells), whereas, expression of HTRA2/Omi, which was generally associated with SMAC/Diablo, did not change during diosgenin-induced megakaryocyte differentiation in HEL cells and during the subsequent apoptosis of mature megakaryocytes (Fig. 1B(c)).

OVEREXPRESSION OF DEATH RECEPTORS IN DIOSGENIN-INDUCED MEGAKARYOCYTE DIFFERENTIATION IN HEL CELLS

We examined the expression of two TRAIL receptor isoforms : TRAIL R1 (DR4) and TRAIL R2 (DR5; Fig. 2A). In diosgenin-induced megakaryocyte differentiation of HEL cells, TRAIL R1 was most strongly expressed, whereas expression of TRAIL R2 did not change during treatment : TRAIL R1 expression increased significantly after 192 h diosgenin treatment (+1.7-fold vs. untreated cells).

We then studied Fas expression (Fig. 2A) which increased over time with diosgenin treatment to reach maximum levels after 192 h treatment (+3-fold vs. untreated cells).

Finally, we examined TNF R1 (TNF receptor family) expression (Fig. 2B). This expression was largely up-regulated after 192 h 10 μM diosgenin treatment (+10.5-fold vs. control cells).

LINK BETWEEN EXTRINSIC AND INTRINSIC APOPTOSIS AT LATER STAGES OF DIOSGENIN-INDUCED MEGAKARYOCYTE DIFFERENTIATION IN HEL CELLS

We analyzed the link between intrinsic and extrinsic apoptosis by evaluating caspase-8 and -9 activation and Bid cleavage. We observed an increase in caspase-8 and -9 activation after 48 h 10 μM diosgenin treatment (+1.9 and +2-fold, respectively, vs. untreated cells; Fig. 3A). At the same time, we saw a large amount of truncated Bid (t-Bid) versus Bid expression (Fig. 3B). However, after 192 h treatment, activation of these two caspases was not significant compared to untreated cells; furthermore, the normal form of Bid was almost undetectable. We hypothesize that, after 192 h treatment, mature megakaryocytes were in a later stage of apoptosis (executive phase).

APOPTOSIS EXECUTIVE PHASE OF MATURE MEGAKARYOCYTES INDUCED BY DIOSGENIN TREATMENT IN HEL CELLS

In our conditions, we observed an increase in HSP60 expression following 1 h diosgenin treatment (+1.3-fold vs. untreated cells) and with a maximum after 192 h (+1.8-fold vs. control cells;

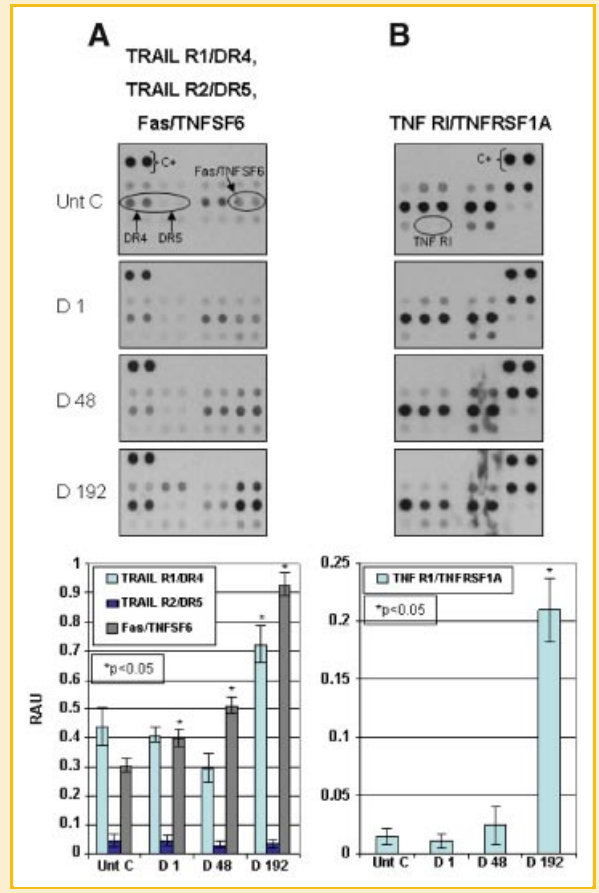

Fig. 2. Effect of differentiating dose of diosgenin (10 μM) on cell death receptor expression during differentiation of HEL cells into megakaryocytes. HEL cells were treated or not (Unt C = Untreated cells) with 10 μM diosgenin (D) for 1, 48 and 192 h. Protein extracts prepared from cells were subjected to Human Apoptosis Array Kit (R&D Systems) to determine TRAIL receptors (TRAIL R1 and TRAIL R2), Fas (A) and TNF receptor (TNF R1) (B) expression during diosgenin-induced megakaryocyte differentiation of erythroleukemia cells. Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown.

Fig. 4A). In early stages of diosgenin-induced megakaryocyte differentiation of HEL cells (1 h), expression of the inactive form of caspase-3 (pro-caspase-3) was increased (+2.4-fold vs. untreated cells). Afterwards, pro-caspase-3 expression decreased after 192 h (−1.3-fold vs. untreated cells) and the cleaved form appeared at the same time (Fig. 4B).

INVOLVEMENT OF PHOSPHORYLATED KINASES IN SUBSEQUENT APOPTOSIS IN DIOSGENIN-INDUCED MEGAKARYOCYTE DIFFERENTIATION IN HEL CELLS

In this study, by using the Human Phospho-Kinase Array Kit, we observed a decrease in phospho-Akt expression after 48 and 192 h diosgenin treatment (−1.6- and −1.5-fold vs. control cells,

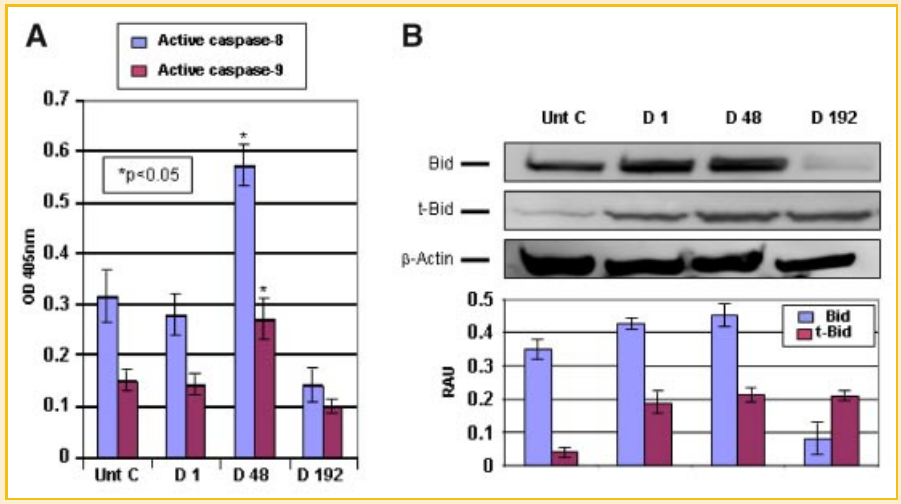

Fig. 3. Effect of diosgenin on caspase-8 and -9 activities and Bid cleavage. HEL cells were treated with 10 μM diosgenin (D) or not (Unt C = Untreated cells) for 1, 48 and 192 h. Caspase-8 and caspase-9 activities were respectively assayed using caspase-8 and caspase-9 colorimetric assay (R&D Systems) and quantified spectrophotometrically at a wavelength of 405 nm (A). Data are expressed as a mean ± SD of three experiments. $^{*}P < 0.05$ (Fisher's PLSD test) was considered to indicate significant differences compared to respective controls. On the other hand, with same treatment over time, Bid cleavage was analyzed (B). Protein extracts prepared from cells were subjected to Western blotting and cellular expressions of Bid, t-Bid and β-actin were estimated using specific antibodies as described in Materials and Methods Section. Quantification of each band was performed by densitometry analysis software and results are expressed as the ratio (Bid/β-Actin or t-Bid/β-actin) in relative arbitrary units (RAU). One representative of three independent experiments is shown.

respectively; Fig. 5A). In later stages of diosgenin-induced megakaryocyte differentiation in HEL cells (192 h treatment), phospho-Src expression decreased (−2.6-fold vs. untreated cells) as shown in Figure 5B after western blot analysis and proteome array. Phospho-STAT5 and phospho-Tor expression decreased after 192 h diosgenin treatment (−4.1- and −2.5-fold vs. untreated cells, respectively; Fig. 5C).

In subsequent apoptosis of diosgenin-induced megakaryocyte differentiation, expression of phospho-RSK (Fig. 6A) and phospho-CREB (Fig. 6B) was downregulated (−2- and −2.1-fold vs. controls respectively). In contrast, phospho-Chk2 expression increased markedly after 48 h diosgenin treatment (+14.5-fold vs. untreated cells; Fig. 6B).

DISCUSSION

Diosgenin is a steroidal saponin with two opposing effects when used on the human erytholeukemia cell line HEL: 40 μM diosgenin induced apoptosis [Leger et al., 2004a,b] while 10 μM induced megakaryocyte differentiation [Beneytout et al., 1995; Leger et al., 2004b, 2006a,b, 2007]. While the apoptotic concentration has already been explored in our laboratory, in this study we examined the molecular mechanisms of subsequent apoptosis after diosgenin-induced megakaryocyte differentiation in HEL cells using a proteomic approach.

Physiologically, terminal megakaryocytic differentiation requires caspase-3 activation for the budding of pro-platelets [Clarke et al., 2003]. Proplatelet formation in response to thrombopoietin (TPO) in cultured megakaryocytes derived from $CD34^{+}$ bone marrow cells has been reported to be a consequence of caspase-3 and -9 activation dependent on mitochondrial release of the pro-apoptogenic factor cytochrome *c*, indicative of an intrinsic program of cell death [De Botton et al., 2002]. As previously described, for 192 h 10 μM diosgenin treatment, HEL cells underwent megakaryocytic differentiation with an increase in megakaryocyte marker (CD41) expression and a decrease in erythroid marker expression (Glycophorin A) [Leger et al., 2006a,b]. In the later stage of this differentiation, mature megakaryocytes died by apoptosis and non-functional proplatelets were liberated. Our aim in this study was, first, to define pathways implicated in the subsequent apoptosis of mature megakaryocytes after diosgenin-induced megakaryocyte differentiation in HEL cells. Then, we focused on the regulation of this subsequent apoptosis by analysing the expression of specific phospho-kinases using a proteomic approach.

The activated form of p53 is already known for its implication in erythroid differentiation of the erythroleukemia cell line K562 [Chylicki et al., 2000]. Phospho-p53 is known to activate pro-apoptotic Bcl-2 family protein members, especially Bax [Zinkel et al., 2006]. In this study, we showed that phospho-p53 (S392) and Bax expression increased after 192 h diosgenin treatment in contrast to Bcl-2 expression. Ogilvy et al. [1999] demonstrated the importance of Bcl-2 expression in hematopoietic progenitor cells: they showed that constitutive Bcl-2 expression reduced the production of blood platelets by half in vivo.

In the intrinsic program of cell death, up-regulation of the pro-apoptogenic factor Bax/Bcl-2 results in cytochrome *c* liberation. Furthermore, HSP27 can down regulate this liberation [Creagh et al., 2000]. There is an overexpression of HSP27 and HSP60

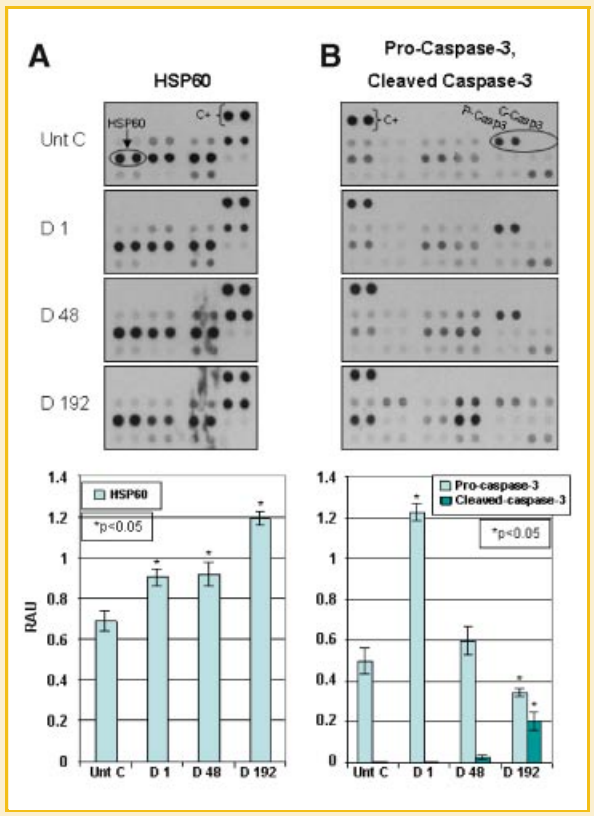

Fig. 4. Activation of caspase-3 and HSP60 during subsequent apoptosis of mature megakaryocytes after diosgenin-induced differentiation in HEL cells. HEL cells were treated or not (Unt C = Untreated cells) with 10 μM diosgenin (D) for 1, 48 and 192 h. Protein extracts prepared from cells were subjected to Human Apoptosis Array Kit (R&D Systems) to determine HSP60 (A), pro-caspase-3 and cleaved-caspase-3 (B) expression during diosgenin-induced differentiation of erythroleukemia cells and subsequent apoptosis. Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown.

in patients with a poor prognosis in myelodysplastic syndromes [Duval et al., 2006]. We demonstrated that cytochrome *c* rate increased after diosgenin treatment correlated with down-regulation of HSP27.

Apoptosis is strongly controlled by IAPs which negatively regulate caspase-3 activation, and by the two pro-apoptotic proteins SMAC/Diablo and HTRA2/Omi which are down regulated by pro-apoptotic Bcl-2 family protein members. In the human leukemia cell line U937, Kim et al. [2008] demonstrated that piceatannol, a polyphenol with anti-leukemic activity, induced apoptosis with down-regulation of anti-apoptotic Bcl-2 and cIAP-2 expression. In addition, premature death of megakaryocytes in myelodysplastic syndrome was accompanied by mitochondrial release of cytochrome *c* and Smac/DIABLO as well as loss of mitochondrial membrane potential. Thus, a stereotyped pattern of mitochondrial alterations was associated with differentiation-associated megakaryocyte death in myelodysplastic syndromes [Braun et al., 2007]. In our study, decreased Bcl-2 expression in conjunction with increased Bax expression and with a general pro-apoptotic tendency of intrinsic apoptosis regulators, especially cIAP1 and SMAC/Diablo expression, could explain the liberation of cytochrome *c* and subsequent apoptosis of mature megakaryocytes.

We previously described that intrinsic apoptosis could be implicated in subsequent apoptosis after diosgenin-induced megakaryocyte differentiation toward Bcl-xL expression [Leger et al., 2006a]. For the first time, we analysed the implication of the extrinsic apoptosis pathway in this model using a proteomic approach. Cell death receptors are a family of transmembrane domain proteins which initialise extrinsic apoptosis when they are activated by their ligands. Studies have revealed that pro-apoptotic stimuli such as nitric oxide can, in concert with potentially pro-apoptotic cytokines such as TNF-α and IFN-γ, trigger increased release of platelet-like bodies from cultured megakaryocyte cells [Battinelli et al., 2001]. It is known that TRAIL R1 and TRAIL R2 are interchangeable for their ability to promote cell death in tumor cells sensitive to TRAIL-induced apoptosis [Ashkenazi, 2002]. TRAIL R2, but not TRAIL R1, becomes detectable starting from the early phase of megakaryocyte differentiation and persists at later culture times [Zauli and Secchiero, 2006]. In agreement with Melloni et al. [2005], we showed that TRAIL receptors could be implicated in megakaryocyte differentiation. Indeed, after diosgenin treatment, TRAIL R1 was most strongly expressed, whereas expression of TRAIL R2 did not change. However, in the model of Melloni et al. [2005], only TRAIL R2 was implicated in human megakaryocyte development. In another model of megakaryocyte differentiation, HBP1 (HMG-box containing protein 1) overexpression also enhanced differentiation of human erythroleukemia K562 cells towards erythroid and megakaryocyte lineages correlated with increased apoptosis with involvement of the Fas/Fas ligand pathway [Yao et al., 2005]. Clarke et al. [2003] provided evidence that Fas ligation increased platelet production by megakaryocytes. In our model, the maximum levels of Fas expression were observed after 192 h diosgenin treatment when HEL cells differentiated into mature megakaryocytes. These results provide a link between increased expression of extrinsic apoptosis pathway markers (TRAIL R1, Fas and TNF R1) and subsequent apoptosis after diosgenin-induced megakaryocyte differentiation in HEL cells.

There is a link between both types of apoptosis. When caspase-8 is activated by the death receptor [Nieminen et al., 2007], cleaved caspase-8 is able to activate Bid which is a pro-apoptotic Bcl-2 family protein member. Cleavage of Bid results in the apparition of truncated Bid (t-Bid) which is known to active intrinsic apoptosis. We suggested that both intrinsic and extrinsic apoptosis were activated by prolonged exposure to 10 μM diosgenin and that caspase-8 and Bid provided a link between these two apoptotic pathways. Caspase-3 is known to be the effector caspase. Cleavage of this pro-caspase is one of later stages of apoptosis and it can be up-regulated by a heat shock protein, HSP60 [Creagh et al., 2000]. In our study, the cleaved form of caspase-3 appeared only after 192 h diosgenin treatment correlated with an increase in HSP60 expression. Leger et al. [2006a] also demonstrated the importance of caspase-3 activation in subsequent apoptosis during differentia-

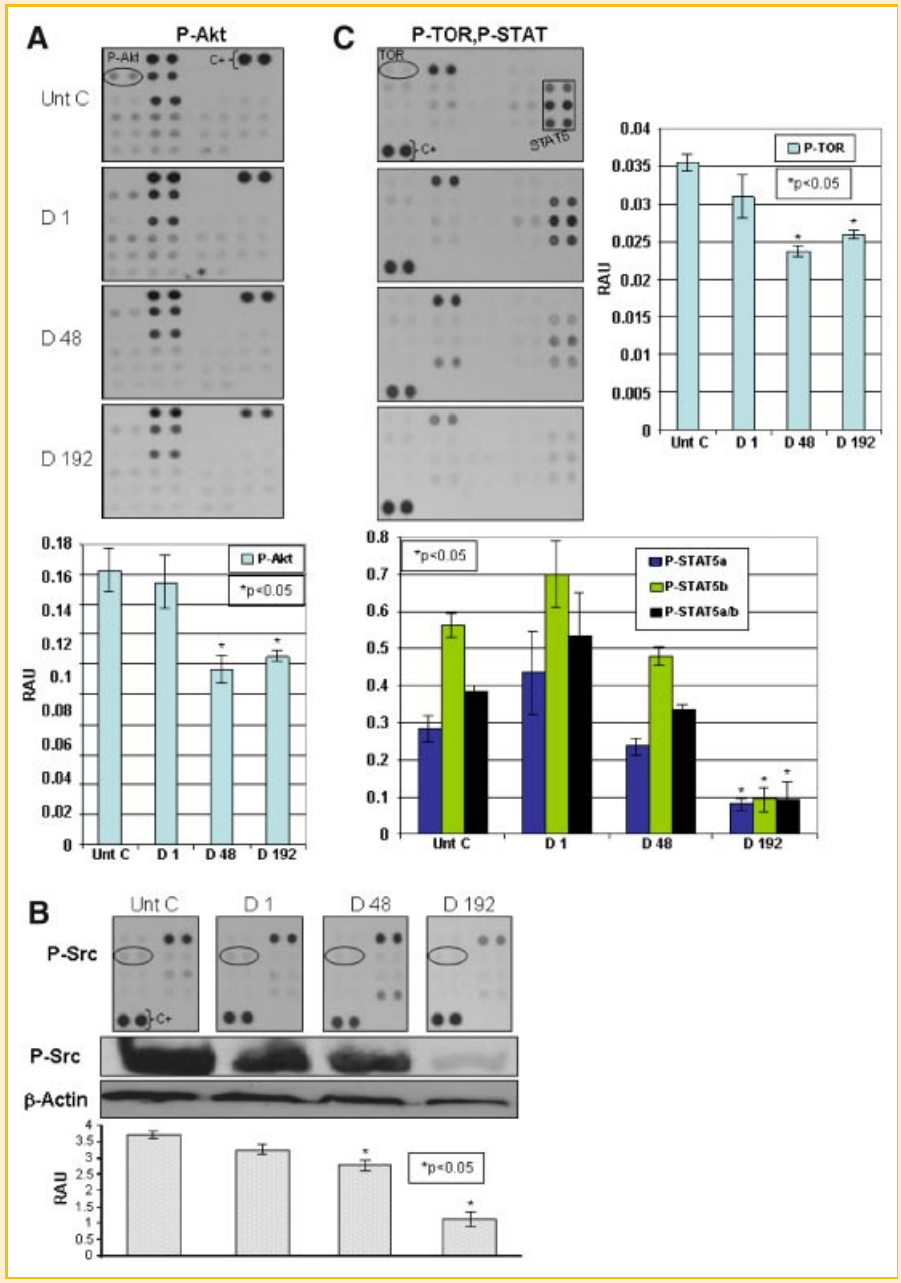

Fig. 5. Down-regulation of Akt (A), Src (B), TOR and STAT (C) phosphorylation during subsequent apoptosis of megakaryocytes after diosgenin-induced differentiation in HEL cells. HEL cells were treated or not (Unt C = Untreated cells) with 10 μM diosgenin (D) for 1, 48 and 192 h and cell lysates were subjected to Western blot analysis using P-Src antibody and β-actin antibody. Quantification of each band was performed by densitometry analysis software, and results are expressed as the ratio (P-Src/β-actin) in relative arbitrary units (RAU). One representative of three independent experiments is shown. Protein extracts prepared from cells were subjected to Phospho-Kinase Array Kit (R&D Systems) to determine phosphorylation rate of Src, TOR, STAT and Akt. Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown.

tion of HEL cells into mature megakaryocytes after diosgenin treatment.

Phospho-Akt is a central protein in the regulation of cell cycle and apoptosis. When Akt is activated, it is responsible for cytokine stem cell factor mediated protection of megakaroycyte progenitors against cell death [Zeuner et al., 2007]. In our model, the decrease in activated-Akt may promote apoptosis of megakaryocytes. Previous studies have demonstrated that diosgenin inhibits Akt-mediated survival signalling in different types of cancers [Chiang et al., 2007; Lee et al., 2007].

Src is known to activate STAT proteins which activate Akt. Lannutti et al. [2005] reported that Src inhibition induced polyploidisation of leukemic cells. Expression of phospho-Src in differentiated HEL cells decreased with diosgenin treatment. This

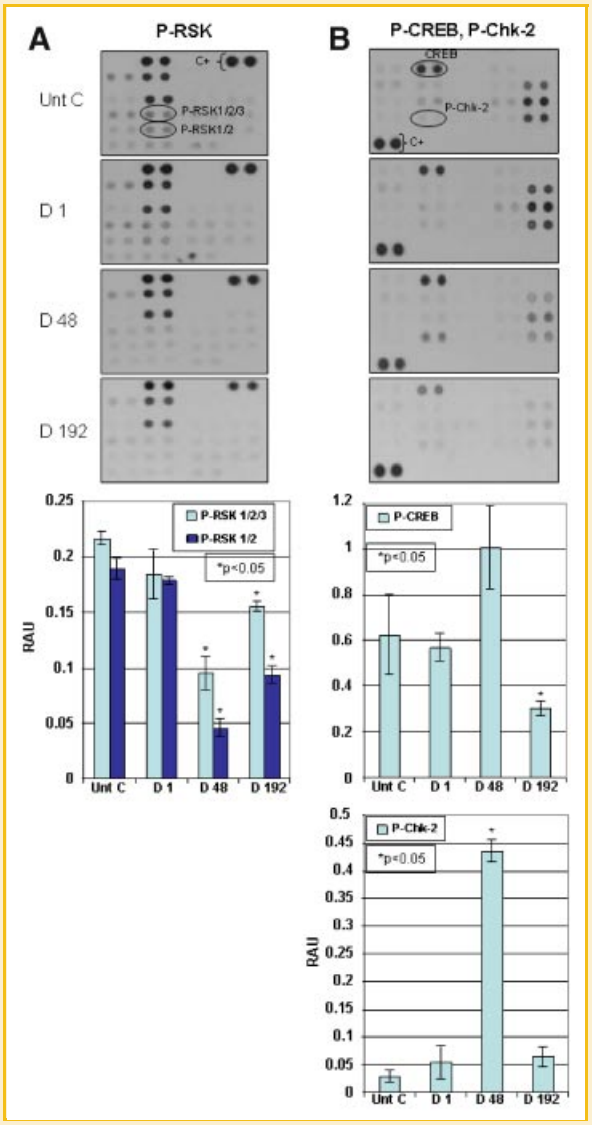

Fig. 6. Diosgenin modulates activation of RSK, CREB and Chk-2 kinases in differentiated HEL cells. After 1, 48 and 192 h 10 μM diosgenin treatment (D) or not (Unt C = Untreated cells), protein extracts prepared from cells were subjected to Phospho-Kinase Array Kit (R&D Systems) to determine phosphorylation rate of RSK (A), CREB and Chk-2 (B). Quantification of each spot was performed by densitometry analysis software, and protein expression was normalized to a positive control (C+), which was represented in each membrane, in relative arbitrary units (RAU). One representative of three independent experiments is shown.

result was correlated with polyploidisation of HEL cells after diosgenin treatment as previously described [Leger et al., 2006a,b, 2007; Cailleteau et al., 2008]. It has been shown that Tor regulated both Akt and STAT phosphorylation [Nicholson and Anderson, 2002]. We showed that phospho-STAT5 and phospho-Tor expression decreased after 192 h diosgenin treatment. STAT and Tor have already been implicated in megakaryocyte differentiation [Jeanpierre et al., 2008]: BMP4 (bone morphogenetic protein 4; a member of the TGF-beta family), like TPO, exerts its effects on human megakaryocytopoiesis through the JAK/STAT and Tor pathways.

Akt is also able to regulate other protein kinases including ERK which is implicated in diosgenin-induced megakaryocyte differentiation [Leger et al., 2006a]. RSK and CREB are targets of ERK and have an important role in cell survival [Frödin and Gammeltoft, 1999]. Furthermore, CREB activation induces survival signals in human erythroleukemia K562 cells [Cataldi et al., 2006]. Phospho-RSK and phospho-CREB expression was decreased in subsequent apoptosis of diosgenin-induced megakaryocyte differentiation. On the other hand, Bad expression increased in our conditions. Akt and RSK are thought to stimulate cell survival through Bad inactivation [Kraus et al., 2002; Hauge and Frödin, 2006].

Checkpoint kinase 2 (Chk2) is implicated in erythrocytic differentiation of human leukemia K562 cells [Takagaki et al., 2005]. Freshly isolated acute myeloid leukemia (AML) cells showed response patterns to gemtuzumab ozogamicin (a humanized anti-CD33 antibody conjugated to the anticancer agent calicheamicin) that included Chk2 phosphorylation and caspase-3 activation [Amico et al., 2003]. We showed here Chk2 activation during HEL cell differentiation. Furthermore, as previously described above, cleaved caspase-3 appeared at this time and its rate increased significantly at 192 h diosgenin treatment.

In conclusion, based on our new results summarized in Figure 7A, we showed that both the intrinsic and extrinsic pathways were implicated in subsequent apoptosis of mature megakaryocytes after diosgenin-induced megakaryocyte differentiation of HEL cells. Using a proteomic approach, we demonstrated that p53 phosphorylation was specific for S392 and correlated with a dramatic increase in Bax and Bad expression compared to Bcl-2 expression. Furthermore, the rate of cytochrome *c* was increased and HSP27 expression, which can regulate its liberation, was down-regulated by 10 μM diosgenin. On the other hand, SMAC/Diablo expression, another pro-apoptotic marker, was increased whereas specific down-regulation of cIAP-1 and XIAP expression was observed. Following this signalling cascade, caspase-9 activation was detected (Fig. 7A).

For extrinsic signalling pathway, cell death receptor expression was increased following diosgenin treatment and active caspase-8 was detected. Furthermore, we characterized the link between the two apoptosis pathways (extrinsic and intrinsic) by the Bid cleavage.

In the executive phase of subsequent apoptosis of mature megakaryocytes after diosgenin treatment of HEL cells, we observed a progressive disappearance of pro-caspase-3 in favour of active caspase-3. We also observed that HSP60 expression, a possible up-regulator of pro-caspase-3 cleavage, was increased (Fig. 7A).

For the first time, using a proteomic approach, we also examined kinase activation signalling involved in differentiation of HEL cells into megakaryocytes after diosgenin treatment but also involved in subsequent apoptosis of mature megakaryocytes (Fig. 7B). In summary, a differentiating dose of diosgenin (10 μM) blocked phosphorylation of different kinases such as Src, Tor, Akt, CREB, RSK and activated phosphorylation of Chk2.

Phospho-Src and phospho-Tor down-regulation could explain the down-regulation of phospho-STAT in our model. In addition,

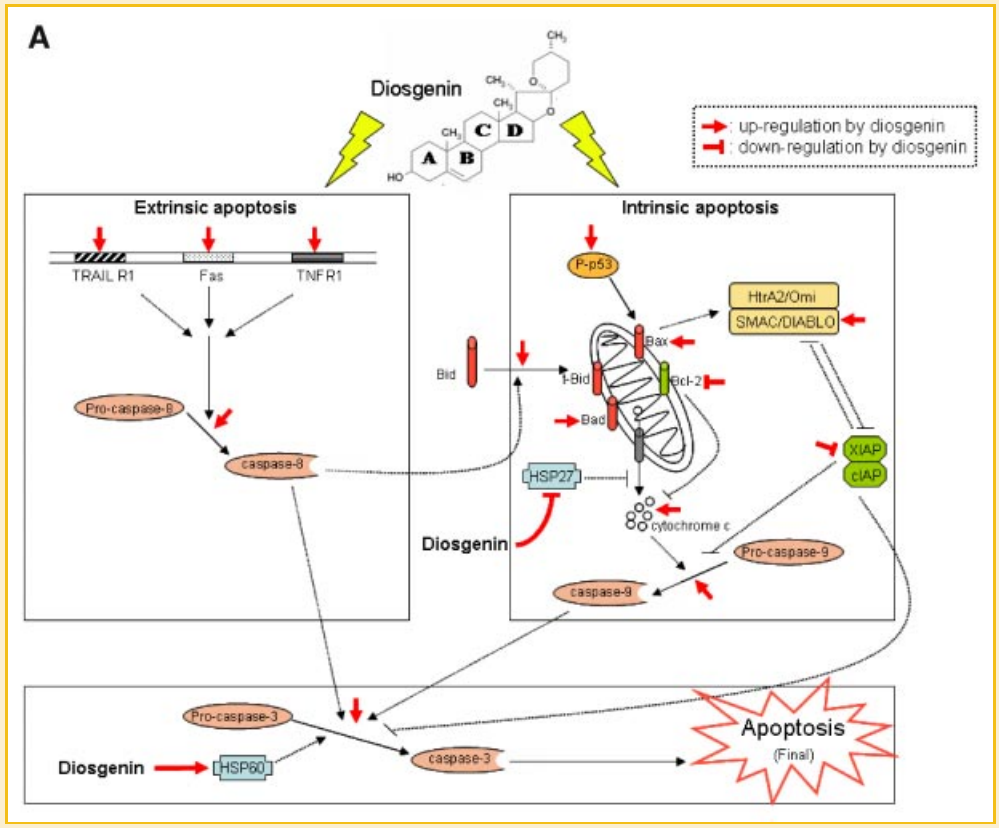

Fig. 7. Possible mechanisms of diosgenin during subsequent apoptosis of mature megakaryocytes after differentiation in human erythroleukemia cells. A: Representation of diosgenin targets in extrinsic and intrinsic apoptosis pathways. Diosgenin regulates proteins of the extrinsic pathway, like death receptors (TRAIL R1, Fas, TNFR1) and caspase-8 as well as the intrinsic mitochondrial pathway including p53, Bax, Bcl-2, Bad, SMAC/DIABLO, XIAP, IAP, cytochrome c and caspase-9. Diosgenin induces Bid cleavage providing a link between both pathways. Furthermore, diosgenin increases caspase-3 activity. B: Kinase signalling pathways implicated in diosgenin-induced differentiation. Subsequent apoptosis of mature megakaryocytes after differentiation of HEL cells induced by diosgenin is associated with regulation of the phosphorylation rate of Src, STAT, TOR, Akt, Chk2, RSK and CREB. *Previous results demonstrated down-regulation of ERK and Bcl-xL, two pro-survival factors [Leger et al., 2006a].

decreased phospho-Akt expression in contrast to Chk2 activation could result in phospho-p53 activation. Furthermore, the down-regulation observed for XIAP could be correlated with decreased phospho-Akt expression.

As previously described, diosgenin induced differentiation of HEL cells into megakaryocytes with decreased ERK phosphorylation over time [Leger et al., 2006a]. In new results, we showed a down-regulation of phospho-RSK and phospho-CREB. In the signalling cascade, ERK inactivation could be involved in the regulation of RSK and CREB. Furthermore, RSK inactivation was correlated with decreased Bcl-xL, a survival factor, which was observed previously [Leger et al., 2006a]. On the other hand, Bcl-2 expression down-regulation, as described above, was correlated with decreased phospho-CREB expression (Fig. 7B).

Taken together, these new results provide, for the first time, strong evidence that extrinsic and intrinsic signalling pathways were involved in subsequent apoptosis of mature megakaryocytes after diosgenin treatment of HEL cells. Furthermore, using a proteomic approach, we characterized the modulation of specific kinases implicated in our model of megakaryocyte differentiation of HEL cells.

More recently, Raju and Mehta [2009] summarized the therapeutic effects of diosgenin. It appears that diosgenin's effect against several cancers is not through any single mechanism; however, diosgenin's activity involves multiple cellular and molecular primary targets. In our model of megakaryocytic differentiation of HEL cells induced by diosgenin, nuclear factor-κB (NF-κB) could be one of primary targets for regulation. Indeed, we showed previously that diosgenin progressively inhibited NF-κB activation during differentiation of HEL cells into mature megakaryocytes [Leger et al., 2006a]. Furthermore, Chen et al. [2007] described that the kinetics of NF-κB activity suggested an important role for the NF-κB program in megakaryocyte apoptosis and differentiation. In order to firmly establish the role of NF-κB in diosgenin-induced megakaryocyte differentiation, future studies will be needed to investigate the effect of forced NF-κB signalling disturbances on cultured HEL cells, as well as on elucidating signalling upstream and downstream from NF-κB.

ACKNOWLEDGMENTS

The authors are grateful to Dr. J. Cook-Moreau for corrections in the preparation of this manuscript. The expenses of this work were defrayed in part by the Ministère de l'Education Nationale, de la

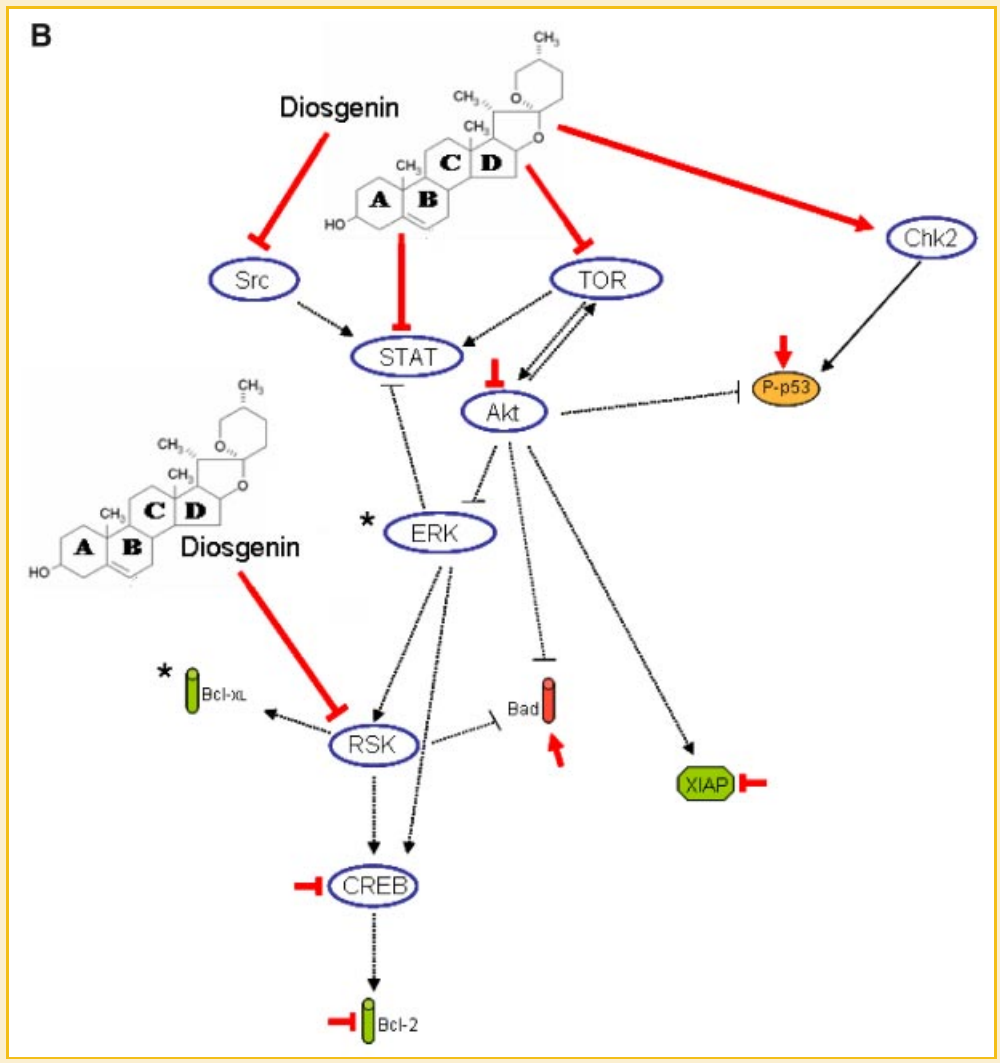

Fig. 7. (*Continued*)

Recherche et de la Technologie and by the Conseil Régional du Limousin.

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