Activation of protein kinase C relays distinct signaling pathways in the same cell type: differentiation and caspase-mediated apoptosis

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Abstract

Activation of PKC with 5 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 72 h in human U937 myeloid leukemia cells is associated with induction of adherence, followed by monocytic differentiation and G_0/G_1 cell cycle arrest. In this study, we demonstrate that in addition to these effects about 25% of U937 cells accumulated in an apoptotic subG$_1$ phase after TPA treatment. The appearance of these apoptotic suspension cells was detectable throughout the time course of the culture and was independent of TPA concentrations between 0.5 and 500 nM. Experiments with cells synchronized by centrifugal elutriation revealed dominant susceptibility of G_1-phase cells to TPA-mediated apoptosis. While adherent cells expressed differentiation markers including the integrin CD11c, this effect was less pronounced in the TPA-treated suspension fraction. Moreover, previous work has demonstrated cell cycle arrest in differentiating U937 cells. Accordingly, PKC activation by TPA treatment was associated with a significant expression of the cdk/cyclin inhibitor p21$^{WAF/CIP/sdi-1}$ in the adherent population and subsequent G_0/G_1 cell cycle arrest. In contrast, suspension cells failed to induce significant levels of p21$^{WAF/CIP/sdi-1}$ after TPA stimulation. Immunoblotting experiments demonstrated no difference in the expression of the pro-apoptotic factors Bax, Bad, and Bak in either control U937 and TPA-treated adherent or suspension cells, respectively. However, anti-apoptotic factors including Bcl-2, Bcl-x$_L$, and Mcl-1 were significantly induced in the adherent population whereas no induction was detectable in the suspension cells. In this context, incubation with the caspase-3/caspase-7 specific tetrapeptide inhibitor DEVD prior to TPA treatment prevented an accumulation of cells in subG$_1$, respectively, demonstrating an involvement of these caspases. Taken together, these data suggest that PKC activation can relay distinct signaling pathways such as induction of adherence coupled with monocytic differentiation and growth arrest, or induction of caspase-mediated apoptosis coupled with the failure to adhere and to differentiate. *Cell Death and Differentiation* (2000) **7**, 795 – 803.

Introduction

Protein kinase C (PKC) is a family of serine-/threonine-specific protein kinases that has been implicated in a wide variety of cellular responses such as cell proliferation, differentiation, regulation of gene expression, protection from apoptosis and, most recently, induction of apoptosis.[1,2] According to the structure and biochemical properties, PKCs can be divided into conventional or calcium-dependent (α, β_I, β_{II}, and γ), novel or calcium-independent (δ, ε, η, and θ), and atypical (λ/ι, μ, ζ) isoenzymes. With the exception of atypical PKCs, conventional and novel PKCs can be activated by interaction with the endogenous ligand diacylglycerol or pharmacological compounds such as phorbol esters.[3]

The diverse responses mediated by PKCs have been attributed to cell type-specific expression and function of distinct isoforms. Previous work has demonstrated that differentiation of murine 32D hematopoietic cells is associated with activation of PKCα and PKCδ,[4] whereas in a human myeloid leukemic cell line enhanced expression of PKCζ promotes changes along a differentiation pathway.[5] Overexpression and activation of PKCδ in CHO cells results in cell cycle arrest and accumulation in G_2/M phase.[6] In contrast, induced expression of PKCε in NIH3T3 cells contributes to neoplastic transformation, as demonstrated by tumor formation in nude mice.[7] Overexpression of PKCι but not PKCζ in K562 cells was associated with a protection from drug-induced apoptosis.[8] Conversely, an induction of apoptosis was accompanied by a proteolytic activation of PKCδ and PKCθ.[9,10] All in all, these findings suggest that activation of different PKC isoenzymes are involved in the regulation of differentiation pathways and apoptosis in a variety of cell types.

Accordingly, activation of distinct PKC isoenzymes may also affect the proliferative capacity of cells. Indeed, PKC activation in certain leukemic cell lines is associated with growth arrest in G_0/G_1 phase of the cell cycle.[11] This effect is paralleled by transient or terminal differentiation followed by subsequent retrodifferentiation or commitment to

apoptosis, respectively.[11] The G_0/G_1 growth arrest is at least in part mediated by the p21 protein product of the WAF1/CIP1/sdi-1 gene, a member of a family of inhibitors of cyclin-dependent kinases (cdk). Although the induction pathway via PKC activation is still unclear, previous work has demonstrated that induced p21[WAF1/CIP1/sdi-1] can arrest cells in G_1 phase by inhibition of G_1-cyclins/cdk complexes. As a result of p21[WAF1/CIP1/sdi-1] inhibition, the cdks fail to phosphorylate the retinoblastoma protein (p105[Rb]) and consequently, underphosphorylated p105[Rb] remains attached to and thereby inactivates the transcription factor E2F which is required for gene transcription during S-phase.[12]

Recent evidence suggested that features of growth arrest, differentiation, and apoptosis are part of a cellular response to PKC activation in the same cellular clone. Thus, autonomously proliferating U937 myeloid leukemic cells undergo a phorbol ester-mediated differentiation program along the monocytic lineage and growth arrest. Whereas the major part of the differentiated population revert back to the undifferentiated phenotype by a retro-differentiation program, some cells undergo terminal commitment and subsequently exhibit internucleosomal DNA cleavage during G_0/G_1 arrest, a hallmark of apoptosis.[13] In this context, it is interesting to note that a subclone of U937 cells with reduced expression of PKCβ_{II}, designated TUR, fails to undergo TPA-induced differentiation, growth arrest and internucleosomal DNA fragmentation, respectively.[14] Consequently, we were interested to investigate a possible relationship between induction of differentiation and apoptosis in response to PKC activation.

Furthermore, PKC-mediated differentiation of myeloid cells along the monocytic pathway is accompanied by increased expression of leukocyte integrins, such as CD11a, b, and c which can form heterodimers with the common β-subunit CD18 and therefore functional cell attachment complexes for intercellular communication processes i.e. via ICAMs. In this context, our previous work has demonstrated enhanced integrin expression, such as CD11c associated with cell attachment in human U937 leukemic cells upon phorbol ester-activated PKC.[15] However, the contribution of induced cellular adherence to subsequent differentiation and apoptotic pathways remains unclear. In the present study, we therefore investigated distinct effects of phorbol ester-induced PKC activation on the induction of cellular adherence and the promotion of a differentiation program *versus* induction of apoptotic pathways in a non-adherent population.

Results

TPA-treated U937 cells undergo both differentiation and apoptosis

Previous studies have demonstrated that activation of protein kinase C by treatment with the phorbol ester TPA in human U937 myeloid leukemia cells is associated with development of a monocytic phenotype and cell cycle arrest.[15] During this differentiation process, the U937 cells became adherent after 10–12 h following TPA treatment. A significant amount of

cells, however, failed to adhere and remained in suspension as single cells. These suspension cells were characterized by cellular morphology (visualized by phase contrast microscopy) and nuclear morphology (visualized by fluorescent microscopy after staining with 4,6-diamidin-2-phenylindol-dihydrochlorid [DAPI]) and demonstrated apoptotic features such as nuclear blebbing (data not shown). In order to quantify the extent of apoptosis, U937 control cells and cells treated with 5 nM TPA for 72 h were analyzed for cell cycle distribution by flow cytometry. U937 cells demonstrated proliferation throughout the different phases of the cell cycle and less than 2% of cells in a subG$_1$ phase which represents an apoptotic population (Figure 1a). In contrast, TPA-treated U937 cells exhibited a significant accumulation of growth-arrested cells in G_0/G_1 phase and the amount of cells in the subG$_1$ phase was elevated to about 27% (Figure 1a). In this context, increasing concentrations of the phorbol ester at 0.5, 5, 50, and 500 nM demonstrated no significant differences in the number of apoptotic subG$_1$ phase cells after 72 h, whereas TPA concentrations as low as 0.05 nM were ineffective to induce apoptosis (Figure 1b). Following a time course, a significant increase in the amount of subG$_1$ cells was detectable in U937 cells after 24 h of TPA treatment and
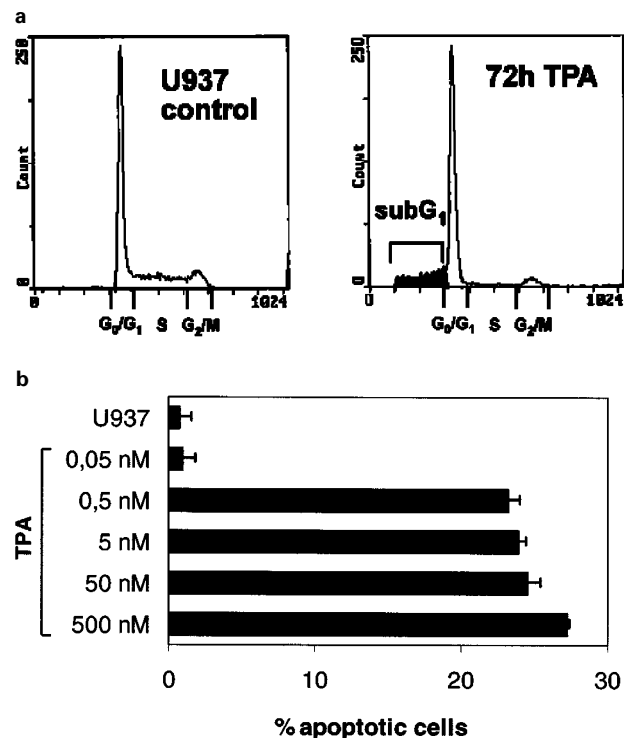

Figure 1 Analysis of TPA-induced apoptosis in U937 cells. Apoptotic cells were quantified using propidium iodide staining of fixed cells by flow cytometry. (**a**) Treatment of U937 cells with 5 nM TPA for 72 h demonstrated an accumulation of cells in the subG$_1$ phase and a corresponding reduction of cells in the S- and G$_2$/M cell cycle phase. One representative experiment out of five similar ones is presented. (**b**) Threshold effect of TPA-induced apoptosis: TPA concentrations over a range of three logs induce comparable amounts of apoptosis in U937 cells. The columns represent the means of at least three independent experiments ± s.e. measuring subG$_1$ (hypodiploid) DNA

reached a plateau after 48 h (Figure 2a). Moreover, adherent cells demonstrated less than 7% of cells in subG$_1$ phase after 72 h, whereas suspension cells demonstrated a progressively increasing population in subG$_1$ phase which reached 60% of apoptotic cells after 48 h of TPA exposure (Figure 2a). Further analysis in TPA-treated suspension cells confirmed apoptotic processes by simultaneous analysis of annexin V binding to extracellular phosphatidylserine which was performed in unfixed cells by flow cytometry. This double staining allowed to distinguish early, intermediate and late states of apoptosis and thus, the amount of suspension cells displaying subG$_1$-DNA was confirmed by the amount of annexin V-positive cells (Figure 2b).

We next asked the question of whether the observed apoptosis in the suspension cells occurs after complete attachment and monocytic differentiation of all cells in a certain population subsequently detaching, or of whether the apoptosis is related to an initial attachment failure accompanied by a derailed differentiation program. Serial measurements at different time points following TPA stimulation demonstrated a majority of cells undergoing differentiation-related adherence at approximately 12 h after PKC activation. However, at none of the time-points tested, a complete adherence of all cells was observed (Figure 2c). Thus, a progressive decline in the number of suspension cells was observed reaching lowest values 24 h after TPA treatment. Thereafter, the number of suspension cells started to increase. In parallel, a significant decline of viable cells in the suspension population and a corresponding increase of apoptotic cells was detected (Figure 2c). These data demonstrated, that a certain population of cells failed to adhere and consequently initiated apoptosis without prior differentiation-related adherence.

Further analysis should determine as to whether additional apoptosis occurs in the differentiating, adherent cell population, which may contribute to the increasing number of apoptotic cells throughout the time course. Therefore, U937 cells were initially treated with 5 nM TPA for 12, 24, 36, 48 or 60 h, respectively. Thereafter, the cells in suspension were removed in all samples and the adherent cells maintained in TPA-containing culture medium. At different time-points following removal of the suspension cells according to the scheme in Figure 2d, aliquots of the cells were harvested, fixed and stained with propidium iodide to quantify apoptotic subG$_1$ populations by FACS analysis.

The results demonstrated that in addition to the cells that undergo immediate apoptosis without prior differentiation-related adherence a progressively decreasing number of cells underwent apoptosis after induced adherence and beginning differentiation. Taken together, these data demonstrate that the predominant apoptotic effects resulted from the induced suspension cells with the inability to adhere and differentiate (Figure 2d).

G$_1$-phase cells are predominantly susceptible to TPA-induced apoptosis

Apoptosis sensitivity can be related to certain cell cycle states. In this context, malignant cells demonstrate a predominant sensitivity to chemotherapy-induced cell death when the compound is added in the S-phase of the cell cycle. To evaluate the cell cycle-dependent sensitivity to TPA, U937 cells were synchronized by centrifugal elutriation. Highly enriched fractions of cells in G$_1$-, S-, and G$_2$/M-phase, respectively, were stimulated with 5 nM of the phorbol ester (Figure 3a). In contrast to chemotherapy-induced apoptosis, our data demonstrated predominant susceptibility of G$_1$ phase cells to TPa-induced apoptosis. Whereas unstimulated G$_1$ phase-enriched cells demonstrated only background levels of sub-G$_1$ DNA, 58% of this population became apoptotic after 72 h of TPA treatment, compared to 36 and 35% of apoptotic cells when TPA was added in S- and G$_2$/M-phase, respectively (Figure 3a,b).

Expression of β_2-integrins in the differentiated adherent population, but not in the suspension cells

Previous studies have demonstrated that phorbol ester-induced monocytic differentiation of U937 cells is associated with a significantly increased expression of β_2-integrins, including the CD11c/CD18 heterodimer.[15] Consequently, we investigated the expression of CD11c in both TPA treated adherent and suspension cells gating separately for viable and apoptotic cells. Whereas the viable cells in both, adherent and suspension populations rapidly induced CD11c expression with maximum levels after 24 h, the apoptotic cells demonstrated only a moderate increase in CD11c expression (Figure 4a). Similar results were obtained for the expression of CD18 (data not shown). The CD11c expression of cells remaining in the suspension population in response to TPA can be increased by preincubation with the caspase-3/-7 tetrapeptide inhibitor DEVD (Figure 4b). In addition, the number of cells in suspension becoming adherent after the addition of DEVD increases (data not shown). These findings indicated that apoptosis observed in response to TPA stimulation is accompanied with impaired expression of differentiation markers such as the β_2-integrin CD11c.

Differential expression of pro- and anti-apoptotic proteins and p21$^{WAF/sdi-1}$

The execution of apoptotic signals is regulated by a variety of factors including the Bcl-2 protein family. Overexpression of Bcl-2 or Bcl-x$_L$ is known to confer resistance to apoptosis, whereas overexpression of proteins like Bax, Bak or Bad results in increased apoptosis, either directly or indirectly by sequestering anti-apoptotic proteins. We therefore determined the expression of various pro- and anti-apoptotic proteins of the Bcl-2 protein family in both adherent and suspension cells following 12 h of TPA treatment. Whereas the levels of the pro-apoptotic proteins Bad, Bax, and Bak remained unaltered in adherent and suspension cells, respectively, an increased expression of the anti-apoptotic factors Bcl-2, Bcl-x$_L$, and Mcl-1 was observed exclusively in the adherent population (Figure 5).

In this context, induction of the p21$^{WAF/sdi-1}$ protein is associated with differentiation and cell cycle arrest in myeloid cells by inhibition of cyclin D-dependent kinases.
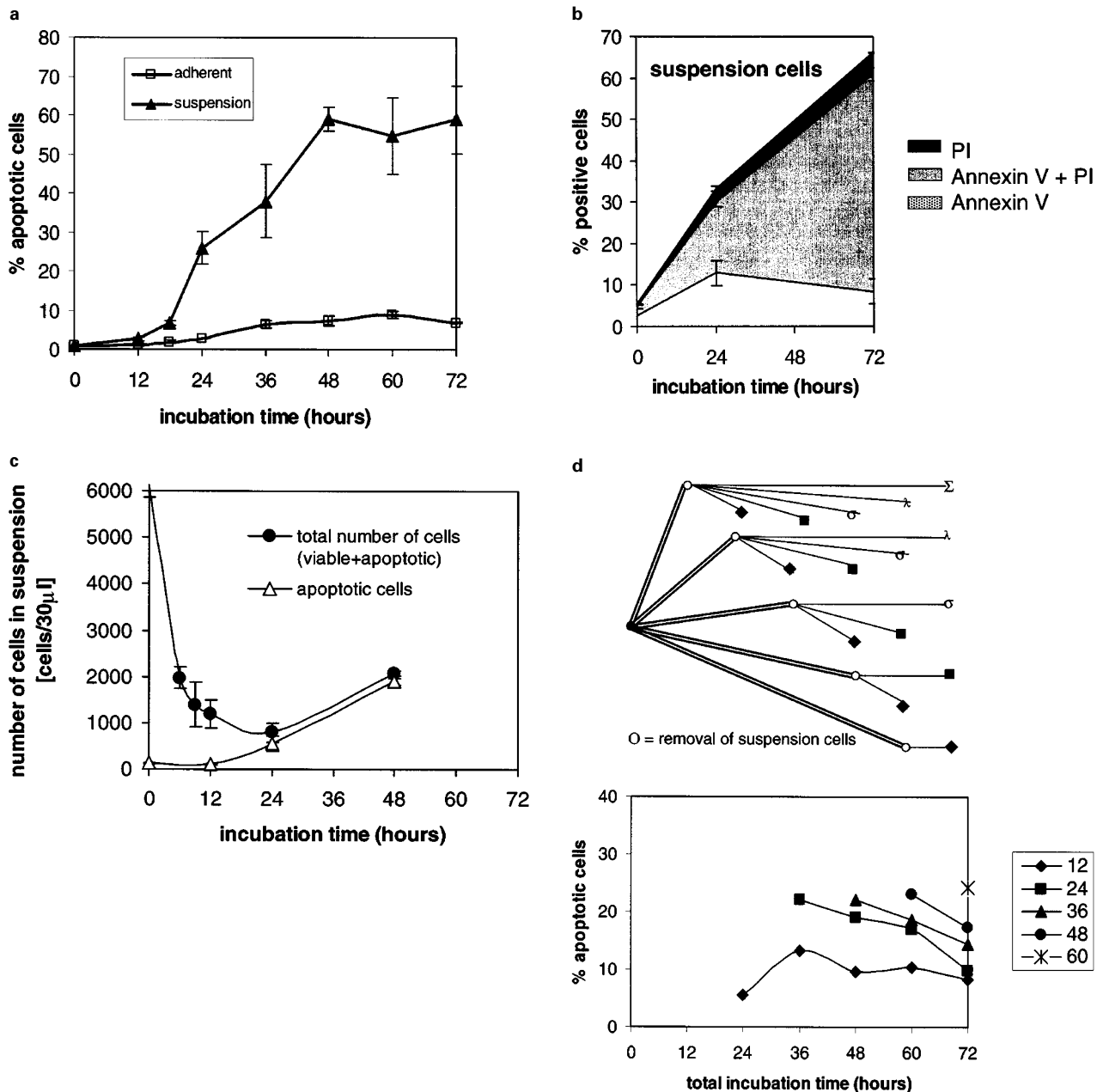
Figure 2 (a) Induction of apoptosis analysed by subG$_1$-DNA containing cells in 5 nM TPA-treated U937 cells was quantified after separation into adherent and non-adherent (suspension) populations at various time-points. All data represent the mean $\pm$ s.d. of at least three independent experiments. (b) The 24 and 72 h time points of U937 suspension cells after TPA treatment were measured for Annexin V binding by flow cytometry. Unfixed cells were counterstained with propidium iodide (PI). Early (Annexin V), intermediate (Annexin V+PI) and late stage (PI) apoptotic cells are depicted as stacked areas. All data represent the mean $\pm$ s.d. of at least three independent experiments. (c) U937 cells treated with 5 nM TPA demonstrate the appearance of suspension cells throughout the culture period. At the time-points indicated, aliquots of the cells in suspension were subjected to flow cytometry to determine the cell number. Concomitantly, aliquots of the cells were fixed and stained with propidium iodide to determine the amount of apoptotic cells after subsequent FACS analysis. All data represent the mean $\pm$ s.d. of at least three independent experiments. (d The culture of TPA-treated adherent cells demonstrate a constant initial rate and a progressively decreasing rate of apoptotic cells. U937 cells were initially treated with 5 nM TPA for 12, 24, 36, 48, or 60 h. Thereafter, the suspension populations were removed and the adherent cells were maintained in TPA-containing culture medium for further 12, 24, 36, 48 or 60 h according to the scheme in the upper panel. At the time points indicated (every 12 h), aliquots of cells were harvested, fixed and stained with propidium iodide to quantify apoptotic subG$_1$ populations by FACS analysis. Samples indicated with ♦ were initially treated with TPA for 12, 24, 36, 48 or 60 h, respectively, and were all kept in culture after removal of suspension for an additional 12 h before harvesting. Samples indicated with ■ were initially treated for 12, 24, 36 or 48 h, respectively, and were kept in culture for an additional 24 h before harvesting. Correspondingly, samples indicated with ▲ were initially treated for 12, 24, or 36 h, respectively, and were then kept in culture for an additional 36 h before harvesting, and so on. Thus, the rate of detachment and cell death due to apoptosis after initial adherence and differentiation was quantified. All data represent the mean of three independent experiments and the s.d. was always below 10%
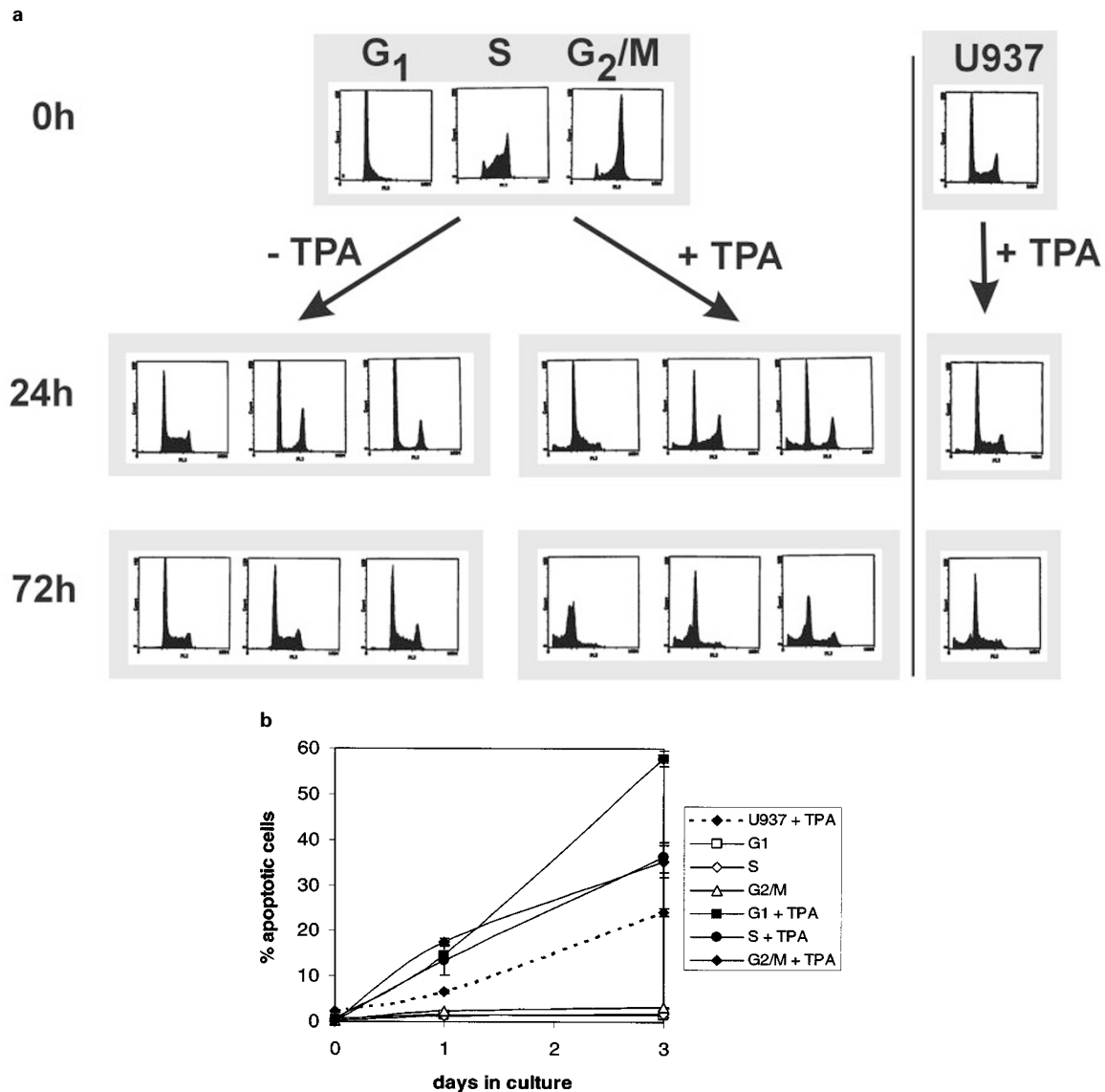
Figure 3 Cells were synchronized by centrifugal elutriation. The fractions containing highly enriched G_1-, S-, and G_2/M-phase cells were treated with 5 nM TPA for 24 and 72 h. (**a**) Histograms of representative samples out of three similar ones analysed by flow cytometry as described in Figure 2 are presented. (**b**) The percentage of apoptotic cells is displayed following stimulation of synchronized cells after centrifugal elutriation with 5 nM TPA. All data represent the mean ± s.d. of at least three independent experiments

While little p21$^{WAF/sdi-1}$ was expressed in proliferating U937 control cells, the adherent fraction demonstrated a strong increase in p21$^{WAF/sdi-1}$ protein expression after 12 h of phorbol ester exposure. In contrast, there was little if any induction of p21$^{WAF/sdi-1}$ in the corresponding suspension population (Figure 5).

TPA-induced apoptosis is accompanied by increased activities of caspase-1 and particularly caspase-3/caspase-7

In order to determine caspases involved in the TPA-induced apoptosis of the suspension population, U937 cells were preincubated with inhibitors of caspase-1 (YVAD) and caspase-3/caspase-7 (DEVD) for 15 min and then TPA

was added and incubated together with the caspase inhibitors for up to 72 h, respectively. Due to an insufficient cell permeability of YVAD and DEVD, 500 μM of these inhibitors were used. There was little if any detectable apoptotic subG$_1$ population after 72 h when the cells were incubated together with either YVAD or DEVD alone (Figure 5a). In contrast, exposure of U937 cells to 5 nM TPA revealed a significant accumulation of about 20% of the population in the subG$_1$ phase after 72 h (Figure 6a). In this context, pretreatment of the cells with YVAD and subsequent TPA exposure reduced the amount of subG$_1$-cells by 25% after 72 h (Figure 6a). This reduction of apoptotic cells was much more pronounced after pretreatment with the caspase-3/caspase-7 inhibitor DEVD followed by incubation with 5 nM TPA which
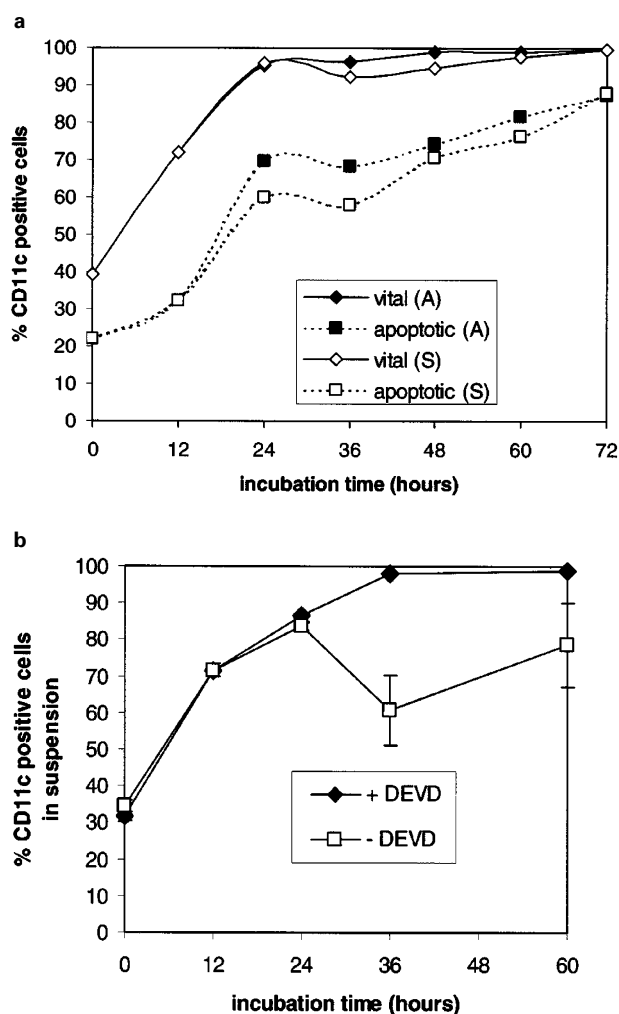
a

b

Figure 4 Expression of CD11c on 5 nM TPA treated-U937 cells as a differentiation marker was determined by flow cytometry. (**a**) Treated cells were separated into adherent and suspension fractions and each fraction into viable and apoptotic cells between 12 to 72 h and analysed for expression levels of CD11c surface markers. All data represent the mean of three independent experiments and the s.d. was always below 10%. (**b**) TPA-treated cells remaining in suspension after 12 h were incubated with 500 μM of the tetrapeptide caspase-3/-7 inhibitor DEVD and analysed for expression levels of CD11c surface markers. All data represent the mean $\pm$ s.d. of two independent experiments

Figure 5 Protein expression levels of pro-apoptotic factors and the cell cycle regulating protein p21$^{WAF1/CIP1.sdi-1}$ in U937 control cells (control) and 72 h TPA-treated U937 cells separated for adherent and suspension population. The Actin blot indicated equal loading of proteins. All blots have been repeated at least three times and one representative set of blots is presented

carried out to determine an additional involvement of caspase-8, which is part of the downstream signaling cascade following stimulation of CD95 or the TNF receptor. Thus, preincubation of U937 cells with the tetrapeptide inhibitor IETD did not result in decreased apoptosis after TPA treatment (data not shown). Taken together, these data demonstrated that YVAD and particularly DEVD can partially block TPA-induced apoptosis. Moreover, while the decrease in apoptotic subG$_1$ cells parallels a corresponding increase in G$_1$ cells during caspase inhibition these data suggested that apoptosis-rescued cells accumulate in G$_1$.

Discussion

Differentiation and cell growth regulation are mutually exclusive phenomena with integral signaling pathways and previous work has demonstrated that phorbol ester-induced PKC activation in human myeloid leukemia cells is one of these key factors to regulate growth arrest and monocytic differentiation.[15] In this context, long term culture of the growth-arrested and differentiated population for 2–3 weeks reveals a reversion of all differentiation markers and a simultaneous re-entry into the proliferative cell cycle in most cells.[16] This phenomenon termed retrodifferentiation, affects about 80 to 85% of the differentiated cells, whereas the remaining 15–20% undergo apoptosis.[13] The process of

reduced the amount of apoptotic subG$_1$-cells by 75% after 72 h (Figure 6a).

According to the subG$_1$ measurements in Figure 6a, similar measurements were performed for the G$_1$ phase. The analysis revealed that the caspase inhibitors YVAD and DEVD did not alter the cell cycle distribution of U937 control cells with about 50% of the population in G$_1$ phase, respectively (Figure 6b). While TPA treatment was associated with an accumulation of about 65% of the cells in G$_0$/G$_1$ (in addition to the 20% in subG$_1$ (Figure 6a)) after 72 h (Figure 6b), there was an increase in G$_0$/G$_1$ to about 75% by preincubation with YVAD and to about 80% by preincubation with DEVD for 15 min prior to the addition of TPA, respectively (Figure 6b). Further experiments were
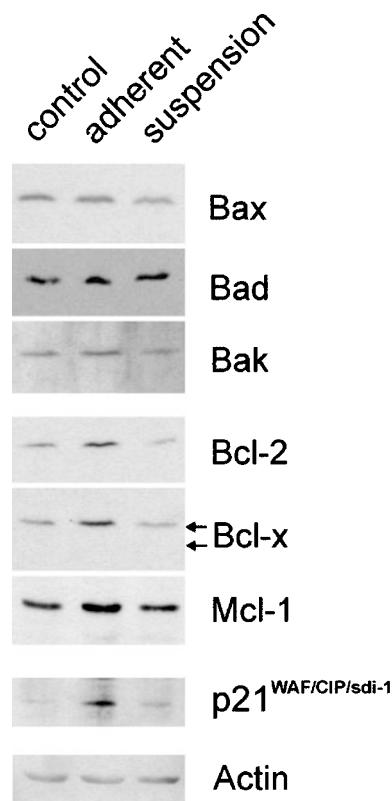
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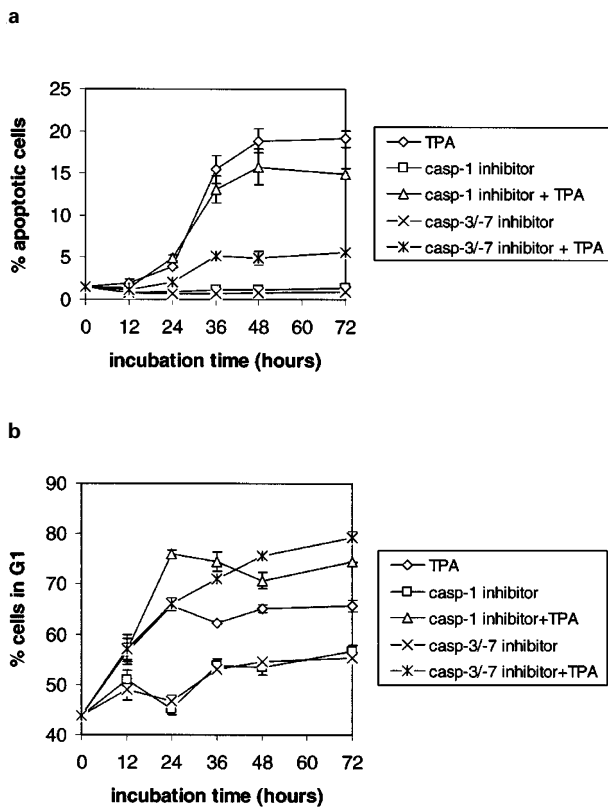G Meinhardt et al

Figure 6 Caspase inhibitors decrease the amount of apoptotic TPA-treated cells in subG$_1$ and thus elevate populations in G$_1$. (**a**) U937 cells were preincubated with inhibitors of caspase-1 (YVAD) or -3/-7 (DEVD) for 15 min prior to addition of 5 nM TPA, respectively. At the time points indicated apoptotic cells in subG$_1$ were quantified by flow cytometry using PI-stained fixed cells. Data represent the mean ± s.d. of three independent experiments. (**b**) Similar measurements were performed for the G$_0$/G$_1$ phase demonstrating an appropriate increase of cells in G$_0$/G$_1$ after caspase inhibition by DEVD and YVAD, respectively. Data represent the mean ± s.d. of three independent experiments

apoptosis as determined by DNA laddering occurs prior to the retrodifferentiation process suggesting that retrodifferentiation and apoptosis represent alternative pathways in the differentiated monocytic cells.[11,13] Moreover, the TPA-mediated induction of differentiation and retrodifferentiation requires an initial threshold of PKC activation which appears to be independent of increasing phorbol ester concentrations.[17] Similar effects were observed for the apoptotic pathway in TPA-treated suspension U937 cells which occurred regardless of TPA concentration. Taken together, these data suggest that a certain threshold of PKC activation is required and sufficient to induce either adherence coupled with monocytic differentiation and growth arrest or to induce apoptotic cell death without prior expression of differentiation-associated markers. In this context, spontaneously apoptotic U937 cells from exponentially growing cultures demonstrate increased PKC-beta and reduced PKC-zeta expression indicating that the expression of specific PKC isoenzymes is modulated during apoptosis.[18]

Although other work has hypothesized that phorbol ester-induced monocytic differentiation can occur indepen-

dently of cell-to-cell adhesion in human U937 cells,[19,20] the present data clearly demonstrate a strong functional relationship between phorbol ester-mediated adherence, integrin expression and subsequent monocytic differentiation. This relationship is also substantiated by the findings that TPA-treated U937 cells which remain in suspension are unable to differentiate and do not induce p21[WAF/CIP/sdi-1]. Thus, the failure of induced cells to adhere may therefore derail integral signaling pathways such as the differentiation program and growth arrest, eventually resulting in apoptosis as observed in about 60% of this population. In this context, our data have demonstrated that at none of the time-points tested, a complete adherence of all cells was observed. Moreover, while apoptosis in the adherent, differentiating populations progressively declined, predominant apoptotic effects resulted from the induced suspension cells with the inability to adhere and differentiate.

It is generally accepted, that the sensitivity of malignant cells to cytotoxic drugs is maximal in cycling cells, particularly in S- and G$_2$/M-phase. Our data using the phorbol ester TPA, however, demonstrate predominant apoptosis of cells in G$_1$-phase. Thus, different mechanisms may be involved in chemotherapy- and phorbol ester-mediated apoptosis. Interestingly, TPA-mediated apoptosis has recently been linked to endogenous production of TNF and activation of an auto- or paracrine TNF/TNF-receptor loop, whereas chemotherapy-induced apoptosis can at least in part be a consequence of a Fas/FasL interaction.[21,22] Moreover, we did not observe a protective effect of caspase-8 inhibition prior to TPA treatment.

Confirmative observations have been obtained in human HL-60 cells which can also be induced to differentiate along the monocytic lineage upon treatment with TPA. Thus, HL-60 cells that fail to adhere undergo apoptosis.[23] However, molecular mechanisms of this effect remain unclear. In this context, it is interesting to note that anti-apoptotic factors, including Bcl-2, Bcl-x$_L$, and Mcl-1 are significantly enhanced expressed in the TPA-treated adherent cells in contrast to the non-induced control cells and the TPA-induced suspension population, respectively. These data suggest that the failure to induce these anti-apoptotic factors upon PKC activation is coupled to altered activation of the caspase system. Our data indicate that the baseline levels of Bcl-2, Bcl-x$_L$, and Mcl-1 protein expression are associated with very low rates of spontaneous apoptosis in culture. In TPA-treated suspension cells, however, these levels may not be sufficient to block the pro-apoptotic signals generated by phorbol ester stimulation. Thus, the adequate increase in the expression of anti-apoptotic factors only in the adherent, differentiating cells may contribute to a protection of this population from death signals induced by PKC activation.

To date, more than 14 different caspases are characterized which are constitutively and ubiquitously expressed as catalytical proenzymes. According to divergent substrate specificities and differences in the length and sequence of the amino-terminal prodomains, several caspase subgroups can be distinguished.[24] Moreover, certain caspases such as caspase-1 are predominantly involved in inflammatory processes and function upstream to initiate proteolytic

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activity in downstream 'executioner' caspases like caspase-3 which then contribute to the cell death pathway by cleaving key intracellular death targets.[25] Thus, TPA-treated suspension U937 cells with an uncoupled PKC-mediated adherence and a subsequently derailed differentiation program appear to relay signals via caspase-3/caspase-7 onto the cell death program. Moreover, inhibition of this caspase pathway with DEVD can prevent the cells to enter the apoptotic subG_1 phase and thus keeps them in a growth-arrested G_0/G_1 phase.

Taken together, the data demonstrate that TPA-induced PKC activation can relay distinct signaling pathways in the same cell type via: (1) induction of adherence coupled with monocytic differentiation and growth arrest; and (2) induction of caspase-mediated apoptosis due to a derailed adherence and differentiation program.

Materials and Methods

Cell lines and reagents

Human U937 myeloid leukemic cells (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) (BioWhittaker, Belgium) supplemented with 2 mM L-glutamine without antibiotics. The cells were incubated in a humidified atmosphere (37°C, 5% CO_2) and treated with various concentrations of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, Germany) for the time points indicated or were preincubated with specific inhibitors of caspase-1, YVAD-CHO, and caspase-3/caspase-7, DEVD-CHO (Biomol, Germany), as well as caspase-8, Z-IETD-fmk (Calbiochem, Germany), respectively. TPA-induced U937 cells were separated into the adherent and suspension population and suspension cells were harvested by centrifugation (800 g/5 min).

Flow cytometry

For analysis of sub-G_1-DNA, untreated and stimulated U937 cells (10^6 cells/ml) were fixed with 70% (w/v) ice-cold ethanol overnight. Adherent cells were removed from the plastic surface of the culture dish with a rubber policeman. After two washes with ice-cold PBS, the fixed cells were resuspended in 1 ml of PBS containing 40 μg/ml propidium iodide (Sigma, Germany) and 500 U/ml RNase A (Boehringer Mannheim). Following incubation for 30 min in the dark the cells were analyzed on a EPICS XL-MCL flow cytometer (Coulter, Germany) using System II software. To identify apoptotic populations, objects with fluorescence values <20% of that of the G1 peak were gated out. As previously described, the propidium-iodide fluorescence signal peak versus the integral was used to discriminate G2/M cells from G_0/G_1 doublets.[26]

For analysis of cell surface markers, 10^6 cells were washed with PBS and resuspended in 1 ml PBS with 2% FCS. One-hundred μl of cells were incubated at 4°C with R-phycoerythrine (PE)-conjugated anti-CD11c (Coulter Immunotech, Germany) for 10 min in the dark. After centrifugation the cells were resuspended in 500 μl of PBS with 2% FCS and flow cytometry was performed.

For analysis of cell number, 0.5 ml of medium containing unstained, unfixed suspension cells was subjected to flow cytometry. For each sample, an acquisition volume of 30 μl was used. Debris and dead cells were discriminated and eliminated and, respectively, by live gating on events with forward and side scatter characteristics of viable U937 cells. For all time-points, at least three independent samples were measured.

Synchronization and cell cycle analysis

U937 cells in logarithmic growth phase were subjected to centrifugal elutriation using the JE-5.0 elutriation system (Beckman Inc., Palo Alto, CA, USA). Briefly, 2×10^8 cells were applied to the standard chamber (1600 r.p.m. at 27°C) using a digital flow controller (Cole-Palmer Instruments Inc., Chicago, IL, USA). The calibrated pump speed was increased from 10 to 30 ml/min. Enriched cell populations from the different phases of the cell cycle were elutriated in 100 ml aliquots of RPMI 1640 medium containing 1% FBS. Aliquots (1 ml) of the cells were fixed and analyzed as described above.

Immunoblot analysis

Untreated and stimulated U937 cells were washed three times in ice-cold PBS and lysed in a buffer containing 10 mM Tris-HCl (pH 7.6), 137 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM EDTA, 1% (v/v) NP-40 with the addition of 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride (PMSF). Protein concentration was adjusted using a colorimetric assay. Controls for equal loading of protein were performed using an antibody against β-actin. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Germany). The transfer buffer contained 25 mM Tris-HCl, 192 mM glycine, 0.037 (w/v) SDS and 20% (v/v) methanol. The membranes were blocked with PBS containing 5% dried milk and 0.05% Tween-20 (PBS/Tween). After washing four times with PBS/Tween, the membranes were incubated with appropriate antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized by autoradiography using the ECL-detection kit (Amersham, Germany).

Acknowledgements

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