Lysophosphatidic acid promotes phorbol-ester-induced apoptosis in TF-1 cells by interfering with adhesion

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When exposed to PMA, the erythroblastic cell line TF-1 and its cytokine-independent variant D2 cells can be induced to undergo differentiation and apoptosis. In this study we investigated the mechanism responsible for the differential responses to PMA induction and show that serum present in the medium has a major role in promoting PMA-induced apoptosis in TF-1 and D2 cells. Interestingly, lysophosphatidic acid (LPA) could replace serum to co-operate with PMA in inducing apoptosis via the Rho-dependent pathway. The expression of a constitutively active form of RhoA also increased PMA-induced apoptosis. However, by inhibiting adhesion, most cells underwent PMA-induced apoptosis even in the absence of LPA or serum, indicating that adhesion is required for PMA-induced differentiation. Given that LPA could prevent adhesion for cells maintained in the serum-free medium, here we propose that RhoA has a switching role in determining whether TF-1 and D2 cells undergo differentiation or apoptosis in response to PMA, by modulating cell adhesion.

Key words: differentiation, protein kinase C activation, Rho activation.

INTRODUCTION

PMA, a phorbol ester, is a potent activator of protein kinase C (PKC) and is known to induce the differentiation of several promyeloleukaemia cell lines and to suppress apoptosis [1–7]. In contrast, activation of PKC with phorbol ester has been shown to induce apoptosis in other cells, such as prostate cancer cells, human Burkitt's lymphoma cells, promyeloleukaemia HL-60 cells, U937 cells and murine thymocytes [8–13]. It is still not certain whether there is a concurrent signal pathway that can influence the cell fate in terms of apoptosis and differentiation induced by the PKC activation pathway.

The growth of myeloid progenitor TF-1 cells is dependent on granulocyte/macrophage colony-stimulating factor (GM-CSF) or interleukin 3 [14]. On treatment with PMA, approx. 15% of the cells differentiate into macrophage-like cells and attach to the culture flask, whereas the other 85% of the population remain in suspension and become apoptotic [15]. A few TF-1 variants, which grow autonomously in medium without supplementation with GM-CSF or interleukin 3, have previously been selected and characterized [15]. In one of the variant cell lines, D2, when treated with PMA, 50% of the cells undergo differentiation and the other 50% are apoptotic [15]. In the present study we attempted to explore why there are two different outcomes for these cells in response to induction with PMA. We demonstrated that the removal of serum from the medium prevents PMA-induced apoptosis and that lysophosphatidic acid (1-oleoyl-2-*sn*-glycero-3-phosphate; LPA) could replace serum to promote PMA-induced apoptosis. Furthermore, we observed that the RhoA signalling pathway influences the outcome of induction with PMA in serum-containing medium. However, our results also showed that PMA-induced apoptosis does not necessarily require an LPA-mediated signal. Rather, cell adhesion can prevent the occurrence of PMA-induced apoptosis. Taking these results together, we propose that LPA can promote PMA-induced apoptosis by interfering with cell adhesion via the RhoA-dependent pathway.

EXPERIMENTAL

Chemicals

LPA was purchased from Fluka (Buchs, Switzerland). LPA was dissolved in PBS containing 10 mg/ml fatty-acid-free albumin. PMA was purchased from Sigma Chemicals (St Louis, MO, U.S.A.) and was dissolved in DMSO.

Cell culture

TF-1 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 i.u./ml penicillin G, 100 i.u./ml streptomycin and 1 ng/ml GM-CSF. Human GM-CSF was kindly provided by Schering-Plough Ltd (Taipei, Taiwan). D2 cells were maintained in the same medium without GM-CSF.

Caspase-3 assays

Caspase-3 activity was assayed in 100 μl reaction mixtures with colorimetric reporter substrate peptides [acetyl-Asp-Glu-Val-

Abbreviations used: FBS, fetal bovine serum; GM-CSF, granulocyte/macrophage colony-stimulating factor; GFP, green fluorescent protein; GST, glutathione S-transferase; LPA, lysophosphatidic acid; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; RBD, Rho-binding domain.

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Asp-*p*-nitroanilide ('Ac-DEVD-pNA'); Biosource International, Nivelles, Belgium] in accordance with a procedure recommended by the manufacturer. In brief, cells were lysed in buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.5 % sodium deoxycholate/0.1 % SDS/1 mM EDTA/10 mM EGTA/1 mM dithiothreitol/1 % (v/v) Nonidet P40/1 mM PMSF/1 μg/ml leupeptin/1 μg/ml aprotinin], after which equal amounts of lysates containing 100 μg of protein were incubated with the substrate peptide (1 mM) at 37 °C for 2 h, followed by measurement of A_{405} with a spectrophotometer.

SDS/PAGE and immunoblotting

The preparation of particulate and soluble fractions for the PKC translocation assay was performed as described previously [16]. Samples containing equal amounts of protein were separated by SDS/PAGE [10 % (w/v) gel] and transferred electrophoretically to a PVDF membrane (Millipore). The antibodies used and their dilutions were as follows: rabbit polyclonal antibodies against PKC-α, β, δ, γ, θ, ϵ (Transduction Laboratories) (1:5000) and antibodies against human poly(ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology) (1:5000); alkaline-phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (Promega) were used for the detection of the primary antibodies. Colour development of alkaline phosphatase was performed in accordance with the vender's instructions.

Plasmids

$G\alpha_{13}Q226L$, $G\alpha_{q}Q209L$, $G\alpha_{12}Q229L$ and $G\alpha_{11}Q209L$ in pCMV expression vectors were kindly provided by Dr Dianqing Wu (University of Rochester, Rochester, NY, U.S.A.) as described previously [17]. RhoAV14 in pUHD expression vector and C3 in pEF1α expression vector [18] were kindly provided by Dr Tzuu.-Shuh Jou (National Taiwan University, Taipei, Taiwan). The RhoAV14 in the pCMV vector was constructed by subcloning the cDNA of RhoAV14 into pCDNA3. The pEGFP expression plasmid was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The pGST–RBD plasmid (in which GST stands for glutathione S-transferase and RBD for Rho-binding domain) was provided by Dr M. A. Schwartz (Scripps Research Institute, La Jolla, CA, U.S.A.).

Transient transfection

Cells (5×10^6) were washed twice with STBS buffer [25 mM Tris/HCl (pH 7.4)/5 mM KCl/0.7 mM $CaCl_2$/137 mM NaCl/0.6 mM Na_2HPO_4/0.5 mM $MgCl_2$]. The cell pellet was resuspended in 250 μl of STBS containing 400 μg of DEAE-dextran and 4 μg of plasmid DNA consisting of 3 μg of expression plasmid plus 1 μg of pEGFP. After incubation for 20 min at 37 °C, 5 ml of STBS buffer was added to the transfection mixture. Cells were then centrifuged and resuspended in 10 ml of RPMI 1640 medium containing 10 % (v/v) heat-inactivated FBS and GM-CSF, then incubated for 48 h at 37 °C before treatment.

Fluorescence microscopy

After transfection for 48 h, cells were pelleted and resuspended for 8 h in fresh RPMI 1640 medium containing either PMA or PMA plus LPA. Cells remaining in suspension were then collected by centrifugation at 800 ***g*** for 6 min. Cells expressing green fluorescent protein (GFP) were counted with an Olympus AX-70 fluorescence microscope.

Endogenous RhoA activity assay

A method of affinity-precipitation of cellular GTP-RhoA was used to assay endogenous RhoA activity as described by Ren et al. [19]. In brief, cells were washed and lysed in a buffer containing 50 mM Tris/HCl, pH 7.2, 1 % (v/v) Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. After centrifugation at 13000 ***g*** at 4 °C for 5 min, the supernatants of the lysates were incubated for 2 h with GST–RBD-coupled glutathione-agarose beads. The beads were washed with buffer containing 50 mM Tris/HCl, pH 7.2, 1 % (v/v) Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. The bound RhoA protein was detected by Western blotting with a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

RESULTS

The addition of PMA to culture medium induced 80 % of TF-1 cells to undergo cell death in 12 h, whereas the other 20 % cells became adherent and differentiated to the macrophage phenotype (Figure 1A). As indicated by the increase in caspase-3 activity, cells remaining in suspension became apoptotic in 12 h. Another cell line, D2, which is a cytokine-independent variant derived from TF-1 cells [15], exhibited 50 % cell death when treated with PMA for 8 h; the remaining viable cells adhered to the plate and became spreading, with a monocytic differentiation phenotype (Figure 1B). Caspase-3 activity also increased in the suspended D2 cells during treatment with PMA. D2 cells that were treated with PMA for 2 h in suspension could no longer reattach to a fresh culture dish and all eventually became apoptotic (results not shown). It is therefore likely that PMA induced an irreversible change in half of the D2 cells, leading to cell death.

Because the PMA-induced apoptotic and differentiating fractions were evenly distributed in D2 cells, we then examined whether PKC was differentially regulated in these two fractions. By analysing the translocation of various isoforms of PKC in these two cell populations, we did not find a significant difference in the translocation of PKC isoforms α, β, γ, δ, ϵ and θ (Figure 2). Clearly, these PKC isoforms were well activated by PMA in both attached and suspended cells.

We then investigated whether there were different signalling pathways that co-operated with PKC activation to affect the outcome of PMA induction, by searching for a condition that could prevent the occurrence of PMA-induced apoptosis. We found that when TF-1 and D2 cells were treated with PMA in serum-free medium, most of the cells became adherent to the flask and viable. It is therefore possible that one or more factors in serum were responsible for signal transduction in promoting PMA-activated apoptosis. Insulin, PDGF and EGF were each added to serum-free medium to test their possible involvement in PMA-induced apoptosis; however, none of these growth factors replaced serum in inducing apoptosis in the presence of PMA (results not shown). Because LPA is a normal constituent of serum and the cellular response to albumin-bound LPA has in fact been shown to be similar to that induced by serum (reviewed in [20]), we then examined the effect of LPA on induction with PMA in serum-free medium. Interestingly, the addition of LPA and PMA to TF-1 or D2 cells in the absence of serum reinstated apoptotic induction (Figure 3A). Furthermore, LPA in the range 0.5–20 μM exerted dose dependence, in co-operation with PMA, to induce cell death in TF-1 cells (Figure 3B). With the use of PARP protein cleavage analyses, we found that LPA in com-

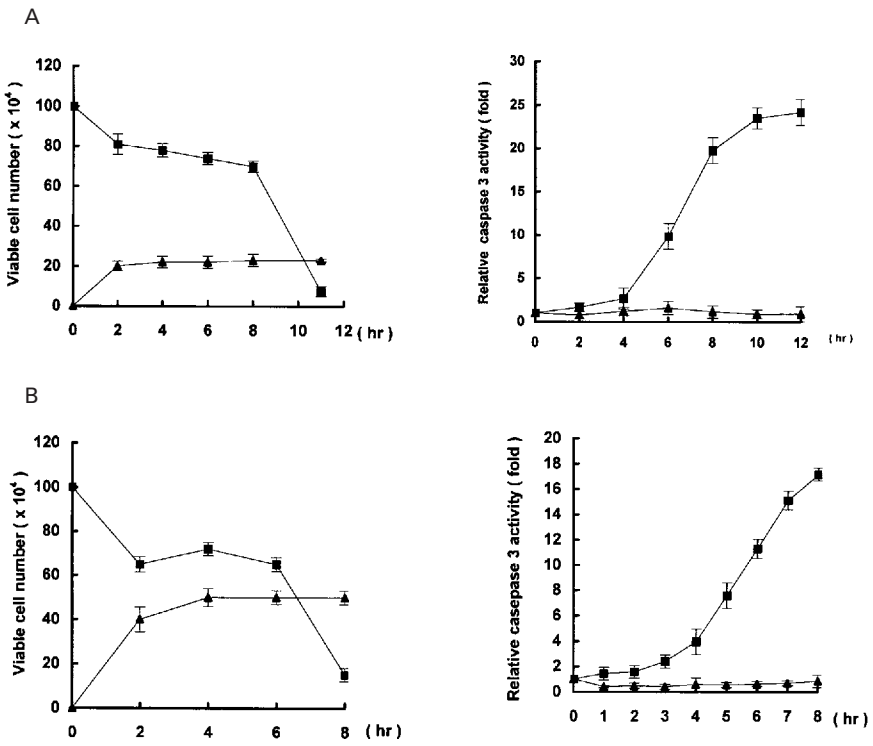

Figure 1 Effect of PMA treatment on TF-1 and D2 cells

(**A**) Left panel: TF-1 cells (10^6) were treated with PMA (32 nM) in 10% (v/v) heat-inactivated FBS-containing medium in the presence of GM-CSF. Viable cells that attached to the flask (■) or remained in suspension (▲) were counted by the Trypan Blue exclusion method. Results are means ± S.D. for four experiments. Right panel: extracts containing 100 μg of protein from cells in suspension after treatment with PMA were analysed for caspase-3 activity as described in the Experimental section. Results are presented as the relative activity as compared to the untreated control and are means ± S.D. for two independent experiments. (**B**) A similar experiment was performed with D2 cells, except that the medium did not contain GM-CSF.

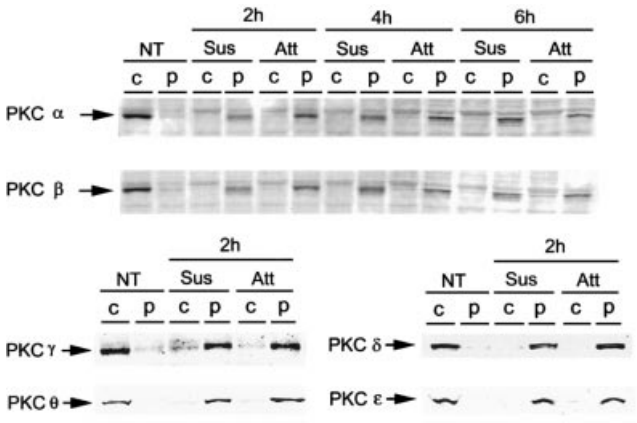

Figure 2 Translocation of PKC isoforms induced by PMA in the attached and pro-apoptotic fractions

D2 cells were treated with PMA as described in the legend to Figure 1. Particulate (p) and cytoplasmic (c) fractions of cell lysates were extracted. Protein lysates (35 μg) from each sample were separated by SDS/PAGE [10% (w/v) gel] and analysed by immunobloting with antibodies specific for the PKC α, β, γ, δ, ϵ, and θ forms. Abbreviations: NT, non-treated; Sus, in suspension; Att, attached.

bination with PMA triggered apoptosis in both TF-1 and D2 cells (Figure 3C). Thus our results suggested that an LPA-linked pathway promoted PMA-induced apoptosis.

The cellular transducers for LPA have been identified as G_i, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (reviewed in [21]). It is known that phospholipase β is the effector for $G\alpha_{q/11}$ [22], tyrosine kinase for G_i [23] and the small G-protein Rho for $G\alpha_{13}$ [24,25]. To determine whether the activation of $G\alpha_{q/11}$ and $G\alpha_{12/13}$ was involved in promoting PMA-induced apoptosis, we transfected cells with constitutively active mutants of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$. Successfully transfected cells were identified with a co-transfected GFP expression plasmid, which indicated that the transfection efficiency was approx. 5–10% in different experiments. We found that PMA induced apoptosis in cells overexpressing consitutive $G\alpha_{12}$ or $G\alpha_{13}$, but not $G\alpha_q$ or $G\alpha_{11}$, in serum-free medium (Figure 4A). Because the small G-protein Rho is the effector for $G\alpha_{12/13}$, we then expressed C3, which is an inhibitor of Rho in intact cells [26], to test whether disruption of the Rho-dependent pathway could change the effect of LPA on PMA-induced apoptosis. After transfection, cells were treated for 8 h with PMA in the presence or absence of LPA in serum-free medium. After treatment with PMA, cells attached to the plastic dish were revealed and counted under a fluorescence microscope for photography. Transfection of the C3 plasmid markedly increased the number of adherent cells in the culture treated with PMA and LPA but not in that treated with PMA alone (Figure 4B). This result indicated that the enhancement of PMA-induced apoptosis by LPA is through the Rho-dependent pathway. The expression vector of RhoAV14, which is a constitutively active form of RhoA, together with the GFP expression vector, were also transfected to TF-1 cells to examine whether PMA-induced adhesion in serum-free medium could be affected by the

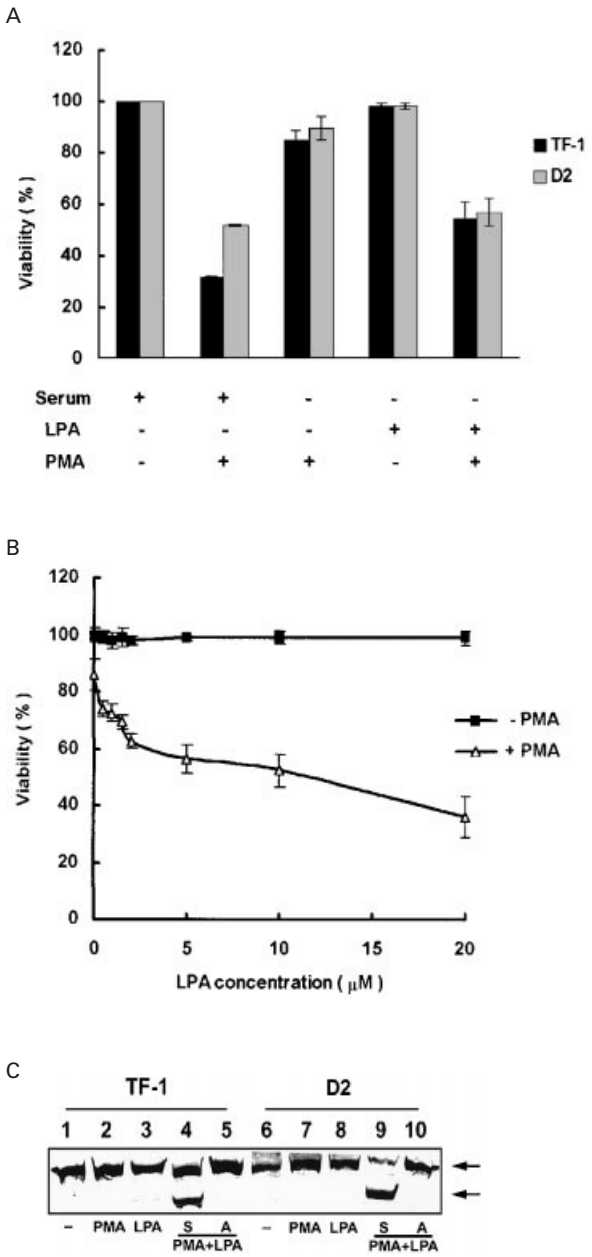

Figure 3 PMA cooperates with serum or LPA to induce apoptosis in TF-1 and D2 cells

TF-1 and D2 cells (2×10^5/ml) were suspended in RPMI-1640 medium with and without GM-CSF respectively. (**A**) PMA (32 nM), 10% (v/v) heat-inactivated FBS or LPA (5 μM) was added to the medium separately or in different combinations as indicated. After 12 h for TF-1 cells (solid bars) and 8 h for D2 cells (hatched bars), viability was determined for each sample by the Trypan Blue exclusion method and is expressed as viability relative to that of cells incubated with serum only. Results are means $\pm$ S.D. ($n = 3$). (**B**) TF-1 cells were incubated in serum-free medium containing LPA at various concentrations in the absence (■) or presence (△) of PMA (32 nM). Cell viability was then determined as above. (**C**) Immunoblot analysis of PARP in TF-1 cells (lanes 1–5) and D2 cells (lanes 6–10), in serum-free medium, treated with PMA (32 nM) in the absence or presence of LPA (5 μM), as indicated, for 12 h. Cells treated with LPA plus PMA were separated into suspension (S) and attached (A) fractions as indicated. Cell extracts (each containing 50 μg of protein) were subjected to SDS/PAGE [10% (w/v) gel] for immunoblot analysis with antibody against PARP. Arrows point to full-length PARP (upper) and cleaved PARP (lower).

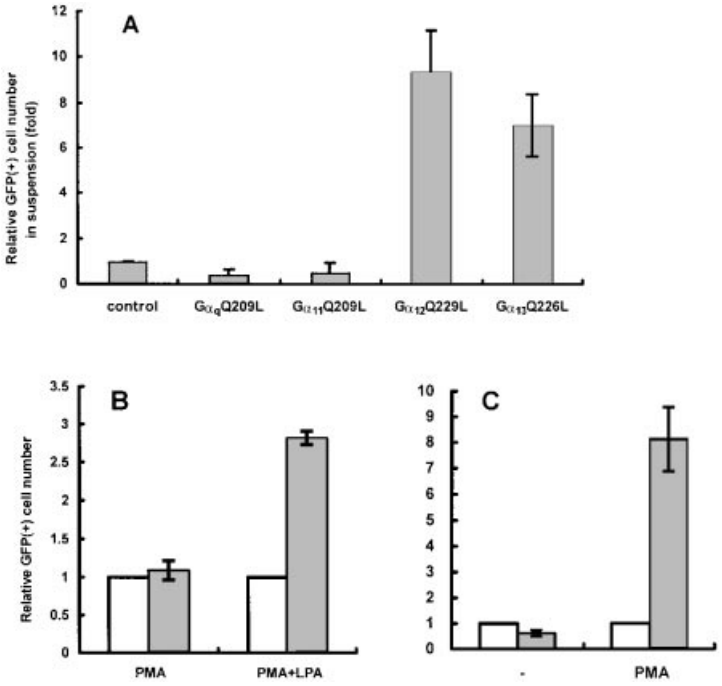

Figure 4 Involvement of the Gα12/13-linked Rho-dependent pathway in PMA-induced apoptosis

All cells were transiently transfected with 1 μg of GFP expression vector and 3 μg of the indicated plasmid or control vector as described in the Experimental section. After transfection for 48 h, cells were washed twice with PBS, which was then replaced with serum-free medium containing GM-CSF. (**A**) Cells transfected with the indicated Gα subunit plasmid or control vector were incubated with PMA (32 nM). At 8 h after treatment with PMA, cells remaining in suspension were collected. GFP-positive cell numbers in suspension were determined and are expressed relative to the cells transfected with the control vector. The transfection efficiency was 5–10% in all experiments. (**B**) Cells transfected with C3 (filled bars) or the control plasmid (open bars) were treated with PMA (32 nM) or PMA (32 nM) plus LPA (5 μM). GFP-positive cell attached to the plate were counted from 10 randomly chosen fields and are expressed relative to the cells transfected with the control vector. (**C**) Cells transfected with the expression vector of RhoAV14 (filled bars) or the control vector (open bars) were suspended in serum-free medium with or without PMA (32 nM). GFP-positive cell numbers in suspension were determined and are expressed relative to the cells transfected with the control vector. All results are means $\pm$ S.D. ($n = 2$).

activation of RhoA. Consistently, the expression of RhoAV14 significantly increased the cell number in suspension after the addition of PMA to the serum-free medium (Figure 4C). All cells in suspension were apoptotic, as judged by chromosome condensation by staining with 4,6-diamidino-2-phenylindole.

We next determined whether the endogenous RhoA activity in TF-1 and D2 cells was highly responsive to serum removal and was correlated with susceptibility to PMA-induced apoptosis. Because Rho effectors interact only with GTP-bound Rho, the endogenous Rho activity was assayed by analysing the amounts of RhoA associated with RBD derived from the effector protein Rhotekin [19]. GST–RBD protein coupled to glutathione-agarose beads was incubated with the lysates to detect GTP-bound RhoA by Western blot analysis. As shown in Figure 5, there was a significant decrease in RhoA activity in the attached D2 cells, which were incubated in serum-free medium in the presence or absence of PMA for 30 min, in comparison with that in the suspended cells that were maintained in serum-containing medium. Thus the endogenous RhoA activity in D2 cells is highly sensitive to serum and the cells containing an elevated activity of RhoA are susceptible to PMA-induced apoptosis. In TF-1 cells, endogenous RhoA activity also remained elevated in serum-containing medium. TF-1 cells exhibited adhesion when incubated in serum-free medium with or without PMA for 2 h, at

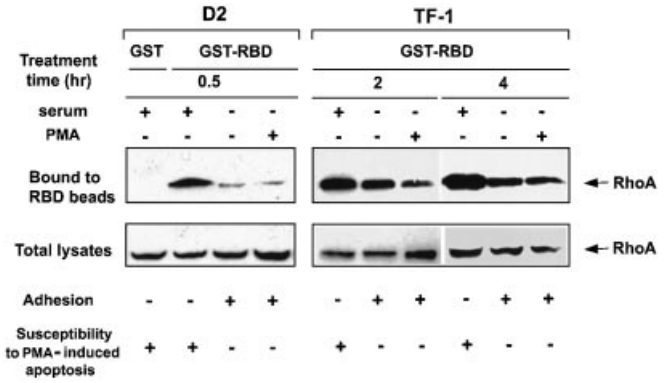

Figure 5 Changes in endogenous RhoA activity, cell adhesion and susceptibility to PMA-induced apoptosis in response to serum removal

D2 and TF-1 cells were washed twice with PBS and were either maintained in suspension in serum-containing medium or incubated in serum-free medium with or without PMA (32 nM) for the times indicated. Cell lysates were incubated with GST–RBD glutathione beads. After being washed, the bound proteins and total lysates (15 μg of protein) were analysed by Western blotting with anti-RhoA antibody.

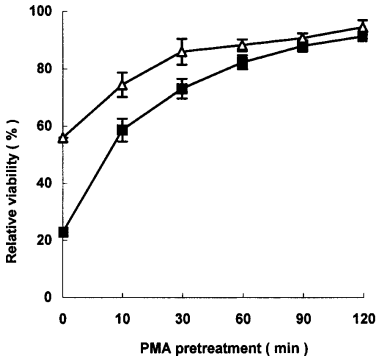

Figure 7 PMA-induced adhesion prevents apoptotic activation

TF-1 cells were washed and suspended in serum-free medium containing 32 nM PMA for the times indicated, followed by the addition of LPA (20 μM, △) or serum (5%, ■) and incubation for a further 10 h. Viability was determined by the Trypan Blue exclusion method. Results are means ± S.D. for two experiments.

which time RhoA activity was decreased. Similar results were also obtained in those cells with the same treatment for 4 h. Because total amounts of RhoA protein in cells were unchanged, it is clear that, in D2 and TF-1 cells, RhoA activation is dependent on serum and is inversely correlated with adhesion capability. Furthermore, the results also indicated that PMA could induce apoptosis in suspended TF-1 and D2 cells containing activated RhoA but not in adherent cells in serum-free medium containing down-regulated RhoA.

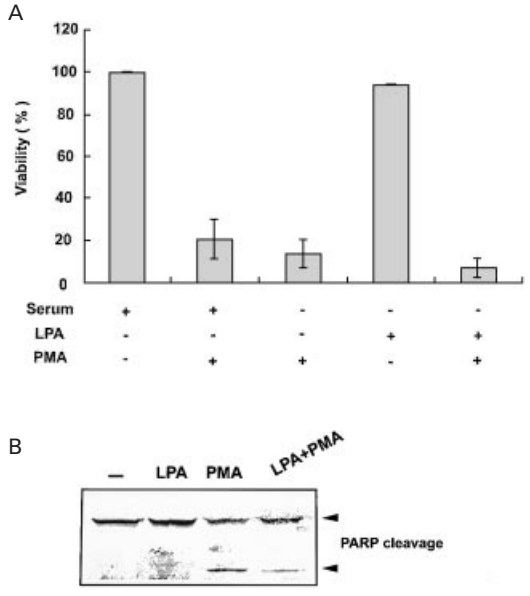

Figure 6 Effect of PMA on TF-1 cells plated on a low-attachment plate

TF-1 cells were plated for 12 h on an ultra-low-attachment plate (Corning, Corning, NY, U.S.A.) in serum-free medium in the presence or absence of serum (10%, v/v), PMA (32 nM) or LPA (5 μM) as indicated. Cell viability (**A**) and PARP cleavage (**B**) were performed as described in the legend to Figure 3.

Because only those cells without adhesion were susceptible to PMA-induced apotosis, we sought to determine whether all cells in serum-free medium were induced to apoptosis by PMA when maintained in suspension. To this end we seeded TF-1 cells on low-attachment plates with a hydrogel coating. In this way, cell adhesion could be prevented during the PMA treatment. In contrast with the results obtained from the adherent plate (Figure 3), on the low-attachment plate PMA induced most TF-1 cells to undergo apoptosis in the absence of serum or LPA (Figure 6). Expression of C3 did not overcome cell death induced by PMA under the same conditions (results not shown). It was therefore likely that the presence of adhesion can prevent TF-1 cells from becoming apoptotic in response to PMA-activated signal(s) in serum-free medium. The results also indicated that PMA-induced apoptosis could always occur in the cells when the adhesion signal was absent.

To prove that the early adhesion event can protect cells from apoptosis, TF-1 cells were pretreated with PMA in the absence of serum for 0–120 min to ensure that PMA-induced adhesion and spreading occurred in advance. LPA or serum was then added to the cultures at the indicated time for a determination of viability (Figure 7). As expected, neither LPA nor serum promoted PMA-induced apoptosis for these adherent cells pretreated with PMA for 30 min, suggesting that the pre-existing adhesion signal allowed cells to undergo the PMA-induced differentiation program, a process that could not be reversed by the LPA-mediated signal.

The above findings suggested that the Rho signalling pathway activated by LPA probably exerted a negative effect on adhesion rather than having a direct role in PMA-induced apoptosis. Because most TF-1 and D2 cells in serum-free medium became readily adherent to the plastic flask without cell spreading, we then examined whether the presence of LPA in serum-free medium could directly affect cell adhesion. As shown in Figure 8, most TF-1 cells remained in suspension when incubated in serum-free medium containing LPA. Furthermore, adherent cells maintained in serum-free medium became detached again when LPA was added to medium for 60 min. Similar results were obtained for D2 cells (results not shown). Clearly, LPA was able to transduce the signal to modulate cell adhesion negatively for TF-1 and D2 cells in serum-free medium.

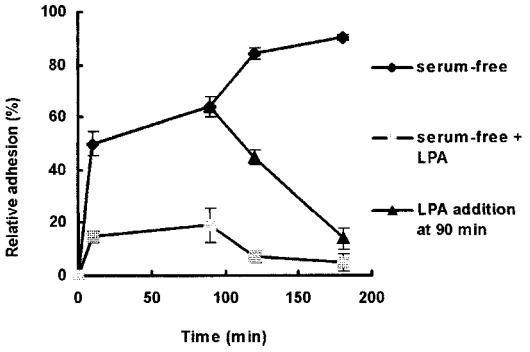

Figure 8 LPA interferes with cell adhesion in serum-free medium

TF-1 cells (5×10^5/ml) were plated in 35 mm culture dishes and incubated in serum-free medium containing GM-CSF in the presence (■) or absence (◆) of LPA (10 μM). For a parallel set of cultures, LPA was added after cells had been preincubated in serum-free medium for 90 min (▲). Cells that remained in suspension were counted at the indicated times. Results are means ± S.D. for two experiments.

DISCUSSION

In TF-1 and D2 cells, exposure to PMA induces cells to undergo differentiation or to become apoptotic. Here we have addressed the question of why these cells respond to PMA so differently under the same conditions. Our results indicated that the adhesion signal present in the cells affects whether or not the apoptotic pathway can be activated by PMA. We proposed that LPA or serum in the growth medium attenuates the adhesion capability of TF-1 and D2 cells, in which the PMA-induced apoptosis can be freely triggered. For those cells in which the adhesion signal occurs earlier, the PMA-induced apoptotic pathway is aborted, so that the differentiation programme is preferentially activated.

Our results also suggest that the interference of LPA in adhesion is probably through activation of the RhoA-dependent pathway. It has been well documented that Rho activation by LPA is mediated by $G\alpha_{12/13}$, leading to cytoskeletal reorganizations [27,28]. In fibroblasts, RhoA has been shown to trigger the assembly of focal contacts and stress fibre. However, another study has shown that RhoA activation by LPA is well detected in both adherent and detached Swiss 3T3 cells [19], indicating that the activation of RhoA by LPA does not necessarily lead to adhesion. Here we showed that C3 expression specifically promotes cell adhesion induced by PMA treatment in the presence of LPA, suggesting that Rho activation negatively modulates the cell adhesion event induced by PMA. It has been shown consistently that RhoA is a negative regulator of cell adhesion in the monocytic cell lines THP1 and U937 [29,30]. It therefore seems that this component of the RhoA activation pathway functions differently in haemopoietic myeloid cells, whose cellular contexts are different from those of fibroblasts. In the present study, we obtained further evidence that the constitutively active form of RhoA, when expressed in TF-1 and D2 cells, promotes PMA-induced apoptosis in serum-free medium, which might be relevant to the inhibition of adhesion. We also provided new evidence that endogenous RhoA activity is decreased in both TF-1 and D2 cells in response to serum removal, which is always associated with immediate cell adhesion. In other words, there is a temporal relationship between the down-regulation of RhoA activity and cell adhesion in TF-1 and D2 cells during incubation in serum-free medium. Given that the addition of LPA to serum-free medium attenuates cell adhesion, our results suggested that the activation of endogenous RhoA is closely related to cell detachment in TF-1 cells.

Because PMA can induce apoptosis in most cells in serum-free medium when cells are kept in suspension, it is logical to assume that the presence of LPA in medium promotes PMA-induced apoptosis by decreasing the cell adhesion efficiency. However, it is still unclear why only a fraction of cells receive the signal to adhere and differentiate. Because RhoA is a G-protein with a cyclic on–off device, it is possible that only some of the cells contain the temporarily switched-off RhoA at the time of PMA induction, which allows the adhesion signal to occur earlier and block the apoptotic pathway. Here we propose that the switched-on and switched-off RhoA forms might represent one of the subtle molecular differences within the population that affects the outcome of exposure to PMA by modulating the adhesion mechanism.

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