Mechanism of Apoptosis Suppression by Phorbol Ester in IL-6-Starved Murine Plasmacytomas: Role of PKC Modulation and Cell Cycle

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ABSTRACT: We show here that the mode of cell death in IL-6-starved T1165 and T1198 plasmacytoma cell lines is apoptosis, and that it can be suppressed by phorbol ester (PMA) treatment in a protein kinase C (PKC)-mediated process that involves α and/or δ isozymes. PMA-induced PKC activation, but not the depletion that follows it, participates in the suppression of apoptosis. Extended PKC activation is necessary but not sufficient for the apoptosis suppression. In addition, the cells must be in a "competent" state, which appears not to be determined by PKC. We observed two points of "competence" during the time between withdrawal of IL-6 and the start of massive cell death: one, immediately after withdrawal, and another, just before onset of apoptosis, at the time corresponding to maximal accumulation of cells in a G0/G1 block imposed by IL-6 withdrawal. Treatment with PMA and other PKC activators resulted in a shift of the cell population to S phase, lifting the G0/G1 block. We propose a model in which cells are rescued in a certain stage of the G1 phase of cell cycle. Death suppression occurs when a transient PMA- induced PKC activation occurs when a significant number of cells are in this part of G1, allowing them to pass the restriction point safely without initiating the cell death program.

Apoptosis is a process of active cellular self-destruction that plays an indispensable role in the development and maintenance of homeostasis in multicellular organisms (Yuan & Horvitz, 1990). It is thought that the death program is activated in order to eliminate cells whose survival would be detrimental to the host. The characteristic features of apoptosis are shrinkage of cell volume, chromatin condensation, and degradation of DNA into ladder-like patterns (Kerr et al., 1972). Apoptosis can be induced by a variety of physiological and nonphysiological means such as withdrawal of serum or growth factors, or treatment with glucocorticoids, calcium ionophores, and DNA-damaging agents (Tomei et al., 1988; McConkey et al., 1990, 1991; Benhamou et al., 1990; Perotti et al., 1990; Valentine & Licciardi, 1992). It has been shown that phorbol esters can modulate apoptosis, induced by deprivation of growth factors and other stimuli, in many cell systems. In some cases, they enhance apoptotic cell death (Iwata et al., 1990); in others, they suppress it (Valentine & Licciardi, 1992; Jarvis et al., 1994). These effects are presumably mediated through PKC,[1] a major high-affinity receptor of phorbol esters (Castagna et al., 1982).

In most cases, there is no information on the mechanism of the phorbol ester effect on apoptosis. Its analysis is complicated by the following: (i) the pathways triggering apoptosis are multiple and divergent; (ii) patterns of PKC expression differ among different cell types; (iii) the impact of individual isozymes on cellular processes can be different and even opposite; (iv) phorbol esters generally have a biphasic effect on PKC: short-term activation is followed by depletion of phorbol ester-responsive isozymes as a result of protein degradation; (v) different isozymes have different kinetics of down-regulation by phorbol esters; (vi) PKC responses to phorbol esters may depend on the cell type (e.g., the same isozyme is depleted at different paces in different cell lines); and (vii) there may be other receptors for phorbol esters in the cell [e.g., vav (Gulbins et al., 1994), n-chimerin (Hall et al., 1993), etc.].

So far, few studies have addressed the mechanisms of apoptosis modulation by phorbol esters in a manner adequate to the complexity of this system. The crucial role of PKC activation in this process has been demonstrated in a few cases (Milner et al., 1993; Walker et al., 1993; Jarvis et al., 1994). However, the involvement of different isozymes was not investigated, and the role for phorbol ester-mediated down-regulation of certain isozymes, perhaps in combination with activation of others, was not ruled out. In this study, we report a new cell model of PMA- suppressed apoptosis in which sustained PKC activation, but not depletion, participates in this effect, and we show that PKC isozymes α and/or δ are involved. The ability of cells to be rescued is linked to the cell cycle and is associated with PMA-induced abrogation of G1/G0 block imposed by growth factor withdrawal.

MATERIALS AND METHODS

Cell Culture. IL-6-dependent murine PCT cell lines T1165 and T1198 were maintained in RPMI 1640 medium (Whitaker/MA Bioproducts, Walkersville, MD) supple-

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[1] Abbreviations: PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PBS, phosphate-buffered saline; ATP, adenosine 5′-triphosphate; PCT, plasmacytoma; cell line TEPC1165, T1165; cell line, TEPC1198, T1198.

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mented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, 50 μg/mL gentamicin, and 100 PCT growth factor units/mL (10 ng/mL) of recombinant murine IL-6. To withdraw IL-6, cells were centrifuged at 180*g* and washed 3 times with IL-6-free medium. For growth/apoptosis studies, cells were seeded at a concentration of 1×10^5/mL in the same medium. Treatment of T1165 and T1198 cells with PKC activators or inhibitors was initiated either immediately or at 18 (T1165) and 33 h (T1198) after withdrawal of IL-6. These activators and inhibitors were used at concentrations that did not affect cell viability in the presence of IL-6. Cell viability was assessed by trypan blue exclusion (Gibco BRL) or MTT assay (Sigma) at 24 or 48 h after IL-6 deprivation for T1165 and T1198, respectively. To ensure reproducibility, all experiments were repeated independently at least 3 times, and each one was run in triplicate. Means and standard errors of typical experiments are shown in the figures. In a separate series of experiments, we determined that measurements of viability obtained with direct cell counts and MTT assay parallelled each other in the presence and absence of IL-6, PMA, and PKC inhibitors (not shown). The apoptotic morphology was visualized by vital staining of cells with acridine orange at a concentration of 0.1 mg/mL.

PKC Stimulators, Inhibitors. Reagents used in our experiments were from Calbiochem, unless specified otherwise. PKC activators used: PMA and its inactive analog (4-α PMA), (−)-7-octylindolactam V, and thymeleatoxin. PKC inhibitors used: GF109203X and chelerythrine chloride (LC laboratory).

DNA Extraction and Analysis. A total of 5×10^5 cells were harvested, washed once in PBS, and incubated for 4 h at 37 °C in 400 μL of lysis buffer (200 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 μg/mL proteinase K, and 1% SDS) followed by a 1 h treatment with 50 μg/mL RNase A. After phenol−chloroform extraction and ethanol precipitation, the DNA was rehydrated in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). Five micrograms of DNA per lane was electrophoresed in 1.4% agarose gels and then stained with ethidium bromide.

PKC Assay. Whole cell lysates were prepared from 1×10^7 cells. Cells were washed 3 times in PBS, lysed in buffer A (10 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 0.5 mM EGTA, and 1 mM PMSF) containing 0.1% Triton X-100, and sonicated for 10 s. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the PKC substrate (RFARKGSLRQKNV) in the presence of phospholipid vesicles (20% PS/80% PC) at 30 °C for 30 min (Nakadate et al., 1987). One micromolar PMA was used to activate PKC in the assay. This allows evaluation of the level of PMA-responsive isozymes by their activity. Incorporation of ^{32}P into substrate was determined as the difference of the values in the presence and absence of the peptide. Activity values were normalized to the protein concentration determined using the BCA protein assay (Pierce).

Western Blots. For Western blot analysis at time points at which a considerable number of dead cells could be expected, we isolated live cells on a ficol gradient (Sigma, Histopaque- 1077). To prepare cytosolic and particulate fractions, 3×10^7 cells were lysed in 200 μL of buffer A, sonicated for 10 s, and spun at 10^5*g* at 4 °C for 1 h. After aspiration of supernatant (cytosolic fraction), the pellet (particulate fraction) was suspended in 200 μL of buffer A containing 1% Triton X-100. The insoluble material was removed by centrifugation at 500*g* for 5 min. Whole cell lysates were prepared in buffer A containing 1% of Triton X-100. Extracts were kept at 4 °C. Twenty micrograms of protein per lane was electrophoresed in an acrylamide gel (National Diagnostics) and blotted on a nitrocellulose membrane. Western blots were probed with polyclonal antibodies against PKC-α, -β, -γ, -δ, -ϵ, and -ζ (Life Technologies BRL, Inc.), PKC-η (Santa Cruz), and PKC-δ (R&D) and monoclonal antibodies against PKC-α, -δ, -μ, -λ and -θ (Transduction Lab.). Cross-reactivity of all isozyme-specific PKC antibodies was controlled in a separate series of experiments using cell lines ectopically expressing different isozymes (not shown). Goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Life Technologies BRL, Inc.) were used as secondary antibodies. The immunoreactive bands were visualized on X-ray film using a chemiluminescent substrate (Kirkegaard and Perry Lab.).

Flow Cytometry. For cell cycle experiments, cells were kept in the log phase of growth for at least 3 days. The percentage of cells in each phase of the cell cycle was determined by flow cytometry using propidium iodine-stained cells. A total of 1×10^6 cells were washed with PBS, and fixed in 0.25% paraformaldehyde for 1 h at 4 °C. Fixed cells were permeabilized in PBS containing 0.05% Tween 20 for 15 min at 37 °C. This was followed by simultaneous treatment with 500 μL of RNase (200 units/mL) and 500 μL of propidium iodine (0.01% w/v propidium iodine, 0.1% v/v Triton X-100, and 0.0037% w/v EDTA) for 30 min at 37 °C. Cell cycle distributions were calculated from histograms of 5×10^4 cells using CellFit software, and data of cell cycle distribution were analyzed using the program RFit (Becton Dickinson Immunocytometry Systems).

RESULTS

Phorbol Ester Treatment Suppresses Apoptosis in IL-6-Deprived PCT Cells. It was shown earlier that the murine PCT cell lines T1165 and T1198 are characterized by a different stringency of IL-6 control over their growth and survival (Neckers & Nordan, 1988). After withdrawal of IL-6, T1165 and T1198 cells die with viability decreasing to 25% within 1 or 2 days, respectively. In the present work, using phase contrast microscopy and vital staining, we found that the typical morphological features of apoptosis accompany cell death in both lines. Cells lost volume, the plasma membrane became ruffled, the chromatin collapsed into crescents along the nuclear envelope, the nucleus broke up into spheres, and, finally, cells were fragmented into apoptotic bodies (not shown). DNA isolated from IL-6-starved PCT cells had typical apoptotic ladders with fragments in multiples of 180−200 base pairs in length, whereas cells maintained on IL-6 yielded intact high molecular weight DNA (Figure 1A).

Addition of PMA to the IL-6-deprived cells markedly attenuated cell death in T1165 (Figure 1A,B) and T1198 (not shown) cells. Analysis of DNA fragmentation showed that IL-6-withdrawn T1165 cells cultured with PMA preserved intact high molecular weight DNA much longer than in the absence of PMA (Figure 1A).

PMA Treatment Abrogates Cell Cycle Arrest Induced by IL-6 Deprivation. Previous results (Neckers & Nordan, 1988) indicated that the cell death in IL-6-deprived T1165

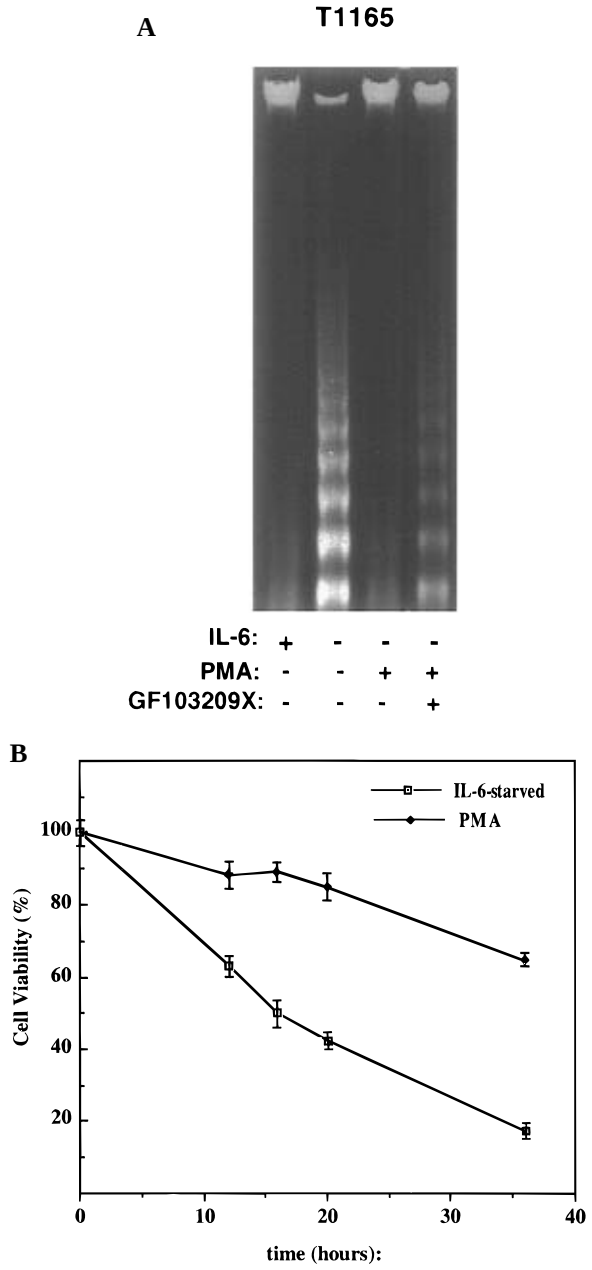

FIGURE 1: Effect of PMA on apoptotic cell death induced by removal of IL-6 from PCT cell lines. Immediately after IL-6 withdrawal, T1165 and/or T1198 cells were incubated in IL-6-free medium or medium with addition of 100 units/mL IL-6, 100 nM PMA, or 1 μM GF109203X in different combinations. (A) DNA degradation patterns induced in T1165 cells by incubation in IL-6-free medium or medium with addition of IL-6, PMA, or PMA and GF109203X simultaneously. It can be seen that DNA degradation is maximal in IL-6-starved cells, is diminished as a result of PMA treatment, and is partially restored if PKC inhibitors are added along with PMA. Also note the differences in the amount of undegraded DNA. (B) Viability of T1165 cells with and without PMA treatment at different times after IL-6 withdrawal.

and T1198 cells is preceded by accumulation of cells in a G0/G1 state, which reaches a maximum at 18 and 33 h, respectively. Readdition of IL-6 at these time points results in a synchronous proceeding of cells into S phase. In the absence of growth factor, cell cycle arrest is followed by massive cell death. Using flow cytometry, we have studied in greater detail how PMA affects cell cycle arrest induced by IL-6 deprivation (Table 1). T1165 cells growing in the presence of IL-6 had the following distribution in cell cycle: 37% in G0/G1, 60% in S phase, and 3.4% in G2/M. Upon the withdrawal of IL-6, cells accumulated in the G0/G1 state with a maximum at 18 h. Elevation of cell numbers in G0/G1 up to 56% was accompanied by the reduction of the S phase of cell cycle to 35%. Treatment of T1165 cells with PMA for the entire period of IL-6 withdrawal lifts the G0/G1 cell block and induces repopulation of S and G2/M stages of cell cycle (Table 1). These changes basically reverse the perturbation in cell cycle distribution induced by IL-6 withdrawal. Thus, abrogation of the G0/G1 block is associated with enhanced cell survival.

Table 1: Effect of IL-6 Withdrawal and PMA Treatment on Cell Cycle Distribution of T1165 Cells

sample	G0/G1 (%)	S (%)	G2/M (%)
+IL-6	37	60	3.4
no IL-6 for 18 h	56	35	9.4
no IL-6 for 18 h, 100 nM PMA for 18 h	36	53	11

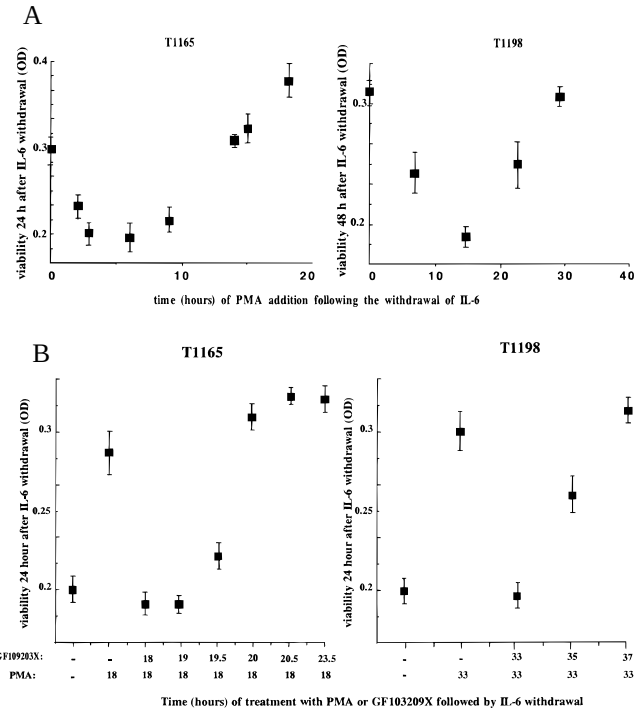

FIGURE 2: (A) Effect of delayed PMA addition on cell death of IL-6-starved T1165 and T1198 cells. PMA treatment (100 nM) was initiated at different times after withdrawing cells from IL-6. (B) Time-dependent effect of GF109203X on PMA-induced suppression of cell death. T1165 and T1198 cells were treated with 100 nM PMA at 18 or 30 h after removal of IL-6. At various times after addition of PMA, 1 μM GF109203X was added to the culture. Cell viability was determined by MTT assay at 24 and 48 h after IL-6 withdrawal for T1165 and T1198 cells, respectively.

Death Suppression by PMA Is Time-Dependent. When PMA treatment was initiated at different times after IL-6 withdrawal, the resulting viability curve was biphasic (Figure 2A). The suppression of cell death by PMA added immediately after IL-6 withdrawal was followed by a period when addition of PMA produced little or no rescue. At later times the treatment became effective again, with death suppression reaching its maximum with addition of PMA at 18 and 33 h after IL-6 withdrawal for T1165 and T1198, respectively. These times roughly coincide with the times of maximal accumulation of cells in G0/G1 block (Neckers & Nordan, 1988). Thus, IL-6-deprived cells could be rescued not at any time, but only when they are in a "competent" state which is achieved at two distinct time points after IL-6 withdrawal.

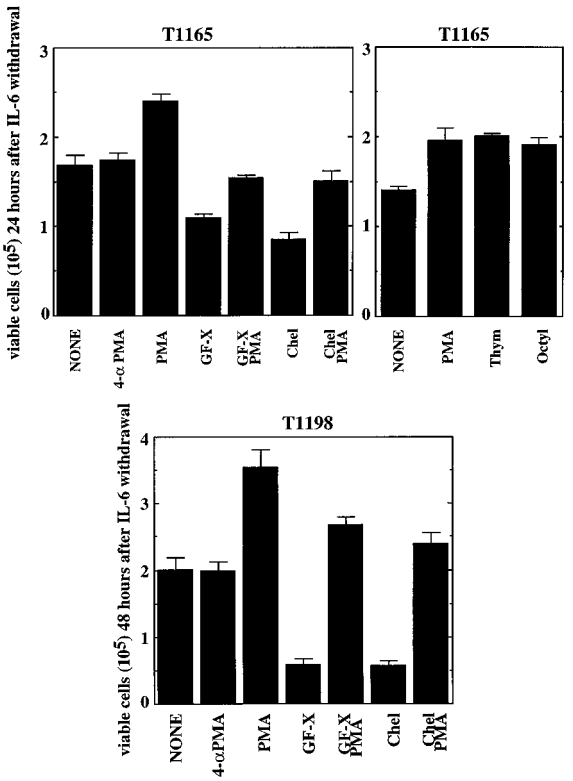

FIGURE 3: Effect of PKC activators and inhibitors on the viability of IL-6-starved PCT cells. Immediately after IL-6 withdrawal, cells were treated with PKC activators: 100 nM PMA, 1 μM thymeleatoxin (Thym), 1 μM (−)-7-octylindolactam V (Octyl); with PKC inhibitors: 1 μM GF109203X (GF-X), 1 μM chelerythrine chloride (Chel); or with combinations of PMA and inhibitors. The inactive PMA analog 4α-PMA was included as a control for PMA. Cells were counted using the trypan blue exclusion method. The data represent a typical result from one of three identical experiments.

Effect of PMA Is Mediated by PKC Activity. As PKC is the major cellular target of phorbol esters, we have evaluated its contribution to the suppression of cell death in IL-6-deprived PCT cells. Along with PMA, we used two PKC-specific activators, (−)-7-octylindolactam V and thymeleatoxin, and two PKC inhibitors, GF109203X and chelerythrine chloride. The inactive PMA analog, 4α-PMA, was included as a control for the PMA effect. Treatment with PKC activators following the IL-6 withdrawal typically results in a 40−80% increase in survival of T1165 and T1198 cells as compared to untreated cells. PKC inhibitors have the opposite effect and counteract activators if added simultaneously (Figure 1A, Figure 3). The inactive analog of PMA, 4-α PMA, failed to suppress apoptosis in this model. Thus, PMA-induced suppression of cell death in PCT cell lines requires active PKC.

PKC-Mediated Death Suppression Is Transient. It is possible that the "competent" state, wherein cells can be rescued from apoptosis by PKC activation, is conferred on the cells by a regulated (e.g., cell-cycle-dependent) expression of the crucial PKC isoenzymes. However, the same pattern of PKC isozyme expression is found in cells at all times after IL-6 withdrawal, and at all times, responsive isozyme(s) can be activated by PMA (data not shown; typical Western pattern is seen in lane 1, Figure 4). The competent state, therefore, is not determined by alterations in PKC expression, but by some other properties of the cells. The PKC activity generated in "non-competent" time points is transient (Figure 2A) and does not persist long enough to rescue cells when the next period of competence is reached. Thus, a short

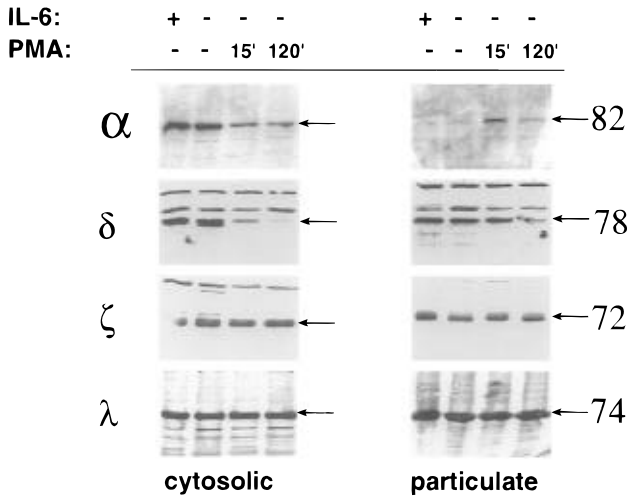

FIGURE 4: Western blot analysis of PKC modulation by PMA following IL-6 withdrawal from T1165 cells. Cytosolic and membrane-bound fractions of PKC were prepared from cells maintained without IL-6 for 18 h and after treatment of these cells with 100 nM PMA for 15 or 120 min. Cells growing in the presence of IL-6 were used as a control. Exposure time with the chemiluminescent substrate was 3 min for blots developed with α, δ, ζ or λ antiserum and 3 h for the others. Bands that represent the PKC isozymes are indicated by arrows.

Table 2: Activity of PKC Induced by Prolonged Treatment of T1165 and T1198 Cells with PMA (×1000 cpm/μg of Protein)[a]

		culture treatments		
cell lines	assay	none	PMA	GF109203X
T1165	PMA	750	96	125
	none	32	91	122
T1198	PMA	476	89	43
	none	144	26	47

[a] Immediately after IL-6 deprivation, T1165 and T1198 cells were treated either with 100 nM PMA or with 1 μM GF109203X for 10 and 15 h, respectively. PMA-dependent PKC activity was determined by performing the assay in the presence or absence of 1 μM PMA as described under Materials and Methods. Results are the average of three replicates from a typical experiment (SEM ≤ 10%).

period of PKC activation is probably followed by PMA-induced down-regulation.

We have tested this hypothesis using a direct kinase assay and have shown that in the absence of IL-6 PMA caused almost complete depletion of PMA-dependent PKC activity within 10−15 h. The extent of PKC down-regulation in these experiments is comparable to the PKC inhibition in cells treated for the same period of time with PKC inhibitor GF109203X (Table 2). Thus, PKC activity responsible for apoptosis suppression is being depleted by long-term PMA treatment. It is likely that effective death suppression occurs only when the transient PKC activation overlaps with the "competent" state.

PKC Activity for an Extended Period of Time after PMA Treatment Is Essential for Suppression of Cell Death. To determine the interval when PKC activity is essential for attenuation of apoptosis, we treated T1165 and T1198 cells with PMA at 18 or 33 h, respectively, after they have been withdrawn from IL-6, and then performed a time course of initiation of PKC inhibitor treatment. GF109203X abolished the effect of PMA only during the initial few hours after PMA stimulation—about 2 h for T1165 and 5 h for T1198 (Figure 2B), indicating that PKC activity is crucial only for this period of time. Thus, although PMA activates PKC very

rapidly, maximal PMA-induced suppression of apoptosis requires extended PKC activation. However, this time is limited, which could reflect either the length of the "competent" state or the eventual depletion of PKC by PMA.

Effect of PMA on PKC Isozymes in PCT Cell Lines. Using Western blot analysis, we checked the expression of 11 PKC isozymes (PKC -α , -βI, -βII, -γ, -δ, -ϵ , -η, -θ , -μ, -ζ, and -λ) in plasmacytoma cells. PKC -α, -δ, -μ, -ζ, and -λ are expressed abundantly in T1165 and T1198 PCT cells, traces of PKC-βII, -γ, and -ϵ isoforms were seen on some blots, and PKC-βI, -η, and -θ were absent. A portion of PKC-δ, -μ, -ζ, and -λ is present in the membrane even in the absence of PMA treatment, presumably due to "constitutive" activation in these cells. There was no significant difference in PKC isozyme expression and membrane localization that could be attributed to the absence or presence of IL-6. Selected data from T1165 are shown in Figure 4.

To assess the effect of PMA on PKC isozymes in PCT cells, we treated IL-6-starved T1165 cells with PMA 18 h after IL-6 withdrawal and studied the kinetics of PKC response over the next 2 h (Figure 4). Treatment with PMA induces the redistribution of PKC-α and -δ within 15 min. This was followed by their down-regulation after 2 h treatment. The response of PKC-μ to PMA could be seen only after 2 h treatment. This is consistent with the previous reports that PKC-μ has a very limited, if any, response to PMA (Johannes et al., 1994). PKC -ζ and -λ, as expected, were not affected by PMA treatment. Thus, the two isozymes apparently responding to PMA were α and δ.

PKC Down-Regulation Does Not Promote the Survival of IL-6-Starved PCT Cells. We considered two models of PKC response to phorbol esters to explain the apoptosis suppression. In the first, apoptosis delay is a result of activation of phorbol ester-responsive isozymes. In the second, "rescuing" PKC activity can reveal itself only if accompanied by the down-regulation of another "counteracting" PKC isozyme. In this case, the rescuing activity would not necessarily need to be PMA-inducible but could be constitutively active, and the role of PMA would be only to deplete the counteracting isoform(s) (see Discussion).

To choose between the proposed models, the following experiments were made. We evaluated the effect of complete depletion of all PKC-responsive isozymes on PMA-induced suppression of apoptosis. In the presence of IL-6, there were no obvious differences in the dose- and time-dependent effect of PMA on PKC-α and -δ in the two PCT lines. Pretreatment with 500 nM PMA for 48 h in the presence of IL-6 resulted in complete PKC depletion in both cell lines as measured by Western blots (Figure 5A). After such pretreatment, T1165 cells were deprived of IL-6 and treated with 100 nM PMA either immediately (not shown) or after 18 h. Neither of the treatments suppressed apoptosis (Figure 5B). These results show that complete depletion of phorbol ester-responsive isozymes is not responsible for attenuation of apoptosis and demonstrate that the crucial activity required for apoptosis delay is, in fact, depletable.

To find out if PKC down-regulation has any role at all in death suppression, we used short-term PMA treatment to avoid PMA-induced PKC depletion. T1165 cells were treated with PMA for 15 or 45 min immediately after or 18 h after IL-6 withdrawal. Similar cell death suppression was obtained in both experiments. Data from the 18 h experiment are shown in Figure 5C. A 15-min PMA treatment yielded a 30% increase in viability compared to the control, whereas

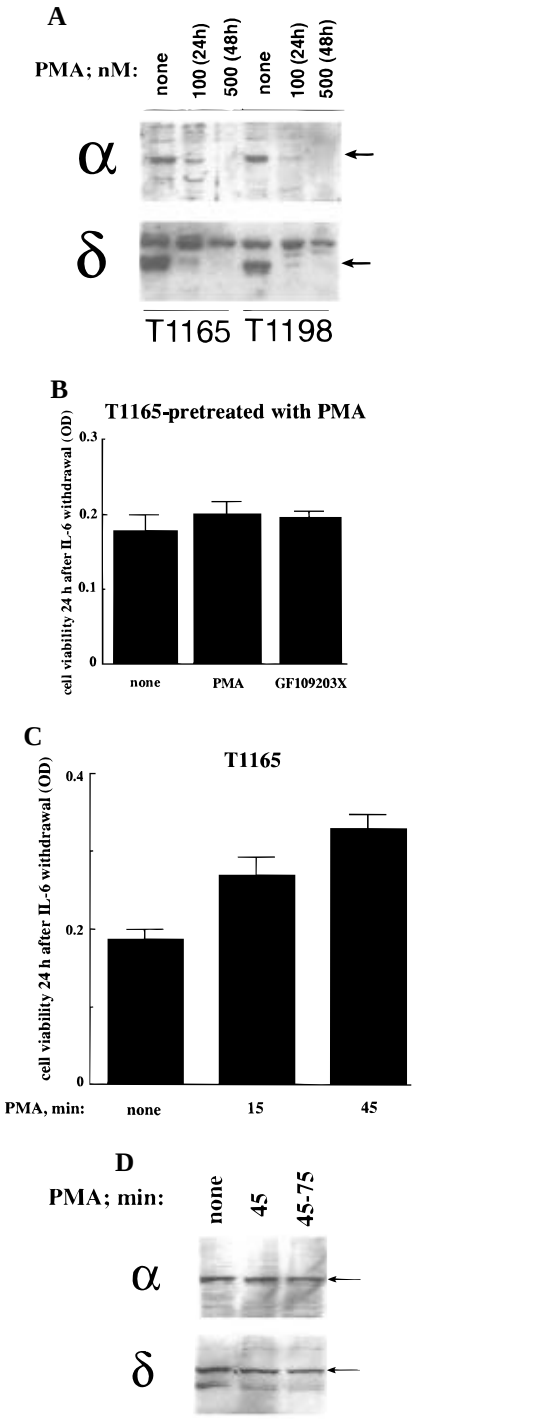

FIGURE 5: Effect of PKC modulation by PMA on cell death in IL-6-withdrawn PCT cells. (A) Western blot analysis of whole cell lysates prepared from T1165 and T1198 cells treated with 100 and 500 nM PMA for 24 and 48 h in the presence of IL-6. (B) T1165 cells were cultured with 500 nM PMA and IL-6 for 48 h. IL-6 and PMA were then withdrawn, and 18 h later, the cells were treated with either 100 nM PMA or 1 μM GF109203X. Cell viability was determined by MTT assay 24 h after withdrawal of IL-6. It can be seen that no death suppression occurs on PMA-pretreated cells. (C) After withdrawal of IL-6, T1165 cells were kept on plain medium or were treated at 18 h with 100 nM PMA for 15 and 45 min. PMA was removed by washing cells 3 times with large volumes of medium without IL-6. Twenty-four hours after removal of IL-6, cell viability was determined by MTT assay. (D) Western blot analysis of whole cell lysates prepared from T1165 cells withdrawn from IL-6 for 18 h and treated acutely with PMA. Lysates were prepared immediately after a 45 min treatment with 100 nM PMA or 75 min after removal of PMA.

the 45-min treatment resulted in a 70% increase in viability. Western blot analysis revealed no obvious down-regulation

of phorbol ester-responsive isozymes neither immediately after the 45-min treatment nor within the following 75 min in which cells were maintained without PMA. The data for PKC-α and -δ are shown in Figure 5D. Thus, attenuation of apoptosis occurs even in the absence of PKC depletion.

DISCUSSION

Model Used. We used the model of IL-6-dependent mouse PCT cells that constitutively overexpress c-myc as a result of chromosomal translocation. Unlike other B-cell tumors and normal B-cells (Shibuya et al., 1992), PCTs are unable to down-regulate their c-myc when deprived of IL-6 (our unpublished observation). Withdrawal of IL-6 from PCT cells causes apoptotic cell death that can be suppressed by phorbol ester treatment. These features display a striking resemblance to a fibroblast system employed in the studies of myc-mediated apoptosis (Evan et al., 1992; Harrington et al., 1994; Hermeking & Eick, 1994; Goruppi et al., 1994). In these works, c-myc was constitutively overexpressed in rodent fibroblast cell lines, and their susceptibility to apoptosis, triggered by serum deprivation, was studied. Cell death was manifested only in the presence of functionally-active overexpressed c-myc, and the rate of apoptosis was proportional to the level of c-myc expression. Addition of purified growth factors or phorbol esters suppressed the death. It was determined in this system that in the absence of serum or growth factors normal fibroblasts rapidly down-regulated their endogenous myc, withdrew from the cell cycle accumulating in a G0/G1 block, but did not die. It is conceivable that myc plays an apoptosis-promoting role in tumors that have unregulated overexpressed myc, e.g., murine PCTs and human Burkitt lymphomas. In fact, it has been shown in Burkitt lymphomas that apoptosis induced by ionomycin treatment or aggregation of surface immunoglobulin is myc-driven (Milner et al., 1993). Among the PCT cell lines used in the current study, T1165, which dies most rapidly after IL-6 withdrawal, expresses an unusually high level of c-myc RNA (Bauer, et al., 1989).

Apoptosis Delay in PCT Cells Is PKC-Mediated. To determine whether suppression of apoptosis by PMA is PKC-mediated, we blocked the effect of PMA with the PKC inhibitors GF109203X and chelerythrine chloride. These chemicals express specificity for different PKC domains and are highly selective for PKC. GF109203X is competitive with respect to ATP, and it had been shown to be a potent inhibitor of all PMA-responsive PKC isozymes (Toullec et al., 1991). Chelerythrine chloride inhibits the catalytic PKC domain, showing competitive kinetics with substrate, and it is much more selective for PKC than for other kinases including tyrosine kinases, cAMP-dependent kinase, and Ca/calmodulin-dependent kinase (Herbert et al., 1990). In our experiments, both PKC inhibitors counteracted the effect of PMA and increased the rate of cell death after IL-6 withdrawal, indicating that suppression of cell death by PMA is PKC-mediated and caused by the activity of one or more PKC isozyme(s).

Depletion versus Activation. Since PKC inhibitors counteract the effect of PMA in PCT apoptosis, it must be that activation rather than depletion/down-regulation of PKC is responsible for the PMA-induced suppression of cell death. However, since the kinetics of PKC down-regulation in response to phorbol esters vary for different isozymes (Mischak et al., 1993; Roivainen et al., 1993), it is possible that the PMA-induced activation of one isozyme could persist after another has been depleted. Thus, one must consider alternative hypotheses such as (i) death suppression by PMA involves activation of one PKC isoform, which would be manifested only after a counteracting isoform is depleted, or (ii) the rescuing activity of PKC is constitutive and PMA-independent, and the sole role for PMA is to deplete a counteracting enzyme. In the latter case, total depletion of PMA-responsive isozymes would result in death suppression. In fact, examples where different isoenzymes have opposite effects on cell growth have been described (Mischak et al., 1993). We evaluated the possible role of PKC depletion in cell death suppression in two ways. In the first approach, the effect of total PKC depletion on the suppression of apoptosis was examined using extended pretreatment of cells with a high dose of phorbol ester. This treatment depleted all phorbol ester-responsive isozymes, and it also effectively abolished all the effects of PKC activators and inhibitors on cell survival. This result indicates that depletion of PKC alone is not responsible for the suppression of apoptosis and, in fact, results in loss of cell rescue. In the second approach, the effect of activation without depletion was examined. Short-term, 15- and 45-min treatments with PMA followed by washout also led to apoptosis suppression, with 45 min being the more effective. No significant down-regulation of depletable isozymes occurred either immediately after the treatment or within the following 75 min, when the cells were maintained without activator. Thus, death suppression can take place in the absence of PKC depletion. Additionally, it is clear that the isozymes which respond to PMA with membrane/cytosol redistribution within 15 min (PKC-α and -δ) are the probable candidates for the rescuing activity.

Our experiments in which PMA treatment was followed by a PKC inhibitor administered at different times additionally demonstrate that prolonged (approximately 2 h for T1165), rather than acute, activation of PKC is required to achieve maximal death suppression. This conclusion is supported by the greater effectiveness of the 45-min vs 15-min short-term PMA treatment. Although in vitro experiments indicate that PKC activation by PMA results in irreversible translocation to the membrane (Nelsestuen & Bazzi, 1991), recent studies (Szallasi et al., 1994) demonstrate that after removal from the medium, PMA leaves the cell with a half-time of 9 min, followed by retranslocation of PKC back to the cytoplasm. As concentrations of PMA between 10 and 100 nM are effective in suppression of cell death, it follows that residual activation after PMA removal would last at least 30 min, which along with the 45-min treatment gives us a time comparable to that required for maximal rescue (2 h). These results support our conclusion that a prolonged PKC activation, without depletion, is responsible for cell rescue.

PMA Effect Is Linked to the Cell Cycle. We observed two "competent" points after IL-6 withdrawal when cells could be rescued by PMA: one immediately after the growth factor was removed, and the other, at the point of accumulation of cells in G0/G1, immediately preceding the onset of massive cell death. It is unclear whether a single mechanism drives apoptosis suppression at both points. One possibility is that at the first competent point, immediately after withdrawal of the cytokine, PKC activation prolongs the effect of a declining IL-6 signal, perhaps by slowing the dephosphorylation of components of the IL-6 signaling cascade. Alternatively, the ability of cells to be rescued by

PMA at both competent points may coincide with their presence at a critical interval within G1. Hence, at the first point, PMA would rescue those cells that in an asynchronous population are caught in the competent position within G1, and, at the second point, it rescues those G1/G0-arrested cells that are in the same competent position. In this case, a single mechanism operates at both competent points; however, one has to explain why the number of competent cells drops at intermediate time points and then rises again. Our previous data show that the dynamics of the whole G1 population do not follow this biphasic survival curve. Neckers and Nordan (1988) found that the total number of cells in G1 remains constant after IL-6 withdrawal and then grows steadily within a few hours up to the time of maximal accumulation at the G1/G0 restriction point and the onset of cell death. They have mapped this restriction point in early G1. We hypothesize that the cells which pass this restriction point in the presence of IL-6 will continue to progress through the rest of G1 and the later parts of the cell cycle with a normal speed, whereas the cells which pass it in the absence of the growth factor will still continue to progress, but at a much slower pace. In this case, the cells will accumulate in a dense group at the restriction point, and there will be a drop in cell numbers beyond it. Later this dense group will slowly migrate into the empty area behind the G1 restriction point and the number of cells there will rise again, generating a byphasic curve. Thus, under the above hypothesis, the ability of cells to be rescued will be identified with their presence in the interval in mid/late G1 behind the early G1 restriction point. However, to test the correctness of this explanation, further studies with molecular markers of various intervals within G1 are needed.

Finally, two scenarios could be proposed to explain the cell-cycle-related action of phorbol ester. In the first one, activation of PKC could slow down or arrest the cells, so that they cannot reach the point where the death program is initiated. Alternatively, it could allow cells to pass this point or a number of such points safely, thus allowing a delay of cell death. We have addressed this question by analyzing the effect of PMA on the cell cycle arrest induced by IL-6 deprivation. Using flow cytometry analysis, we found that PKC activation abrogates G0/G1 arrest, causing repopulation of S and G2/M phases of the cell cycle. This is corroborated by an increase in the number of cells incorporating thymidine (S phase cells) after PMA treatment. This increase is abrogated by PKC inhibition (not shown). Thus, the action of PMA appears to fit the second scenario. This finding clarifies why G0/G1 would be a competent point for rescuing, as this is a position where cells are ready to be moved by phorbol ester through the first cell death "restriction" point, presumably at the G1/S border or in early S phase. By this action, PMA is apparently mimicking some aspect of IL-6 signaling, rather than just "freezing" the death pathway.

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