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Phorbol Ester Synergizes the Dimethyl Sulfoxide–Dependent Programmed Cell Death Through Diacylglycerol Increment

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ABSTRACT: The regulation of cell proliferation or cell death by extracellular factors are the most intensely studied subjects in cell biology. Many conceptual problems remain to be clarified concerning the mechanisms that regulate the programmed cell death. In this work, we focus our attention on the possible role of protein kinase C activation during dimethyl sulfoxide (DMSO)–induced cell death. The present results suggest that the frequency of DMSO-dependent apoptosis of RPMI 8402 thymic lymphoma cells is increased by phorbol ester acetate supplementation. Enhancement of apoptosis can be abolished by cotreatment with the bisindolylmaleimide, a specific PKC inhibitor. The association between PMA and DMSO treatment provokes an early activation of an intracellular signaling mechanism that results, via sustained diacylglycerol elevation, in a possible long-term PKC activation.

KEY WORDS: apoptosis, diacylglycerol, dimethyl sulfoxide, phorbol ester acetate.

I. INTRODUCTION

Apoptosis or programmed cell death (PCD) is a specific form of active cell death characterized by shrinkage of the cell, by dramatic reorganization of the cell nucleus, and, finally, in the fragmentation of the cell into apoptotic bodies. Apoptosis occurs in two physiological stages. In response to a signal, which may be intrinsic or extrinsic, the cells enter an early phase, which is specific for a particular cell type. Afterward, the cells proceed to an execution phase, characterized by a well-recognized pattern of morphological and biochemical changes, a pattern that is generic for different cell types.[1,2] Thus, the process of activating PCD might be exclusive for specific cells, but, once triggered, PCD proceeds by a common mechanism. Eukaryotic cells use a variety of evolutionarily conserved intracellular signaling pathways to respond to changes in their external environment. Among these pathways, those that activate the programmed cell death have received particular attention. The classical *in vitro* system for inducing the apoptosis is to expose the cells to different biochemical and pharmacological agents that appear to trigger the mechanisms inducing the programmed cell death.[2–5] Dimethyl sulfoxide (DMSO) is described as an inducer of differentiation in several cell lines with the ability to arrest cell growth at the G0/G1 phase of the cell cycle.[6–12] In some experimental models, DMSO provokes the PCD, while in others, such as human B cell lines, DMSO can prevent the apoptotic phenomenon.[13–15] Phorbol esters are tumor promoters that bind to and constitutively activate protein kinase C (PKC) and can induce the differentiation of leukemic and normal human hematic cells.[16–18] It was first reported that PMA stimulates phosphatidylcholine (PC) hydrolysis, producing a species of diacylglycerol (DAG) that are effective activators of PKC.[19–21] DAG may exert differential effects on the various isoforms of protein kinase.[22] Previously, through the use of

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different experimental models, phorbol miristate acetate (PMA) has been shown to be antiapoptotic, antagonizing the ceramide-mediated apoptosis.[19,23] In our foregoing work we showed that DMSO, in human RPMI-8402 pre-T leukemic cells, induces a reversible G1 arrest in the cell cycle, followed by apoptotic phenomenon.[13] Therefore, to gain more-detailed information on the potential involvement of lipid second messenger mediates signal transduction in determining and controlling the PCD, we have co-stimulated RPMI 8402 cells with PMA and DMSO.

II. MATERIALS AND METHODS

Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, VA). All materials for cell cultures and biochemical reagents were analytical grade materials from Sigma (St. Louis, MO). Radionucleotides were from Amersham (Milano, Italy).

A. Cell Culture

The human RPMI 8402 cell line described by Huang et al.[24] is a thymic lymphoma showing immunological features of pre-T cells. Cells were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% FBS, 4 m*M* L-glutamine, 100 m*M* NaPyruvate, and 25 m*M* HEPES. Cells were grown at 2.5×10^5/ml, with more than 98% viability as determined by trypan blue exclusion test. During the log growth phase, in order to induce PCD the cells were treated with 1.5% (v/v) of DMSO (gas chromatography grade) with or without 16 n*M* PMA, for modulation of the PKC. Bisindolylmaleimide hydrochloride, known as staurosporine-analog PKC inhibitor, has previously been added to culture medium at a final concentration of 50 μM.[25,26] All treatments have been performed for times ranging from zero to 72 h.

B. Apoptosis Estimation

Apoptosis was established by morphological examinations, at light and electron microscope levels, by DNA laddering assessment, and by flow cytometry as previously described.[13,27]

C. Cell-Cycle Distribution

The measurement of nuclear DNA content was carried out using a CycleTEST PLUS DNA Reagent Kit as described by the manufacturer (Becton Dickinson, Mountain View, CA) with analysis on a FACStar^{plus} flow cytometer (Becton Dickinson). The apoptotic peak is definite as the peak that occurs in channel number lower than G0 as described by others.[28]

D. Lipid Metabolism

Untreated and treated cells were labeled with [^{3}H]Glycerol (10 μCi/ml/1 $\times$ 10^6 cells) for 90 min, which is a labeling time shorter than the half-life of the major phospholipids, to focus attention on newly synthesized material.[29] Cells were precipitated with 10% TCA, and then 10 volumes of chloroform/methanol/concentrated HCl (300/300/1.5) were added and lipids extracted for 20 h at 4°C. After centrifugation, the supernatants were preserved and the pellets were re-extracted twice with 10 volumes of chloroform/methanol/concentrated HCl (400/200/1.5). The combined supernatants were dried under nitrogen, and lipids were dissolved in 100 μl of chloroform and washed three times in four volumes of chloroform/methanol/water (3/48/47). Finally, the samples were dried under a stream of nitrogen and [^{3}H]-labeled lipids were analyzed by TLC on silica gel 60 plates and developed with ether/exane/NH_4 (50/50/0.25). TCL plates were sprayed with EN-hancer (Du Pont, NEN, Germany) and fluorographed at −80°C. Spots corresponding to internal lipid standards were scraped off, extracted with 1.5 ml of 0.6 *N* HCl-methanol (60/40 by volume) for 48 h with gentle stirring and counted using a liquid scintillation counter using 5 ml of Packard Pico-Fluor 40 scintillation cocktail.

III. RESULTS

Apoptosis estimation was evaluated as previously described.[13] DNA fragmentation, morphological changes, and cell growth arrest show the specific apoptotic activity of DMSO in the human pre-T RPMI 8402 cell line. If 16 n*M* PMA is added to DMSO medium culture, a stimulated increase of cell death can be obtained. Morphological analysis (Figure 1) shows the dramatic effect of DMSO/PMA treatment.

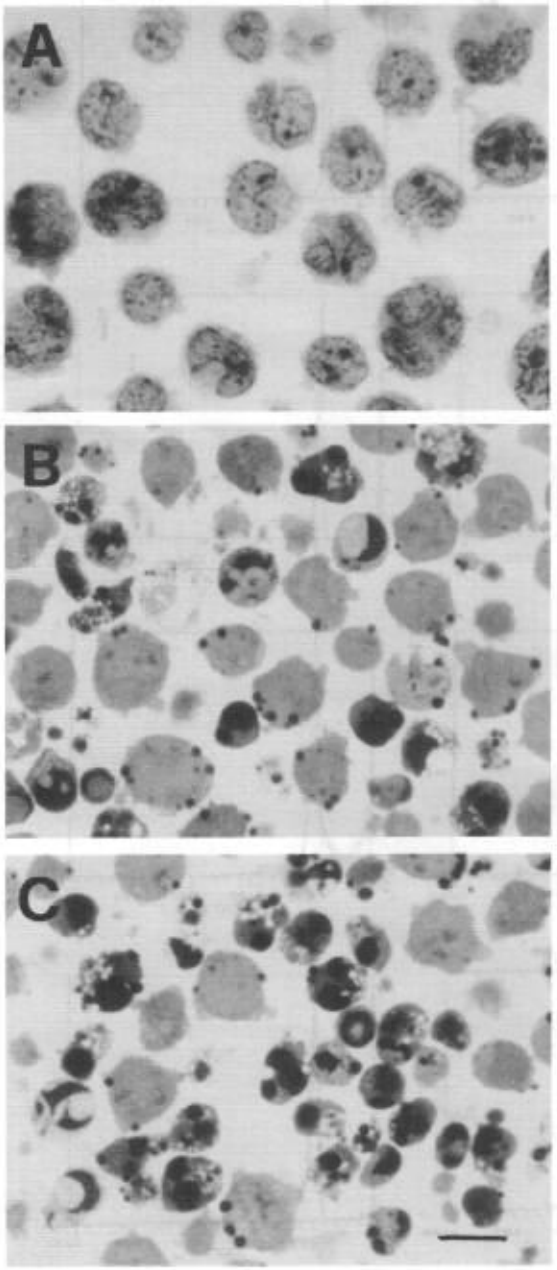

FIGURE 1. Morphological analysis, carried out by light microscopy, of untreated and treated RPMI 8402 cells performed as described in Materials and Methods. (A) The untreated sample; (B) the samples treated with 1.5% DMSO alone; (C) the DMSO/PMA-cotreated cells. In Panels B and C it is possible to identify the presence of typical features of programmed cell death with an increase of cell death in the cotreated sample. Bar, 15 μm.

As reported, a significant increase of apoptotic cells in co-stimulated cells, compared with the DMSO-treated sample, is detectable. Moreover, in DMSO-treated cells the first apoptotis can be morphologically visualized at 48 h of treatment, and after 72 h (Figure 1B) more than 60% of cells are dead by apoptosis. If the DMSO culture medium has been supplemented with 16 n*M* of PMA, increased and accelerated cell death occurs. In fact, the first apoptotic cells can be observed at 24 h of treatment, and at 72 h (Figure 1C) more than 90% of the cells are dead. Analogous to morphological results are the data recognizable by DNA laddering gel (Figure 2). Flow cytometry analyses were carried out to assess the effect of DMSO and DMSO/PMA treatment on cell synchrony and apoptosis (Figures 3 and 4). Figure 3, Panels a, d, and g, shows that in DMSO-treated samples the hypodiploid peak, indicative of apoptosis, can be visualized at 72 h of treatment.

DMSO/PMA treatment induces an increase of cell death, and this phenomenon occurs early in the treatment. In fact, it is possible to recognize the specific apoptotic peak at 48 h of stimulation (Figure 3 b, e, and h). Bisindolylmaleimide, if supplementing DMSO/PMA, provokes the disappearance of PMA-positive effect (Figure 3 c, f, and i). Untreated RPMI

FIGURE 2. Evaluation of programmed cell death by biochemical assay. The characteristic ladder-like pattern of DNA banding indicative of apoptosis is evident in treated samples with an increase of DNA fragmentation in DMSO/PMA-stimulated cells. (A) DMSO-treated cells; (B) DMSO/PMA-treated cells; (C) untreated cells.

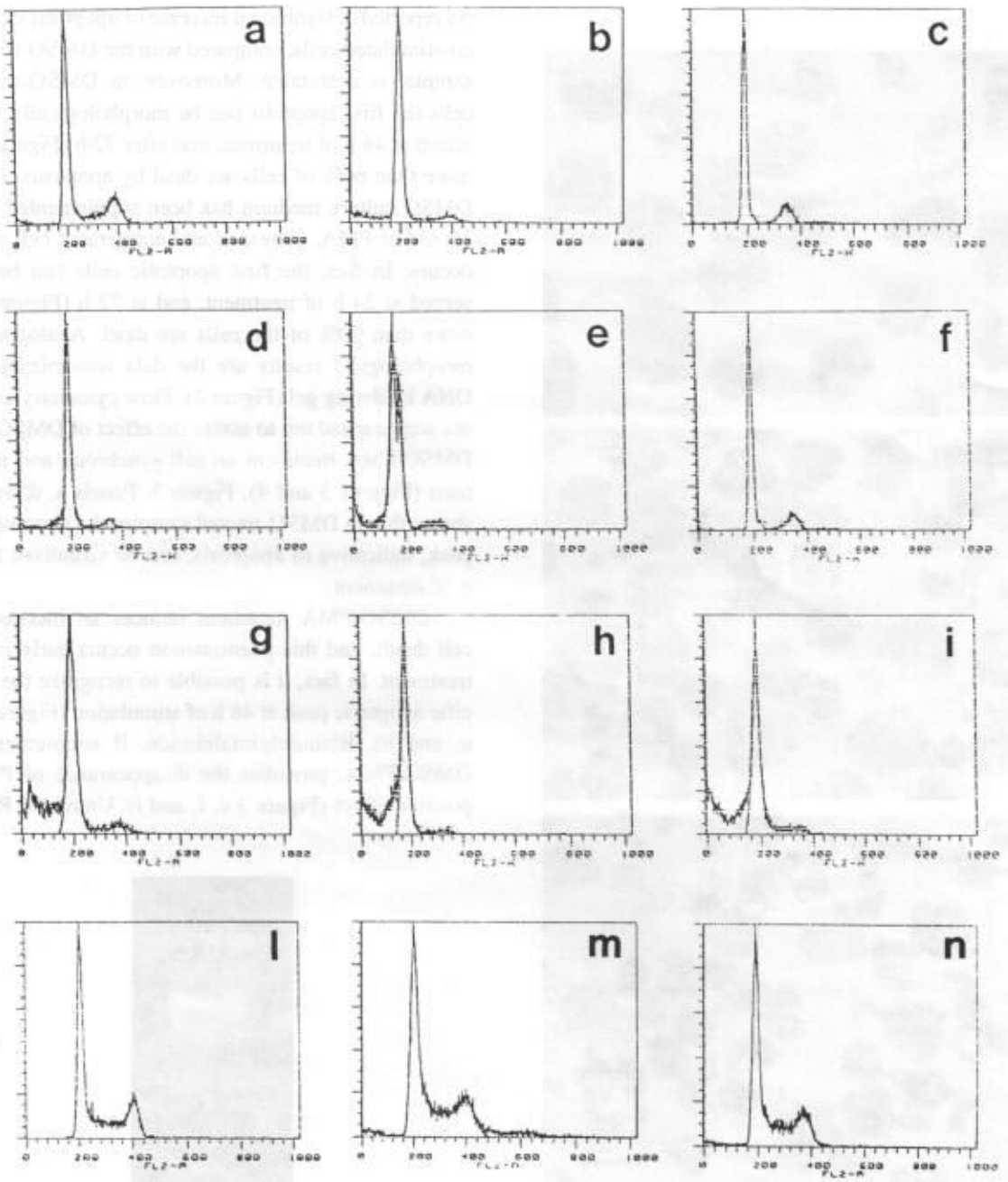

FIGURE 3. Flow cytometry analysis of DMSO-treated samples (a, d, g); DMSO/PMA-costimulated cells (b, e, h); and DMSO/PMA/Bis (bisindolylmaleimide)-treated cells (c, f, i). (a–c) Results obtained at 24 h of treatment; (d–f) results obtained at 48 h; and (g–i) results at 72 h. It is possible to note the forward presence of the peak indicative of apoptosis in DMSO/PMA-treated samples (b, e, h). This effect can be eliminated by bisindolylmaleimide supplementation (c, f, i). Untreated samples, reported in Panels l (24 h), m (48 h), and n (72 h), do not show the presence of apoptotic cells.

8402 cells do not display the presence of spontaneous apoptotis (Figure 3 l, m, and n) or a decrease of cell growth during the time of observation. Cell-cycle distribution of treated or untreated cells, obtained as described above, is reported in Figure 4. Analysis of cell-cycle phases during DMSO treatment, compared with control sample, shows an accumulation of the cells in the G1 phase with a decrease in the S phase.

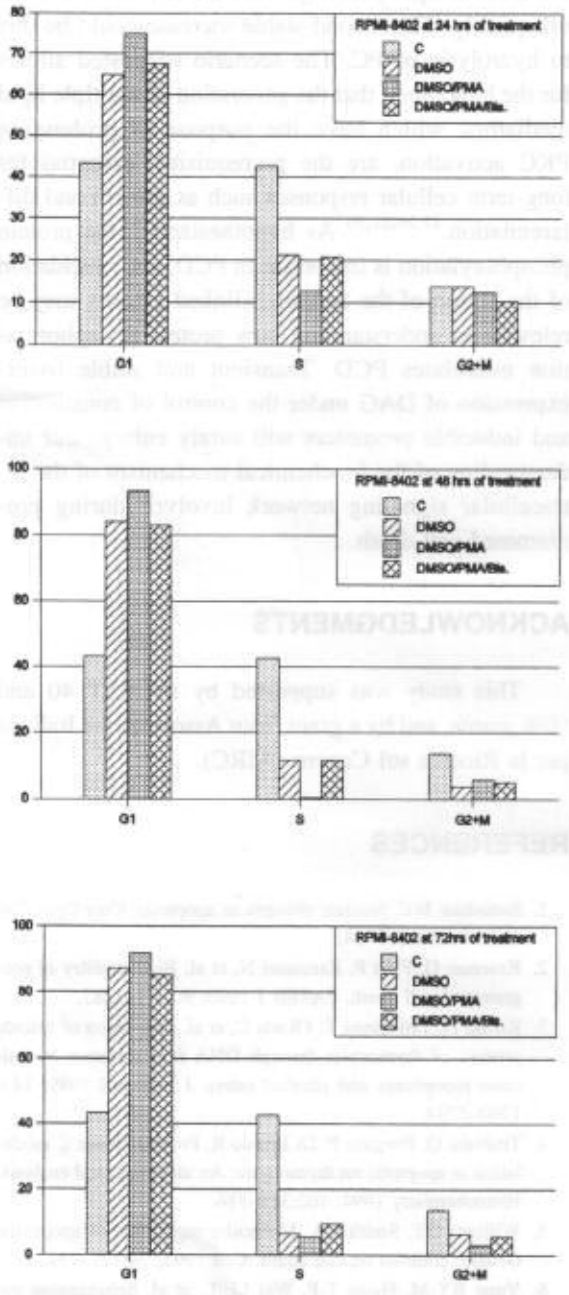

FIGURE 4. Cell-cycle analysis of treated and untreated RPMI 8402 cells. As described in Results, it is possible to observe the different percentages of cells arrested in G1 phase. DMSO- and DMSO/PMA/bisindolylmaleimide-treated samples display the same pattern of cell-cycle distribution. Values are the average of six separate experiments (SD: ±3%) and are expressed as the percentage (*y* axis) of cell-cycle distribution. "C" columns report the percentage of untreated cells.

The cotreatment DMSO/PMA induces an early and increased arrest of cell cycle, while bisindolylmaleimide supplementation induces a modification of cell-cycle progression similar to DMSO treatment alone, also eliminating the PMA effect in cell cycle distribution. Since bisindolylmaleimide can abolish the PKC activity, as measured by phosphorylation of specific kinase substrates including DNA polymerase,[25,26] the results obtained suggest the possibility that PKC plays a critical role in the active phase of apoptosis. Thus, since the PKC is the only clearly defined target for DAG,[18–21] we have specifically analyzed the possible role of DAG in DMSO- and in DMSO/PMA-treated cells. Moreover, we also proved biochemically, using the specific PKC inhibitor, that a PKC-mediated signal transduction pathway modulates the DMSO-induced cell death. The results obtained, reported in Figure 5, show an induction of *de novo* synthesis of DAG. DMSO alone provokes a gradual increment of DAG with a peak after 4 h of treatment. The simultaneous DMSO/PMA treatment induces a great early increase of DAG, recognizable after 90 min, followed by another increase at 8 h of treatment. This dual neo-synthesis produces at the last a prolonged and sustained increment of DAG. Untreated cells (Figure 5) did not display a spontaneous increase of DAG during the follow-up time, while in bisindolylmaleimide/DMSO/PMA-treated cells such an increase was observed only at the peak at 4 h, comparable to cells treated with DMSO alone (data not shown). A review of the reported data provides possible evidence that PMA has a prolonged catalytic effect, via DAG-PKC activation, on DMSO-induced apoptosis.

IV. DISCUSSION

While the role of protein phosphorylation in cell metabolism has been analyzed for many years and has now been elucidated, the role of protein phosphorylation in cell death has been much less studied and the results obtained on this topic are as yet in a primary phase. Many studies have focused on clarifying the biological mechanism of DMSO on cell growth and differentiation.[6–12] It has recently been reported that DMSO positively or negatively affects the apoptotic program depending the cell type.[13–15] The results reported in this paper prove that DMSO-induced pro-

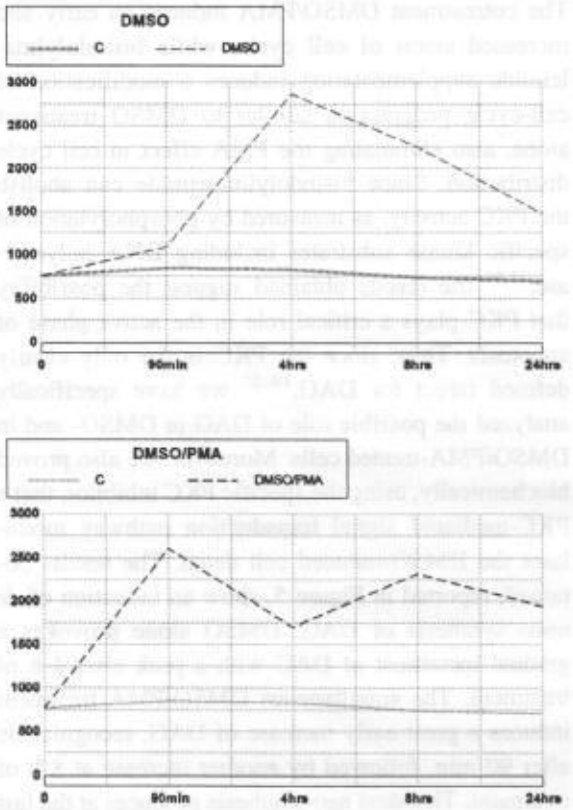

FIGURE 5. Uptake and metabolism of tritium-labeled diacylglycerol in DMSO- and DMSO/PMA-treated RPMI 8402 cells compared with untreated cells (C). The *x* axis shows the time of treatment, while the *y* axis reports the total counts (cpm) for 5×10^6 cells. Values obtained represent the average of three separate experiments (SD: ±4%).

grammed cell death of pre-T human cell line RPMI 8402 can be modulated by phorbol ester supplementation. The increase of cell death can be eliminated by cotreatment with a specific PKC inhibitor. The direct activation of PKC by phorbol ester suggests the consistent idea that it is critically involved not only in cell growth control but also in the regulation of biochemical events of PCD. Previous reports[21] showed that PKC is activated in various fashions depending on different membrane lipid metabolites. The fast and late increases of the newly synthesized DAG obtained in DMSO/PMA-cotreated cells support the hypothesis of the ability of this system to generate a sustained DAG signal. Our study sought to explain the key role of the DAG as pro-apoptotic stimuli and suggests that the DAG accumulation probably results from two different mechanisms. FIrst it is produced immediately via inositol lipid pathway. This DAG molecule turns off quickly. The second stable increase could be due to hydrolysis of PC. The scenario suggested allows for the hypothesis that the generation of multiple lipid mediators, which have the purpose of prolonging PKC activation, are the prerequisites essential for long-term cellular responses such as growth and differentiation.[17,20,21,30] As hypothesized,[31] the protein phosphorylation is important in PCD, and elucidation of the action of the apoptosis-linked kinases may be relevant to understanding how protein phosphorylation modulates PCD. Transient and stable (overexpression of DAG under the control of constitutive and inducible promoters will surely enlarge our understanding of the biochemical mechanism of the intracellular signaling network involved during programmed cell death.

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