Down-modulation of c-myc expression by phorbol ester protects CEM T leukaemia cells from starvation-induced apoptosis: role of ornithine decarboxylase and polyamines

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Abstract

Myc is a transcriptional activator whose deregulated expression not only promotes proliferation but also induces or sensitizes cells to apoptosis. Here we demonstrate that c-myc plays a role in triggering apoptosis in CEM T leukaemia cells exposed to progressive medium exhaustion. Indeed starved cells undergo apoptosis in the presence of constitutively elevated c-myc expression and the phorbol ester, phorbol 12-miristate 13-acetate (PMA), which rescues cells from apoptosis, induces complete c-myc down-regulation. We also investigate the hypothesis that ornithine decarboxylase (ODC), a transcriptional target of c-myc, is a down-stream mediator of c-myc driven apoptosis. We demonstrate that PMA induces in starved cells an earlier and larger decrease in ODC expression (mRNA and activity) and intracellular polyamine content, compared to untreated starved cells. Moreover we show that α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC enzymatic activity, effectively reduces, while exogenous added polyamines enhance apoptosis in starved cells. All these data indicate that ODC and polyamines may act as facilitating factors in triggering apoptosis induced by growth/survival factors withdrawal. Cell Death and Differentiation (2001) **8,** 967–976.

Introduction

The removal of growth/survival factors or essential nutrients in normal cells causes growth arrest by the activation of specific control mechanisms acting at different points of the cell cycle.[1,2] Also interference with or lack of these control mechanisms can lead cells to apoptosis. Protooncogenes such as c-fos and c-jun, are involved in the induction of apoptosis by growth factor deprivation in lymphoid cell lines.[3] The protooncogene c-myc plays a role too, since inappropriate c-myc expression has been shown to induce programmed cell death in fibroblasts[4,5] and in myeloid cells[6] upon serum or survival factor shortage. c-Myc is expressed in normal cells in response to mitogenic stimuli and is down-regulated in many cell types induced to terminal differentiation.[7,8] Indeed, constitutive c-myc expression drives cell-cycle progression and blocks the differentiation of several cell types.[6,9,10] Since c-myc is a transcription factor, it is conceivable that the activation of distinct target genes correlates with these distinct roles. c-Myc is a powerful transactivator of ODC promoter, and enforced c-myc expression results in constitutive expression of ODC gene.[11] ODC, the first and rate-limiting enzyme in polyamine biosynthesis in mammalian cells, is an immediate-early gene, which is rapidly but transiently induced by hormones and growth factors, while it is permanently activated in transformed cells.[12–14] Polyamines have important physiological functions and are essential for normal cell growth and differentiation.[15] Although their role is controversial, polyamines are also implicated in apoptosis. Even if it has been reported that exogenous added polyamines rescue cultured cells from apoptosis induced by different stimuli,[16–18] recent data indicated that ODC induction and increase in polyamine levels directly contributes to apoptosis induced by HGF in a hepatoma cell line.[19] Strikingly, c-myc-induced apoptosis in myeloid cell line 32D.3 after IL-3 withdrawal has been directly linked to ODC induction.[20]

The phorbol ester PMA, which activates PKC, is a potent tumor promoter known to induce proliferation or differentiation in different cell lines.[21] PMA also modulates apoptosis induced by different stimuli, including deprivation of growth factors.[22–24] In several cases, evidences have been provided that the anti-apoptotic effect of PMA is mediated through PKC. It is notable that PMA, which rapidly induces ODC activity in several tissues and cultured cells,[18,25,26] protects a pancreatic cell line against oxidative stress-induced apoptosis by a PKC-dependent induction of ODC activity.[27]

In this study we report that CEM cells undergo starvation-induced apoptosis in the presence of constitu-

tively elevated c-*myc* expression and that PMA prevents it. We therefore investigated whether PMA interferes with apoptosis through modulation of PKC activity, c-*myc* expression and ODC expression. The role of ODC in apoptosis was also assessed by the exposure of CEM cells to DFMO. Our results show that the protective effect of PMA correlates with a marked down-regulation of c-*myc*, ODC and intracellular polyamine levels, and that PMA probably reduces ODC by inhibiting c-*myc* expression. Finally polyamine supplementation or depletion experiments, directly demonstrate that polyamines play a permissive role in the induction of apoptosis by growth/survival factor deprivation, in CEM cells having a deregulated c-*myc* expression.

Results

PMA protects CEM cells from starvation-induced apoptosis

If the medium is replaced every 24 h, CEM cells grow exponentially with a doubling time of almost 24 h, up to 72 – 96 h when a critical cell concentration is reached. Progressive nutrient starvation was obtained culturing CEM cells without medium renewal for 120 h. In these conditions cell growth was comparable to control until 48 h, while signs of cell death were clearly detectable after 72 h. When exposed to a single dose of PMA (100 nM) at the beginning of starvation, cells grew for 96 h and remained viable thereafter (Figure 1A). By biochemical and morphological means we show that starvation of CEM cells induced apoptosis confirming previous data.[28] Moreover we demonstrate that PMA exerts a protective effect. Indeed, we observed a typical oligosomal DNA fragmentation in cells starved for 120 h, which was blocked in the presence of PMA (Figure 1B). Accordingly, upon staining with a fluorescent cell-permeant DNA stain, the typical morphological alterations of apoptosis (i.e. condensation of chromatin and nuclear segmentation) appeared in starved cells, but not in PMA-treated starved cells (Figure 1D). Quantitation of DNA fragmentation shows that soluble low molecular weight DNA was 5% of total DNA in control cells, increased to 39% of total DNA in starved cells but remained at control level (5% of total DNA) in starved cells grown in the presence of PMA (Figure 1C). It should be noted that the inhibition of apoptosis by PMA lasted at least for an additional 2 or 3 days (data not shown).

Cell cycle analysis of CEM cells under progressive nutrient starvation

In several cell types serum withdrawal leads to growth arrest in the G0/G1 phase of the cell cycle and to programmed cell death. On the other hand, CEM cells under conditions of progressive exhaustion of nutrient factors continued to progress through the cell cycle and partially accumulated in the S-phase (Figure 2A,B). Indeed, after 120 h of culture, the percentage of cells in the G0/G1 phase and S-phases was 57 and 27% respectively, in logarithmically growing cells, *versus* 28 and 52% respectively, in starved cells. It is notable that at this time a significant proportion (13%) of cells appeared at a

pre-G0/G1, apoptotic peak. PMA addition did not interfere with the progression of CEM cells through the cell cycle but cancelled the occurrence of the pre-G0/G1 apoptotic peak and partially suppressed S-phase accumulation.

Effects of PMA on PKC activity in CEM cells

As PKC is the main cellular target of PMA, we analyzed the effects of PMA on PKC activity and on the subcellular relocalization of PKC isoenzymes in cells growing under starvation conditions. In logarithmically growing cells, a significant amount of PKC activity was constitutively present in the membrane cell fraction (Figure 3A). In starved cells the cytosol *versus* membrane distribution of PKC activity was not modified compared to unstarved cells (not shown). However after PMA addition, PKC activity was translocated from the cytosol to the membranes by 5 min and this translocation was maintained for at least 30 min. Long-term stimulation with PMA (24 – 72 h) caused a substantial loss of PKC activity, as indicated by the marked decrease in cytosolic PKC activity and the failure to observe a corresponding increase in the membrane-associated PKC activity (Figure 3A).

It is well known that the PKC family consists of different isoenzymes. Interestingly, the activation of certain isoenzymes, perhaps in combination with the down-regulation of others, was demonstrated to be of relevance to the effect of phorbol esters in several cell types.[29] In order to identify which PKC isoenzymes are present in CEM cells and how PMA modulates their activity, we evaluated the presence of specific PKC isoforms in the cytosolic and membrane cell fractions, by Western blot analysis with specific antibodies (Figure 3B). The results indicate that in CEM cells the conventional α and β isoforms, the Ca+-independent θ isoforms and the atypical ι isoforms were predominant. All these isoforms were distributed both in the cytosol and membranes, and medium exhaustion did not modify their subcellular distribution (not shown). PMA induced cytosol to membrane translocation of the α, β and θ isoform, which became evident within 5 min and lasted for at least 30 min. Long exposure to PMA (24 – 96 h) induced down-regulation of the total cellular amount of these PKC isoenzymes, although it should be noted that the decrease in PKC α and β immunoreactivity was more marked in the cytosol as compared to the membranes, where a significant portion of translocated PKC was still present after 72 h stimulation with PMA. Upon PMA treatment, the ι isoform, which is PMA-insensitive, showed neither translocation nor loss of total immunoreactive material. However, it should be noted that after prolonged PMA treatment, the ι isoform's signal appeared as a doublet in the cytosolic fraction of starved cells. This doublet was not present in the cytosolic fraction of control or untreated starved cells (not shown).

Suppression of apoptosis by PMA is associated with down-regulation of c-*myc* expression

Since (i) mitogen withdrawal is accompanied by rapid down-regulation of c-*myc* expression and (ii) the constitutive expression of c-*myc* has been directly correlated with apoptosis after growth/survival factor removal, we analyzed
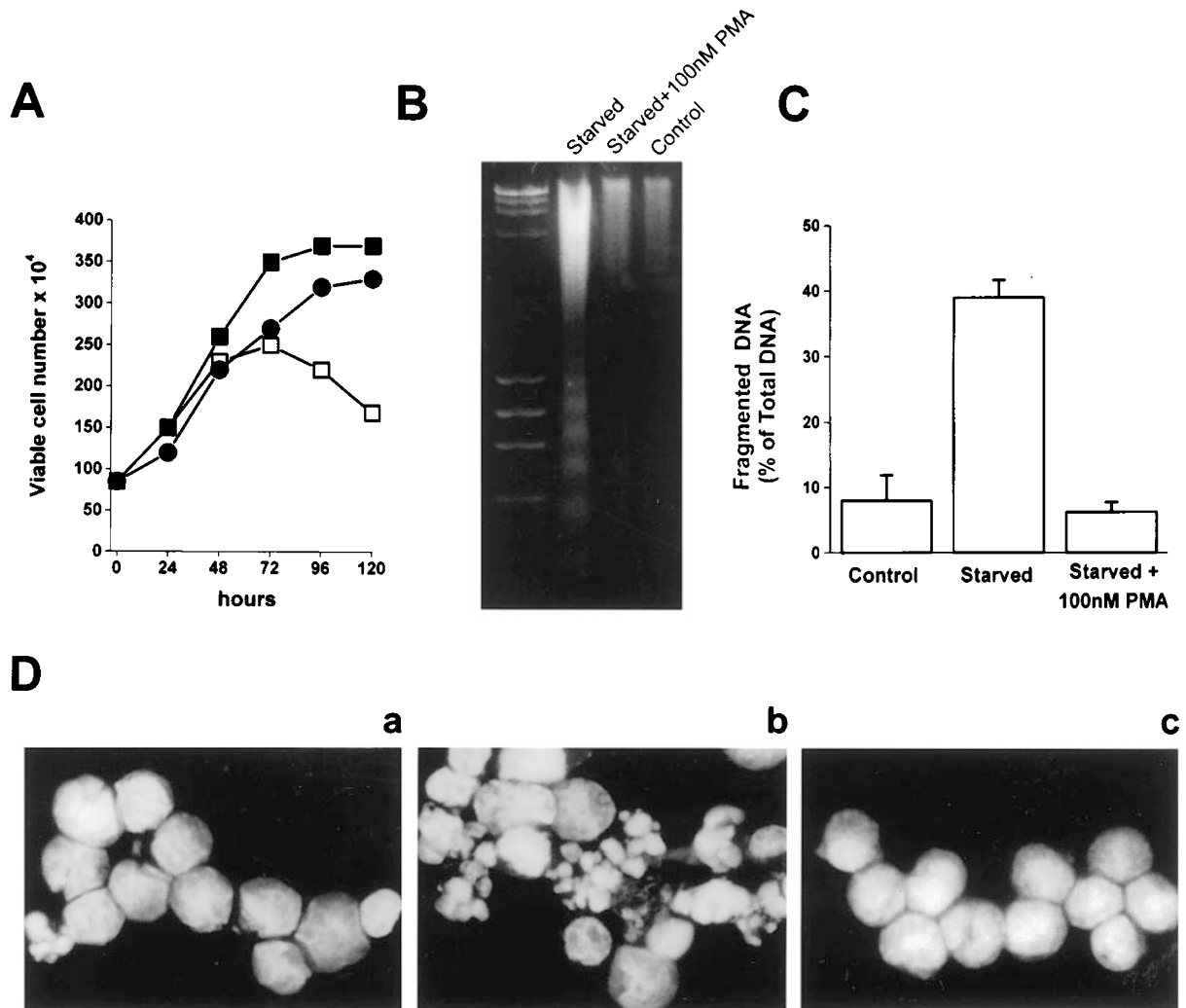
Figure 1 Inhibition of apoptosis by PMA in CEM cells. (**A**) Growth curves. Cells were seeded at a concentration of 0.85×10^5 cells/ml and incubated at $37°C$ in 10% FCS-supplemented RPMI 1640. In control cells (■) the medium was renewed every 24 h. In starved cells (□) the medium was left unchanged. In PMA-treated starved cells 100 nM PMA was added at the beginning of culture (●). At the indicated times the number of viable cells was determined. A typical experiment is reported. (**B**) Apoptosis-associated DNA fragmentation. Control, starved and PMA-treated starved cells were harvested after 120 h of culture and soluble DNA, obtained from equal numbers of cells, was analysed by 1.5% agarose gel electrophoresis. Molecular markers, at the left of the figure, were coelectrophoresed. Representative results from 1 of 6 similar experiments are shown. (**C**) Quantitative analysis of fragmented DNA. Total and fragmented DNA obtained from an equal number of control, starved and PMA-treated starved cells after 120 h of culture, were quantified by using fluorescence DNA dye DAPI as described in the Materials and Methods. The data are reported as % of total cellular DNA recovered as low molecular weight DNA, as described in Materials and Methods. Each value is the mean ± S.D. of six separate determinations. (**D**) Morphological alterations in chromatin. Control (a), starved (b) and PMA-treated starved cells (c) were harvested after 120 h of culture, fixed and stained with DNA-specific fluorochrome DAPI. Magnification of photos 1000 ×

c-*myc* expression in CEM cells exposed to progressive nutrient starvation. The results show that in starved cells c-*myc* mRNA levels were high in the first 24 – 48 h of culture, when no nutritional shortage occurred. c-Myc mRNA was still present after 96 – 120 h of culture when CEM cells were undergoing apoptosis. Addition of PMA dramatically down-regulated c-*myc* expression. Indeed Northern blot analysis indicates that c-*myc* mRNA became undetectable at 24 h after treatment (Figure 4A). Western blot analysis confirmed c-*myc* down-regulation by PMA also at the protein level (Figure 4B). In fact, p62 c-*myc* progressively decreased since 3 h after exposure to PMA and was undetectable at 72 h and 96 h, while it was readily detectable at 72 h and 96 h of culture in the starved cells in the absence of PMA.

PMA treatment down-modulates ODC expression and polyamine levels in CEM cells

In several experimental models ODC gene has been shown to be a transcriptional target of c-*myc*.[11,20,30] Therefore we analyzed ODC mRNA levels, ODC enzymatic activity and intracellular polyamine concentrations in starved cells exhibiting an inappropriately elevated c-*myc* expression, and in PMA-treated starved cells in which c-*myc* expression is inhibited. Northern blot analysis shows that ODC mRNA levels decreased progressively during progressive medium exhaustion and that PMA accentuated this decrease. Levels of ODC mRNA were quantitated by densitometric scanning of Northern blot autoradiograms and were normalized to

A

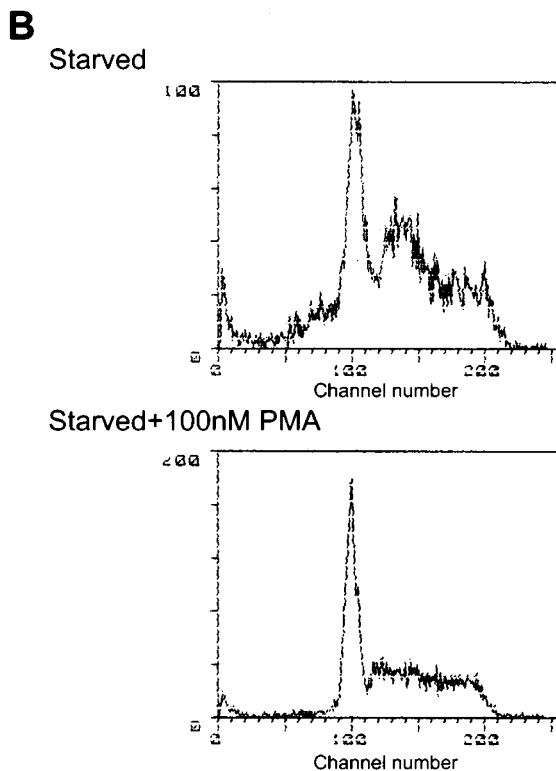| Sample		Sub G0 (%)	G0/G1 (%)	S (%)	G2/M (%)
Starved	24hr	3	57,2	27,3	12,5
	48hr	2,7	57	31,7	8,6
	72hr	3,7	37	50,6	8,7
	96 hr	9,7	32,3	50,5	7,5
	120hr	12,9	28	51,9	7,2
Starved + PMA	24hr	3	59	26,9	11,1
	48hr	3,7	53,4	32,3	10,6
	72hr	4	52,5	34,1	9,4
	96 hr	4	52	32,4	9,6
	120hr	3,8	47,8	38	10,4

B

Starved

Starved+100nM PMA

Figure 2 Effect of nutrient starvation and PMA addition on cell cycle and apoptosis in CEM cells. Cells were grown without medium renewal in the absence (Starved) or in the presence (Starved+PMA) of 100 nM PMA. At the indicated culture times, the cell cycle distributions of propidium-iodide-stained cells were determined by flow cytofluorimetric analysis of DNA. (**A**) Quantitative measure of FACS data using the sum of broadened rectangles (SOBR) model. (**B**) Representative tracings from FACS analysis at 120 h culture time. The results from a typical experiment are shown. The experiment was repeated two times with similar results

A

B

Figure 3 Effect of PMA on PKC in starved CEM cells. Cells were grown without medium renewal in the presence of 100 nM PMA. (**A**) Subcellular distribution of PKC enzymatic activity. At the indicated times after PMA addition, PKC activity was determined with a PKC enzyme assay kit (Amersham) in the cytosolic (Black column) and particulate (open column) cell fractions. Data are the means ± S.D. of triplicate independent determinations from a typical experiment. Another experiment gave essentially identical results. (**B**) Intracellular levels and localization of PKC isoenzymes. At the indicated times after PMA treatment, cells were harvested, lysed, and equal amounts of protein extracts (12.5 μg) from cytosolic (Sol.) and particulate (Mem.) cell fractions, were resolved by SDS–PAGE. Western blotting was performed as described in Material and Methods. These results are representative of three independent experiments

GAPDH mRNA levels on the same filter. The results clearly indicate that down-regulation of ODC mRNA started at 24 h reaching its maximum at 120 h (Figure 5A). Thus ODC mRNA pattern reflects that of c-*myc* mRNA. Figure 5B shows that in starved cells ODC activity remained stable during the first 24 h, then decreased, being almost undetectable at 96 h of culture. In PMA-treated starved cells, ODC activity decreased more promptly, being reduced as early as 6 h after PMA addition. The analysis of intracellular polyamine content
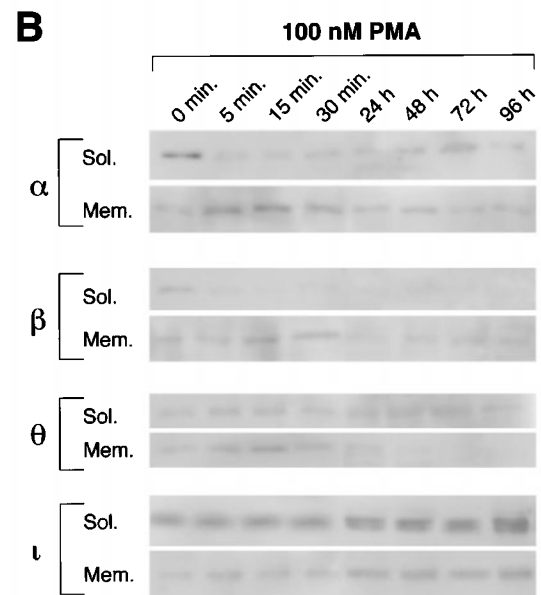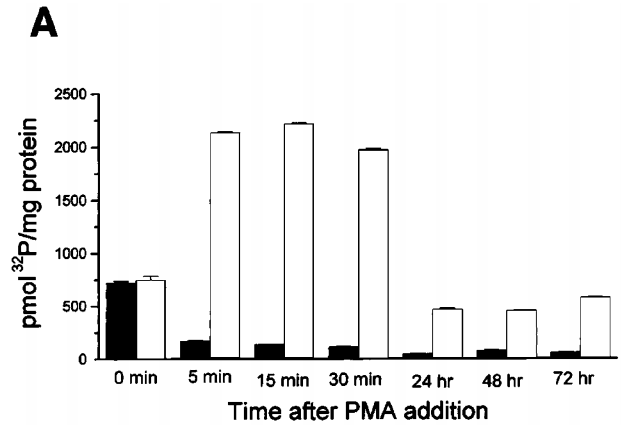
shows that starvation by itself decreased putrescine but did not affect spermidine or spermine concentration. PMA treatment caused a more relevant decrease in putrescine concentration and induced a significant decrease in intracellular spermidine content (Figure 5C). On the whole, these results agree with the data on ODC mRNA levels and ODC enzymatic activity, and suggest that activation of ODC expression by c-*myc* may play a positive role in apoptosis. To directly address the role of ODC activity and polyamine cellular levels in apoptosis, we tested the effect of DFMO, a specific and irreversible inhibitor of ODC activity. Our results
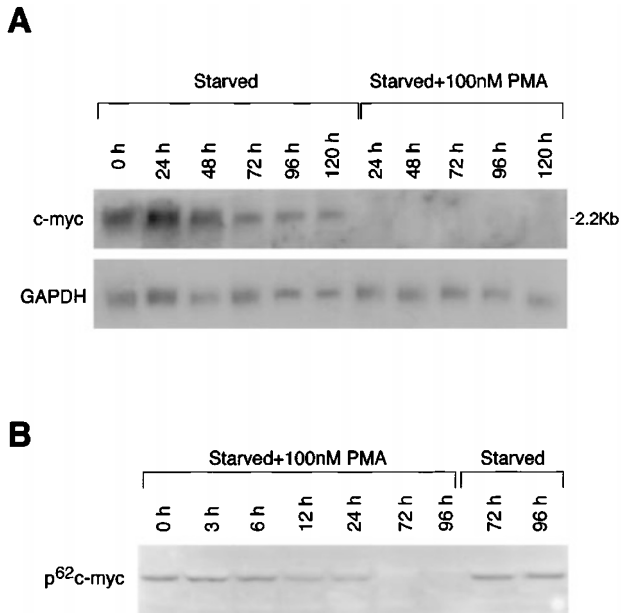
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Figure 4 Effect of PMA on c-*myc* expression in CEM cells. Cells were grown without medium renewal in the absence (Starved) or in the presence (Starved+PMA) of 100 nM PMA. At the indicated culture times, cellular RNAs or proteins were extracted and analyzed for c-*myc* expression. (**A**) Northern blot analysis of total cellular RNA with c-*myc* and GAPDH cDNA probes. (**B**) Western blot analysis of total protein extracts with specific c-*myc* antibodies. Typical autoradiograms and fluorograms from three independent experiments are shown

show that 5 mM DFMO did not interfere with the cell proliferative rate (not shown), but significantly decreased apoptosis as assessed by electrophoretic analysis of oligosomal fragmentation and quantification of fragmented DNA (Figure 6A,B). 10 mM DFMO did not significantly increase the extent of protection compared to 5 mM DFMO (not shown). Indeed, as shown in Table 1, cell treatment with 5 mM DFMO completely blocked ODC activity and greatly reduced intracellular polyamine concentration, particularly putrescine and spermidine. It should be noted that DFMO effect on apoptosis was lower than that obtained with PMA (compare Figures 6 and 1).

Effects of polyamine replacement on CEM cells apoptosis

The positive role of ODC in apoptosis was also elucidated by studying the effects of polyamine replacement. Since supplementation of polyamines has been demonstrated to be protective against apoptosis induced by different conditions in various cell types,[16–18] we added polyamines to the medium of starved cells at the beginning of the culture. Aminoguanidine was also added to prevent polyamine oxidation by serum amine oxidase (SAO).[31]

We tested different polyamine concentrations and found that supplementation with 20 μM putrescine, spermidine or spermine, maintains in starved cells intracellular polyamine levels similar to those present in control unstarved cells (data not shown). Therefore this concentration was used for all supplementation experiments with the different poly-

amines. Contrary from that reported from other cellular systems, polyamine addition did not counteract apoptosis in CEM cells growing under progressive medium exhaustion as demonstrated by the electrophoretic analysis of DNA fragmentation (Figure 7A). Even more strikingly, the data from the quantitation of DNA fragmentation indicate that polyamine supplementation significatively enhanced apoptosis. Indeed soluble DNA was about 34% of total DNA in starved cells grown in the presence of aminoguanidine but it increased up to 53, 50 and 44% of total DNA in starved cells supplemented with putrescine, spermidine or spermine respectively (Figure 7B).

Discussion

Here we discuss that the inability to down-regulate c-*myc* expression, drives CEM cells to apoptosis upon nutrient/growth factors starvation. Indeed, the proliferative signal induced by inappropriate c-*myc* expression could cause a failure to halt cell cycling even in the presence of growth arrest signals, thus resulting in apoptosis.[32] Our results obtained by flow cytometric analysis support this interpretation. We therefore assumed that under growth-limiting conditions PMA may enhance cell survival by suppressing c-*myc* induced apoptosis. The finding that PMA does indeed inhibit c-*myc* expression is a further confirmation of this pivotal role of c-*myc*. We also showed that ODC expression as well as polyamine levels correlate with the inhibition of c-*myc* in PMA treated cells. This is in contrast with other reports showing induction of ODC activity and increase of polyamine levels by PMA in many cell types.[27] However, in this particular cellular context, the inhibitory effect of PMA on polyamine metabolism is probably directly correlated with its primary effect on c-*myc* expression, since ODC is one of the transcriptional targets of c-*myc*. In a recent study it has been demonstrated that ODC acts as a mediator of c-*myc* induced apoptosis[20] and our results agree with this interpretation. A positive role of ODC is also strengthened by the anti-apoptotic effect of DFMO and by the pro-apoptotic effect of exogenous polyamine supplementation. However a direct comparison of the extent of apoptosis in PMA-treated and in DFMO-treated cells, demonstrates that PMA is more active than DFMO in protecting CEM cells from apoptosis. These results therefore suggest that other mediators, in addition to ODC and polyamines, participate in the induction of apoptosis in CEM cells. Among these the activation of caspases and the release of cytochrome *c* from mitochondria by a deregulated c-*myc* expression, could play a role.[33,34]

The role of polyamine in apoptosis has been closely investigated and produced conflicting results. It has been demonstrated that increased intracellular polyamine content could play a protective role against apoptosis induced by different stimuli in other cell types.[15] On the other hand, polyamine depletion obtained by using DFMO prevents apoptosis in at least two different experimental systems.[35,36] High polyamine levels are characteristic of many types of neoplastic cells and tissues. These and other observations have led to intensive investigation into the potential use of polyamine synthesis inhibitors as thera-peutic agents for various proliferative disorders, including cancer.[37] Among
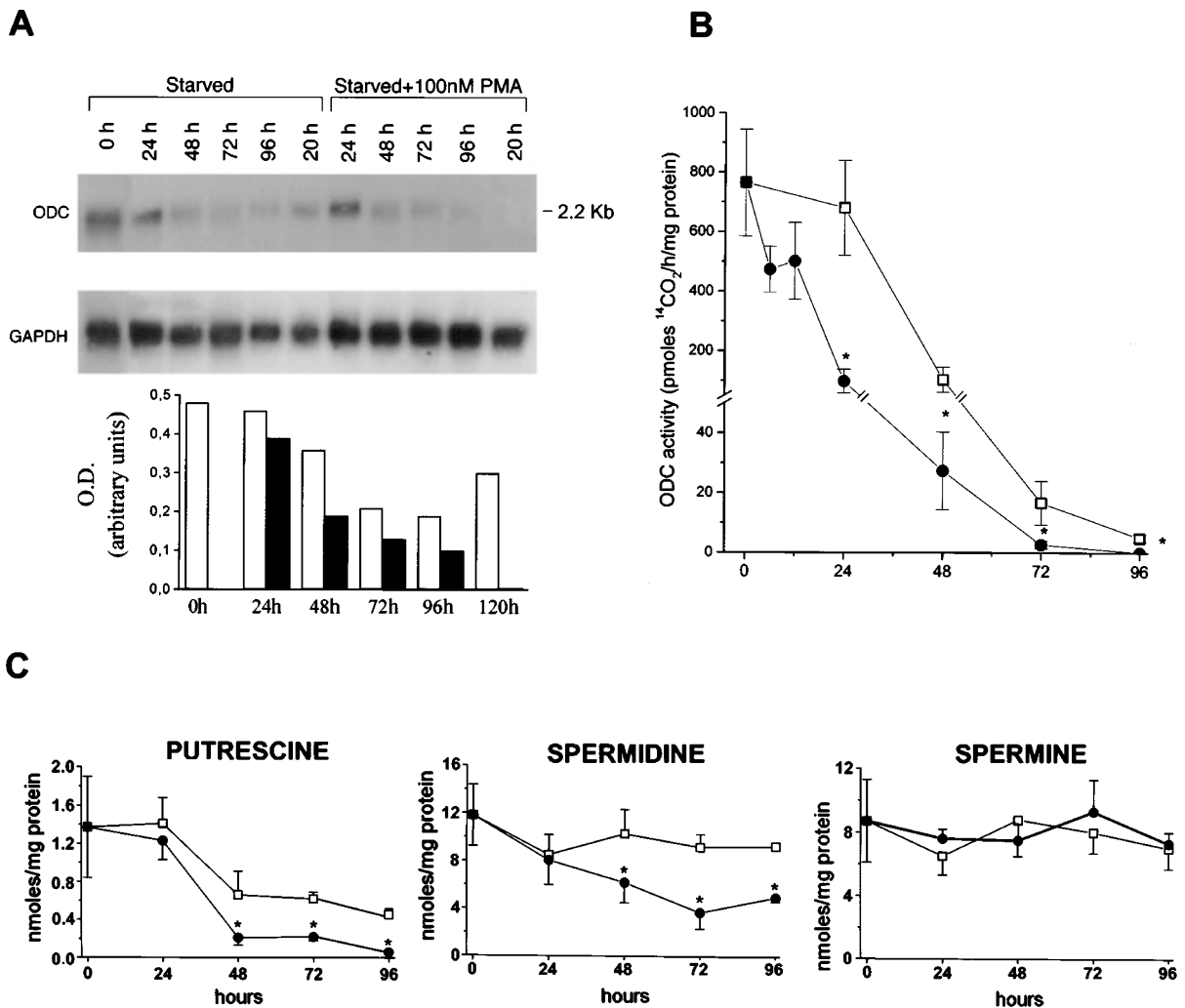
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Figure 5 Effect of PMA on ODC mRNA level, ODC activity and intracellular polyamine concentrations in CEM cells. Cells were grown without medium renewal for the indicated times in the absence or in the presence of 100 nM PMA. (**A**) Northern blot of total cellular RNA from starved and PMA-treated starved cells probed with ^{32}P labeled ODC or GAPDH cDNA. Autoradiograms from a representative experiment are shown. The ODC mRNA levels were quantified by densitometric analysis and normalized to levels of GAPDH mRNA. The experiment was repeated three times with similar results. (**B**) ODC activity in starved (■) and PMA-treated starved cells (●) was measured as $^{14}CO_2$ release, using radiolabelled ornithine as a substrate, as described in Materials and Methods. The data are reported as means ± S.D. of four separate experiments. (**C**) Putrescine, spermidine and spermine intracellular concentrations were assayed in starved (□) and PMA-treated starved cells (●) by HPLC as described in Materials and Methods. The data are reporlted as means ± S.D. of three to four separate experiments. *$P < 0.05$ compared with control at each time-point

others, DFMO has been largely employed in *in vitro* and *in vivo* studies and has proven reasonably effective only as a chemopreventive agent, being ineffective as a chemotherapeutic agent in both animal models and humans.[37] Since several data, including ours, largely show that the depletion of polyamines can protect against specific apoptotic-inducing signals,[35,38,39] caution should be taken when polyamines are chosen as a potential chemotherapeutic target.

There is an extensive literature regarding the role of PKC in suppressing or stimulating apoptosis in various cell types.[24,40] In principle, the different conclusions as to the positive and negative effects of PKC on apoptosis may reflect cell-specific differences, depending on the type of apoptosis-inducing signals. In our experimental model, we observed a transient activation of PMA-responsive PKC

isoforms followed by down-regulation. Our opinion is that the transient activation of PKC, occurring at early times, might not be relevant for cell death, which occurs at late times. It is therefore conceivable that the depletion/down-regulation of PKC (rather than its activation) is responsible for the protective effect of PMA in nutrient-depleted CEM cells. In addition, since c-*myc* expression could be modulated by PKC activators,[41,42] it is tempting to speculate that PKC down-regulation contributes to the inhibition of c-*myc* expression by TPA in starved CEM cells. This would agree with the inhibitory effect of PMA on c-*myc* expression reported in several myeloid leukemia cell lines where phorbol esters act as differentiating agents.[43]

In conclusion, our results indicate that the constitutive activation of c-*myc* and the subsequent activation of ODC
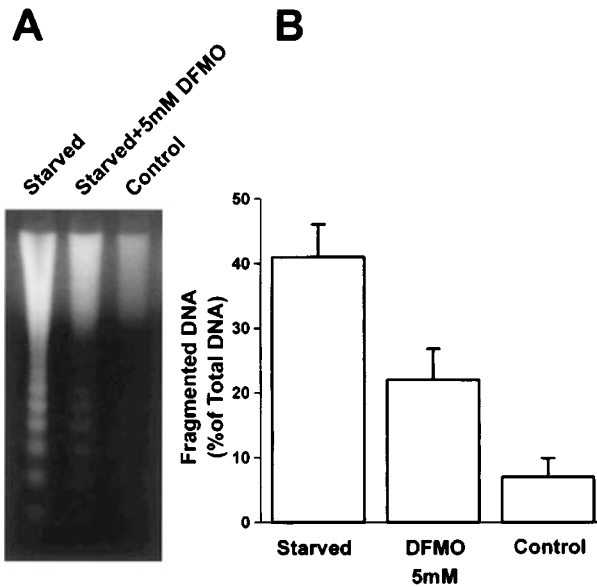
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Figure 6 Effect of DFMO on DNA fragmentation in CEM cells. Control cells were grown by replacing culture medium every 24 h. Starved cells were grown without medium renewal; DFMO (5 mM) was added at the beginning of the culture. (**A**) Apoptosis-associated oligosomal DNA fragmentation. Control, starved and DFMO-treated starved cells were harvested at 120 h of culture. Oligosomal DNA fragmentation was assessed as described in Figure 1. Representative results from one of four similar experiments are shown. (**B**) Quantitative analysis of fragmented DNA. Cells were harvested at 120 h of culture. Other details as reported in Figure 1. The values are means ± S.D. of four independent experiments

expression and polyamine metabolism, probably increases the endogenous apoptotic program of CEM cells under growth-limiting conditions. Indeed the potential of PMA to limit cell death under growth-restricted conditions correlates with its ability to down-regulate c-*myc*, thus counteracting the effect of c-*myc* on polyamine metabolism.

Materials and Methods

Drugs and reagents

PMA, DFMO, putrescine, spermine, spermidine and aminoguanidine were purchased from Sigma.

Cell culture and treatments

CEM cells (lymphoblastic leukemia CD4+ cells), were maintained in RPMI 1640 supplemented with 5% foetal calf serum, 100 IU/ml penicillin and 0.15% streptomycin at 37°C, 5% CO_2. Progressive starvation was obtained as previously described by Petronini.[28] Briefly, exponentially growing cells were collected, seeded at a concentration of 8.5×10^5 cells/ml in RPMI 1640 containing 10% FCS, and grown in the absence (starved cells) or presence of 100 nM PMA (PMA-treated starved cells) without renewal of the medium. In control cultures, the medium was replaced with fresh medium every 24 h. Cells were harvested at the indicated times for each assay. Cell number was evaluated by counting cells in a Burker haemocytometer. Cell viability was assessed by Trypan blue exclusion assay.

Table 1 Effect of DFMO on ODC activity and intracellular polyamine levels in starved CEM cells

Time	ODC activity pmoles $^{14}CO_2$/ h/mg protein	Putrescine nmoles/mg protein	Spermidine nmoles/mg protein	Spermine nmoles/mg protein
0	765 ± 180	1.37 ± 0.53	11.9 ± 2.59	8.8 ± 2.6
1	46.3 ± 13			
24	0	0.21 ± 0.04	2.7 ± 0.50	8.1 ± 1.4
48	0	< 0.1	0.6 ± 0.18	7.3 ± 0.6
72	0	< 0.1	0.4 ± 0.13	7.6 ± 0.9
96	0	< 0.1	0.3 ± 0.06	7.6 ± 0.9

Cells were grown without medium renewal for the indicated time in the presence of 5 mM DFMO added at the beginning of the culture. ODC activity was measured as $^{14}CO_2$ release as described in Materials and Methods. Cellular concentrations of putrescine or spermidine or spermine were assayed by HPLC as described in Materials and Methods. The data are reported as means ± S.D. of three or four separate experiments.
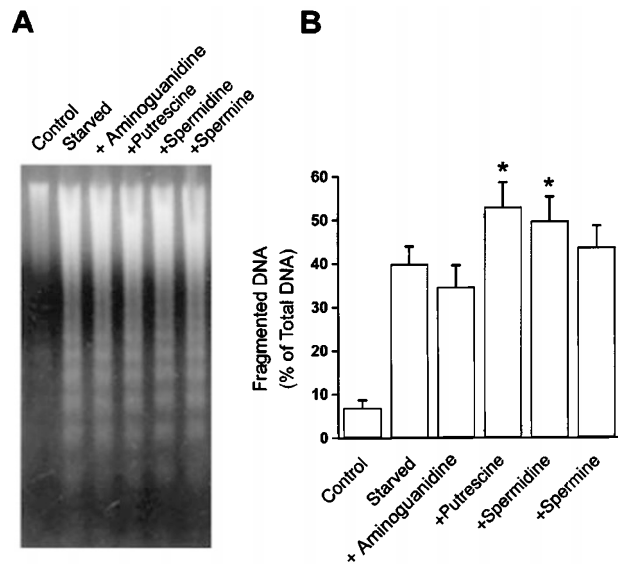
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Figure 7 Effect of polyamine supplementation on DNA fragmentation in CEM cells. Control cells were grown by replacing culture medium every 24 h. Starved cells were grown without medium renewal in the absence or in the presence of polyamines. Putrescine, spermidine or spermine (20 μM) were added at the beginning of culture. Aminoguanidine (1 mM) was also added. (**A**) Apoptosis-associated oligosomal DNA fragmentation. Control, starved and polyamine supplemented starved cells were harvested at 120 h of culture. Oligosomal DNA fragmentation was assessed as described in Figure 1. Representative results from one of four similar experiments are shown. (**B**) Quantitative analysis of fragmented DNA. Cells were harvested at 120 h of culture. Other details are as reported in Figure 1. The values are means ± S.D. of six independent experiments. *$P < 0.05$ compared with aminoguanidine-treated starved cells

Apoptosis assays

DNA fragmentation was evaluated by gel electrophoresis analysis and quantitated by DAPI assay. Cells (2×10^6) were lysed in hypotonic buffer (10 mM Tris/HCl pH 7.6, 1 mM EDTA, 0.2% triton X-100). The lysates were centrifuged and supernatants containing low molecular weight DNA, were collected and used for both gel electrophoretic analysis and quantitation of fragmented DNA. For gel electrophoretic

analysis, low molecular weight DNA was extracted with phenol-chloroform-isoamylalcohol, precipitated in ethanol, resuspended in 12 μl of RNAse A (final concentration of 20 μg/ml) in 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and incubated for 30 min at 37°C. After the addition of loading buffer, samples were heated at 65°C for15 min and electrophoresed in 1.5% agarose gel. For quantitation of DNA fragmentation, total DNA present in the whole cellular lysates and low molecular weight apoptotic DNA present in the supernatants, were quantitated by using the fluorescent DNA dye 4′, 6-diamidino-2-phenilindole (DAPI) (Boehringer) as described by Brunk.[44] Fluorescence was measured in arbitrary units: the excitation wavelength was 360 nm and the emission wavelength was 450 nm. DNA fragmentation was calculated as percentage of total cellular DNA recovered as low molecular weight DNA in the supernatant. To detect morphological alterations, cells (0.5×10^6) were collected by centrifugation, resuspended in DAPI-methanol and incubated for 15 min at 37°C. DAPI-methanol solution was removed by centrifugation, fixed-stained cells were resuspended in PBS, and 10 μl of cell suspension were placed on a glass slide and examined under a fluorescence microscope. Any cells with condensed chromatin and two or more chromatin fragments were considered apoptotic.

Cell cycle analysis

Following the specified culture times, 1×10^6 cells were harvested, washed in PBS, fixed in 1 ml of 70% ethanol, and stored at -20°C. On the day of analysis, the fixation buffer was removed and the cells resuspended in 300 μl of PBS. Cells were stained by propidium iodide and analyzed using a FACScan flow cytometer (Becton Dickinson). Flow cytometric analyzes were performed using an argon laser at 488 nm. Apoptotic cells were evident as cells containing less than 2N DNA.

Preparation of cellular and subcellular protein extracts

Total cellular extracts were obtained incubating 1×10^7 cells in 500 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM Na$_3$VO$_3$, 25 mM β-glycerophosphate, 1 mM PMSF, 20 μg/ml leupeptin, 1 μg/ml pepstatin A). Samples were left for 30 min at 4°C and clarified by centrifugation. For subcellular fractioning, 1×10^7 cells were incubated for 10 min at 4°C in a hypotonic medium (50 mM Tris/HCl pH 7.4, 0.3% (w/v) β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 1 mM PMSF, 20 μg/ml leupeptin and 1 μg/ml pepstatin A), sonicated and centrifuged at 100 000 g for 60 min to obtain a final soluble fraction (cytosol) and a particulate fraction (membranes). Membrane proteins were obtained by dissolving the particulate fraction into the buffer described above containing 1% octilglucoside, and by centrifuging at 100 000 g for 60 min. The different preparations were used for protein kinase activity assay and Western blot analysis as described below.

Protein kinase C activity assay

PKC activity was determined by an *in vitro* phosphorylation assay (PKC enzyme assay system, Amersham) measuring the incorporation of ^{32}P into a peptide which is specific for PKC, in the presence of saturating concentrations of PMA/L-a-phosphatidyl-L-serine. The reaction mixture containing calcium ions, phorbol esters/phospholipids, substrate, DTT, 0.2 μCi [^{32}P]-ATP and 10 μg of membrane proteins or cytosolic proteins, was incubated at 37°C for 15 min. PKC activity was expressed as pmoles of ^{32}P transferred/minute/mg of protein.

Western blot analysis

Total cell (50 μg), cytoplasm (12.5 μg) or membrane (12.5 μg) protein extracts were subjected to SDS – PAGE in 8% reducing gels and elctrophoretically transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in TBST buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% tween 20) and incubated overnight with monoclonal antibodies anti-c-*myc* (1 : 600) (Cambridge Research Biochemicals) or with monoclonal antibody anti-specific PKC isoenzymes (PKC Sampler Kit, Transduction Laboratories), according to the manufacturer's recommended dilutions. Membranes were then washed in TBST buffer, incubated with peroxydase-coupled anti-mouse IgG and developed using the ECL-system (Amersham).

Northern blot analysis

RNA was extracted and Northern blot was performed as previously described.[45] Briefly, equal amounts of RNA, from each sample according to spectrophotometric determination, were electrophoretically separated on 1.2% agarose gel containing formaldehyde, and RNA was transferred to Hybond-N filters (Amersham). The filters were hybridized according to standard methods, with specific [^{32}P]-dCTP labelled cDNA probes generated using a Multiprime DNA Labelling System (Amersham). Inserts from plasmid pRyc-7.4[46] or pODC 10/2H[47] were used as probes for the expression of c-*myc* or ODC mRNA respectively. The signals were detected on Kodak X-OMAT film at -80°C. The blots were reprobed with [^{32}P]-labeled GAPDH cDNA as a control for equal RNA loading. Autoradiographic signals were quantitated using a FUJIFILM Bio-I Imaging Analyser FLA-2000, and expressed as arbitrary units.

ODC activity assay

ODC activity was determined by the release of ^{14}CO$_2$ from L-[1-^{14}C]ornithine, as described by Scalabrino.[48] Briefly, 1×10^7 cells were harvested by centrifugation, washed and suspended in 200 μl of 25 mM Tris/HCl, pH 7.2 containing 0.1 mM EDTA and 1 mM DTT. The cells were disrupted by freezing and thawing, then centrifuged at 12 000 g for 15 min at 4°C. The supernatants (150 μl) were incubated with 100 μl of reaction mixture containing 500 mM Tris/HCl, pH 7.1, 10 mM EDTA, 1 mM DTT, 5 mM L-ornithine, 1 mM piridoxal-phosphate and L-[1-^{14}C] ornithine (0.2 μCi, 57 mCi/mMol; New England Nuclear). After 60 min at 37°C, 250 μl of trichloroacetic acid (50% [w/v]) was added through a syringe in the lid and liberated CO$_2$ was trapped in a paper disk containing 50 μl Soluene-350 (Packard Instruments). After 60 min, paper disks were recovered and radioactivity determined by scintillation counting. ODC assays was standardised for protein content, using a Bradford-modified assay Kit (Pierce).

Polyamine determination

1×10^7 cells were harvested in 0.2 N perchloric acid, and precipitated cellular proteins were removed by centrifugation. The concentration of the amine in the acid extract was analyzed by HPLC (Perkin-Elmer, LC Pump Series 410 BIO) with a fluorescence detector (LS-1) (excitation at 345 and emission at 455 nm) and post-column derivatization with o-phthaldeyde, according to the method of Loser.[49]

Statistical analysis

Statistical analysis was performed using a linear mixed model for repeated measure to evaluate possible significant differences between

the means of control and treated groups at different time points in time-course experiments. In polyamine supplementation experiments data were subjected to one-way ANOVA followed by Bonferroni *t*-test. The statistical package S-plus (MathSoft Inc.) was used to perform the statistical analysis. A $P < 0.05$ was considered statistical significant.

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