Cell Death and Differentiation (1999) 6, 698–709

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Modulation of apoptosis of proliferating and differentiating HL-60 cells by protein kinase inhibitors: suppression of PKC or PKA differently affects cell differentiation and apoptosis

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Received 27.4.98; revised 22.3.99; accepted 14.5.99.
Edited by P. Davies

Abstract

The relationship between RA- or dbcaMP-mediated differentiation and subsequent apoptosis in HL-60 cells was assessed by modulating the levels of differentiation suppressing the activity of PKC and PKA with calphostin C or GF 109203X and H89, respectively. Results demonstrated that (1) RA and dbcAMP caused a dose-dependent increase in apoptosis concomitant with progressive differentiation; (2) the suppression of PKC activity resulted in an increase of apoptosis unrelated to the modulated levels of differentiation; (3) the inhibition of PKA decreased granulocytic differentiation, but did not significantly affect apoptosis; (4) the pretreatment of cells with dbcAMP strongly potentiated RA-mediated differentiation without apparent changes in apoptosis; (5) cell differentiation and apoptosis were associated with cell cycle arrest in G1 phase and G2/M phases, respectively. Our findings indicate that the functional maturity of differentiating cells is not directly related to the apoptotic programme, and suggest that induction of cell differentiation and apoptosis are regulated by separate mechanisms in which PKC and PKA are involved.

Keywords: HL-60 cells; differentiation; apoptosis; protein kinase inhibition

Abbreviations: AO, acridine orange; DMSO, dimethyl sulfoxide; dbcAMP, N^6, 2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; EDTA, ethylenediaminetetraacetic acid; ET, ethidium bromide; GF 109203X, bisindolylmaleimide I; HCl}, Clp, calphostin C; H89, {N-[2((p-Bromocinnamyl) amino) ethyl]-5-isoquinoline sulfonamide; NBT, nitro blue tetrazolium; PI, propidium iodide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RA, all-*trans* retinoic acid; RNAse, ribonuclease; SDS, sodium dodecyl sulfate; TB, trypan blue; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol

Introduction

Neoplastic myeloid cells are derived from cells, which diverse from the natural differentiating pathway at distinct developmental stages, most often as myoblasts[1–6] or promyelocytes.[5,6] The common feature of these neoplastic cells is their capacity to return to the differentiation pathway by exposure to certain chemical agents.[7–9] At certain conditions proliferating or differentiating leukemic cells undergo apoptosis as well.[10–12] Human promyelocytic leukemia HL-60 cells have been used repeatedly to study the control mechanisms of proliferation, differentiation and apoptosis.[5,8,9,11,13,14] Besides compounds such as sodium butyrate and dbcAMP, HL-60 cells can be induced to differentiate to granulocytes by treatment with all-*trans*-retinoic acid (RA) or its isomers.[14] Due to this property retinoids have been proposed as future radical therapeutics for acute promyelocytic leukemias.[15,16] Recently, by *in vitro* experiments, however, it was demonstrated that HL-60 cells induced to differentiate to granulocytes undergo apoptosis.[11,12] A large wave of apoptosis can be expected[17] besides other side effects such as coagulation disorders and hyperleukocytosis. Apoptotic cells and products released during their degradation may cause the hyperleukocytosis. Thus, *in vitro* studies of the relationship between granulocytic differentiation and subsequent apoptosis may be useful for elucidation of the control mechanisms of normal hematopoiesis and for the strategy of differentiation therapy of leukemias as well.

Many evidences indicated remarkable changes in the activity of distinct protein kinases and phosphatases at all the stages of the HL-60 differentiation process.[8,18–37] It has been shown that during granulocytic differentiation the commitment stage is associated with a modulation of the activity of PKC[22,29,34] and PKA[19,35–38] and PKC inhibitors are able to potentiate granulocytic differentiation.[29,36,37] Recently, we have shown that inhibition of protein kinase PKA and PKC exert stage specific and inducer (RA or dbcAMP) dependent effects on HL-60 cell differentiation to granulocytes.[36] Moreover, we have been able to demonstrate the relationship between differentiation mechanisms involving PKA and PKC in uninduced and differentiating HL-60 cells.[37] Data exists that the basal activity of PKC prevents the occurrence of apoptosis in HL-60 cells and MOLT-4 cells.[39,40] In contrast, IPC-81 myeloid leukemia cells undergo apoptosis in response to agents that elevate intracellular cAMP levels.[41] It has also been shown that activation of the conventional isoform of PKC during differentiation of myeloid U937 cells causes apoptosis of differentiating cells.[42]

Earlier we have shown that modulation of PKA or PKC activity causes changes of the level of HL-60 cell differentiation induced by RA and dbcAMP. The present study was undertaken (1) to elucidate the contribution of protein kinase PKA and PKC in promoting or suppressing apoptosis in proliferating and differentiating HL-60 cells; (2) to estimate the correlation between the number of differentiating and apoptotic cells at distinct stages in a differentiating HL-60 cell population and (3) to ascertain at which phase of the cell cycle (G1 or G2/M) cell arrest is more preferable for initiation of apoptosis. Our findings demonstrate that the apoptotic programme of proliferating myeloid cells can be induced by suppression of PKC activity, and that the functional maturity of differentiating HL-60 cells is not always necessary for triggering an apoptotic programme.

Results

Inhibition of PKC or PKA promotes apoptosis in proliferating HL-60 cells at a different extent

HL-60 cells were exposed to two highly specific inhibitors of PKC (calphostin C and GF 109203X) and an inhibitor of PKA (H89). Calphostin C interacts with the regulatory domain of PKC by competing at the binding site of diacylglycerol. GF 109203X interacts with ATP-binding site of PKC. The effective concentrations of each inhibitor causing 50% maximal response (EC 50)[13] were used in our experiments. Quantitative analysis of DNA fragmentation was performed after 72 h of treatment and the appearance of apoptosis was monitored on days 6 and 9 in the proliferating cell cultures. The effects of these protein kinase inhibitors on cell viability were determined by trypan blue exclusion. None of the agents tested potentiated the suppression of cell growth or viability during the period of 6 days. The decrease in cell viability ($<20\%$) was found after co-incubation of the proliferating cells with inhibitors for 9 days (data not shown). Also, the relationship between decrease in cell viability and increase in the number of apoptotic cells was noted.

DNA fragmentation (diphenylamine assay) induced by inhibitors of protein kinase after 3 days of co-incubation was associated with appearance of cells with morphological characteristics of apoptosis (acridine orange/ethidium bromide staining) on day 6 (Table 1). Both inhibitors of PKC promoted extensive DNA fragmentation (37 and 39%) as tested after 72 h. The effect of PKA inhibitor, H89, on DNA fragmentation (23%) was not as potent as of PKC inhibitors. Qualitative analysis of DNA fragmentation was performed using agarose gel electrophoresis and the results were consistent with the quantitative studies of DNA fragmentation (Figure 1, Table 1).

During 72 h of treatment with drugs the cell viability remained high (95–97%). In order to assess relationship between cell viability and apoptosis we stained cells in two parallel aliquots from the same cell culture with (1) acridine orange/ethidium bromide (AO/ET) or (2) trypan blue (TB). This technique allows to identify the cells with apoptotic characteristics among live cells (the membrane of latter is not damaged and ethidium bromide cannot enter the cell). These cells do not stain with trypan blue, but green apoptotic bodies are visible after AO/ET staining, whereas apoptotic bodies in dead cells stain red. Thus, e.g. on the sixth day of differentiation (Table 1), after treatment with PKA inhibitor, H89, the cells were viable (95%, TB exclusion). However, based on AO/ET staining 19% of the cells in aliquots tested had morphological features of apoptotic cells. This included 5% of the cells, which were stained red by AO/ET, and were considered as dead cells.

Table 1 Effects of protein kinase inhibitors on the induction of apoptosis in proliferating HL-60 cells

Treatment	Apoptotic cells (%)	Viability (%)	DNA fragmentation (%)
50 nM H89	19 ± 1.3	95 ± 2.3	23 ± 2.4
100 nM calphostin C	26 ± 3.4	98 ± 1.7	37 ± 3.1
25 nM GF 109203X	25 ± 4.2	97 ± 2.3	39 ± 2.3
Control	8 ± 3.5	95 ± 3.3	9 ± 3.4

Cells were incubated with the indicated protein kinase inhibitor for 6 days. DNA fragmentation was determined (diphenylamine assay) on day 3 of treatment. Apoptotic cells in preparations stained with acridine orange/ethidium bromide were evaluated using fluorescence microscopy on day 6. Cell viability was assayed by exclusion of trypan blue on day 6. Results represent means of three separate experiments

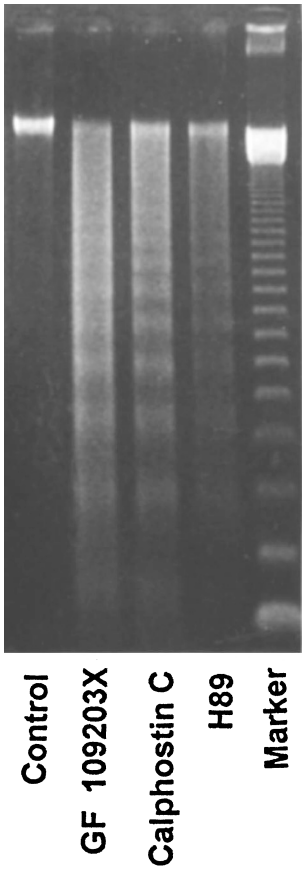

Figure 1 Induction of DNA fragmentation by protein kinase inhibitors in proliferating HL-60 cells. Control cells were treated with protein kinase inhibitors: 20 nM GF 109203X, 100 nM calphostin C and 50 nM H89. DNA was extracted after 72 h of treatment with inhibitor and electrophoresed in 1.5% agarose gel. Marker is the 123 bp DNA ladder

The remaining cells (14%) were considered alive since their apoptotic bodies were stained green by AO only. Therefore, the total population of viable cells in the sample (95%) included the live cells with apoptotic characteristics (14%), as calculated above.

It should be noted that cell death in control culture (no inhibitors used) on day 9 was 73% (data not shown). This was associated with DNA damage and morphological changes characteristic to apoptosis caused by the increased density of the cells in culture and acidification of the medium.

Apoptosis is associated with RA- and dbcAMP-induced differentiation

Granulocytic differentiation of HL-60 cells was induced by dbcAMP (350 μM) or RA (500 nM). These inducers of differentiation at the concentrations used typically results in a reduction of cell growth (25–30%) and viability (20–30%).[36]

In the present study both, dbcAMP and RA, induced cell differentiation in a dose-dependent manner. A dose-dependent increase of granulocytic differentiation was associated with an increase of apoptotic cells in the population (Figure 2). These cells can be recognised by the appearance of nuclear condensation, a marked decrease in cellular volume and formation of apoptotic bodies at the sixth day after induction of differentiation. The maximal concentrations of both inducers (500 μM dbcAMP and 700 nM RA) used in experiments resulted in an increase of the number of differentiated cells by 50% at day 4. Addditional 48 h of co-incubation of the cells with inducers of differentiation resulted in apearance of apoptotic cells in the cultures (29% in dbcAMP-induced and 46% in RA-induced) (Figure 2). In RA-induced differentiating cells internucleosomal DNA fragmentation was clearly visible after 48 and 72 h of induction (Figure 3A). The fraction of PI stained cells, which contained reduced DNA content due to degradation, appeared to be at the left side of the G1 peak on a histogram and was increasing during first 3 days of differentiation in a time-dependent manner (Figure 3B). These observations indicate that in dbcAMP- or RA-induced, into granulocytes differentiating HL-60 cell population part of the cells undergo apoptosis and this event is triggered not later than 48 h after induction of differentiation.

Effects of PK inhibitors on HL-60 cell differentiation and subsequent apoptosis

Further studies were undertaken to determine whether inhibitors of PKC or PKA could affect RA-induced (500 nM) granulocytic cell differentiation and differentiation-accompanying apoptotic events. HL-60 cells were exposed to two highly specific inhibitors of PKC, calphostin C and GF 109203X, and the inhibitor of PKA, H89. The EC 50 values of all inhibitors[13] used in our experiments had no effect on RA-induced cell viability (data not shown). The data presented in Figure 4 indicates that after 4 days of co-incubation of HL-60 cells with RA, 37% of cells were differentiated, while 26% of the

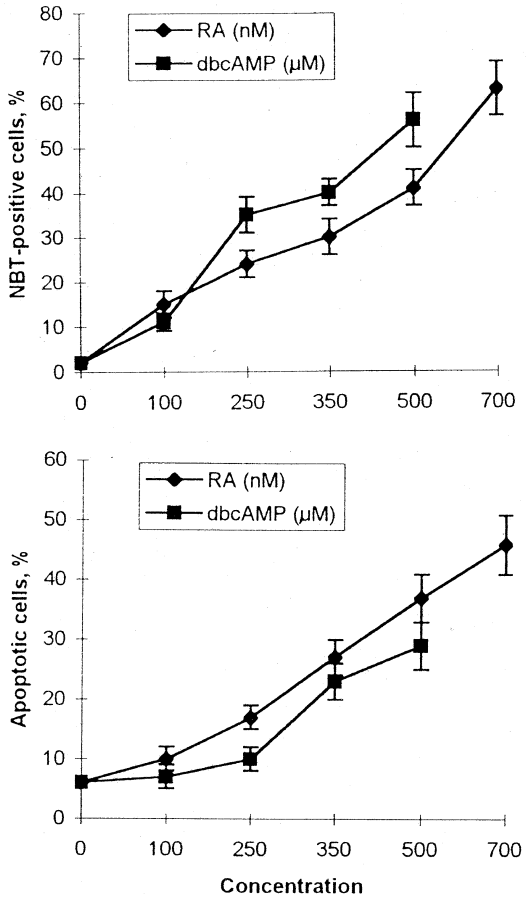

Figure 2 RA and dbcAMP potentiate apoptosis in HL-60 cell culture. Cells (4×10^5 cell/ml) were incubated with different concentrations of RA (nM) and dbcAMP (μM). Cell differentiation was determined by NBT reducing ability on day 4. Apoptotic cells were determined by morphological evidence in preparations stained with acridine orange/ethidium bromide on day 6. Data represent mean $\pm$ S.E.M. of three separate experiments

cells within same population had fragmented DNA at day 3.

Both the inhibitors of PKC (calphostin C and GF 109203X) potentiated RA-mediated cell differentiation and DNA fragmentation with respect to untreated control, while the PKA inhibitor (H89) suppressed cell differentiation and did not alter the DNA fragmentation (Figure 4). The electrophoretic analysis of DNA (Figure 5) obtained from HL-60 cells after 3 days of co-incubation with RA and PKC or PKA inhibitors confirmed the results of DNA fragmentation presented in Figure 4. Thus, the results revealed that correlation between changes in the number of differentiated cells induced by RA and apoptotic cells in this differentiating HL-60 cell population can be altered. To check this phenomenon more precisely, RA- and dbcAMP-mediated differentiation of HL-60 cells have been modulated by using different concentrations of PKC inhibitor, calphostin C, and PKA inhibitor, H89 (Figures 6 and 7). The correlation between the number of differentiated and apoptotic cells was found during RA-induced differentiation when cells were exposed to calphostin C. dbcAMP-mediated differentiation was suppressed by calphostin C to 43%, while the

number of apoptotic cells was increased up to 147%, as compared to the apoptotic cells from the control population (Figure 6). PKA inhibitor, H89, suppressed both RA- and dbcAMP-mediated differentiation, however, the number of apoptotic cells diminished only in the case of dbcAMP, while in RA-differentiated cell population it remained at the

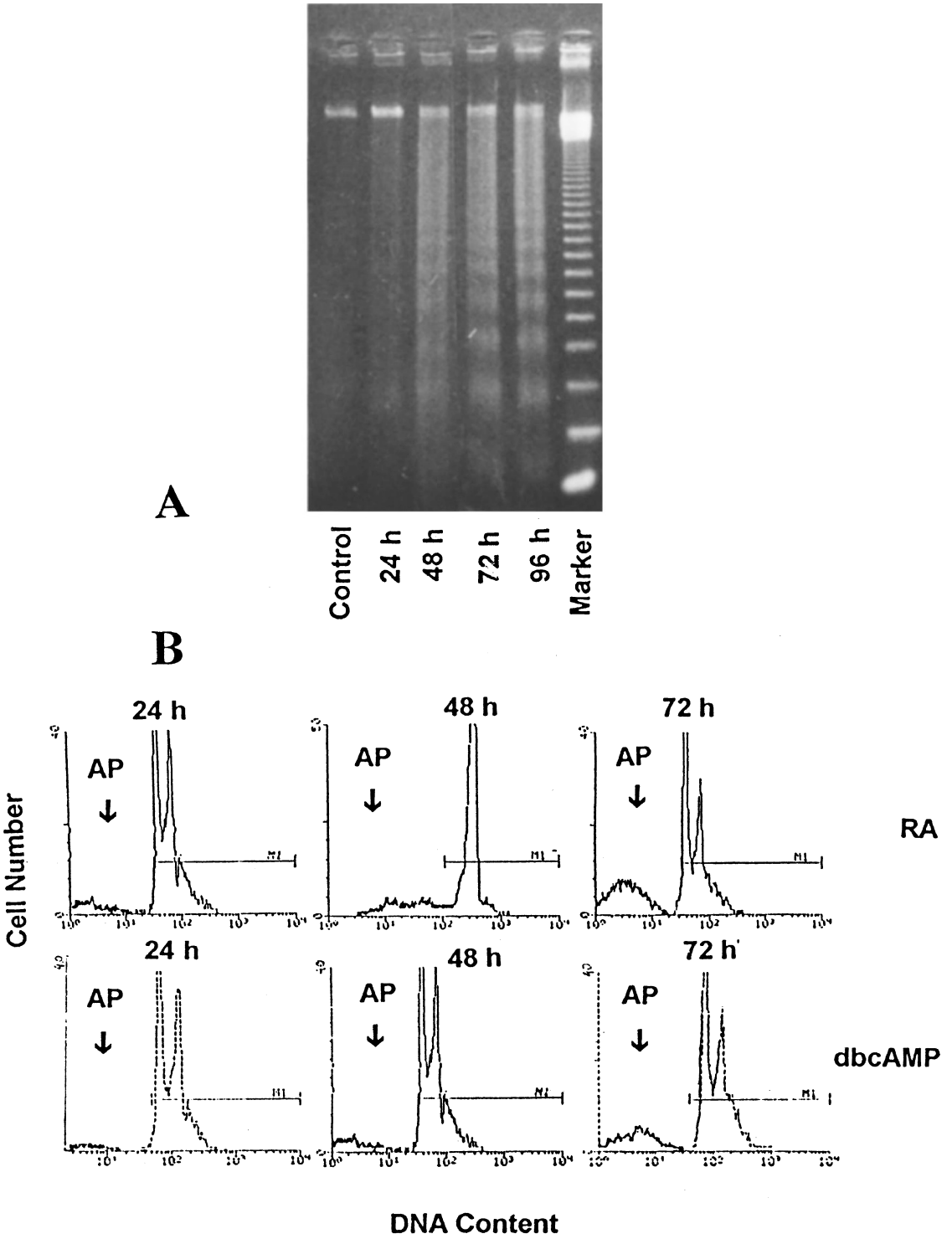

Figure 3 Time course of DNA fragmentation during RA-induced differentiation and flow cytometry analysis of cell cycle distribution in HL-60 differentiating cells. (**A**) HL-60 cells (4×10^5 cells/ml) were treated with 500 nM RA for 4 days. DNA was extracted daily during 96 h of induction and electrophoresed in 1.5% agarose gel. Marker is the 123 bp DNA ladder. (**B**) Representative example of flow cytometric analysis of HL-60 cells treated with 500 nM RA and 350 μM dbcAMP during 72 h. An hypodiploid peak (subG1) represents the amount of apoptotic cells (AP). The percentages of apoptotic nuclei were for RA after 24, 48 and 72 h of induction 12.2 $\pm$ 0.3%, 21.0 $\pm$ 3.3%, 28.2 $\pm$ 2.5%, respectively; for dbcAMP – 11.9 $\pm$ 1.2%, 19.6 $\pm$ 2.7%, 24.2 $\pm$ 3.6%, respectively. Results represent means of three separate experiments

same level (Figure 7). The growth of differentiating HL-60 cells was suppressed with various concentrations of calphostin C or H89 on day 4 (Figures 6 and 7). Protein kinase inhibitors caused a similar degree of growth inhibition as compared to the drug untreated control cells (73% for RA and 77% for dbcAMP). This indicates the cytostatic action of protein kinase inhibitors.

More evidence indicating that apoptosis can be separated from differentiation has been obtained in experiments in which level of differentiation was modulated by the suppression of PKC activity in proliferating HL-60 cells 24 h before induction of differentiation. In these experiments (1) the cells were pretreated with calphostin C for 24 h, washed, and medium supplemented with RA or dbcAMP instead of calphostin C was added (−24–0 h); (2) after 24 h pretreatment with calphostin C the cells were subsequently induced to differentiate with RA or dbcAMP in the presence of calphostin C (−24–96 h); (3) calphostin C was added along with one of the inducers of differentiation (0–96 h). The results presented in Figure 8 clearly demonstrate drastic differences in RA-induced cell differentiation after pretreatment of the cells with calphostin C, i.e. the number of differentiated cells was diminished to 60–75% (−24–0 h, −24–96 h) or increased to 145% (0–96 h) as compared to the control differentiating cells growing in the absence of calphostin C. However, in all of the above mentioned cell populations treated with calphostin C the number of apoptotic cells was increased to 145 to 135%, respectively. In the dbcAMP-induced cell culture any experimental trial to inhibit PKC resulted in the suppression of differentiation to 50–55%, while the number of apoptotic cells was always increased (Figure 8). The fact that apoptosis was induced by PKC inhibitors, calphostin C or GF 109203X, in proliferating cells, and that PKC inhibition before induction of differentiation caused a higher level of apoptosis when compared to PKC inhibition during differentiation, suggests that a fraction of the apoptotic cells arise from proliferating cells or cells being at early stages of differentiation.

Effects of RA pretreatment on dbcAMP-induced differentiation and apoptosis, and effects of dbcAMP pretreatment on RA-induced differentiation and apoptosis

In our previous study[37] we have shown that the pretreatment of HL-60 cells with dbcAMP for the duration

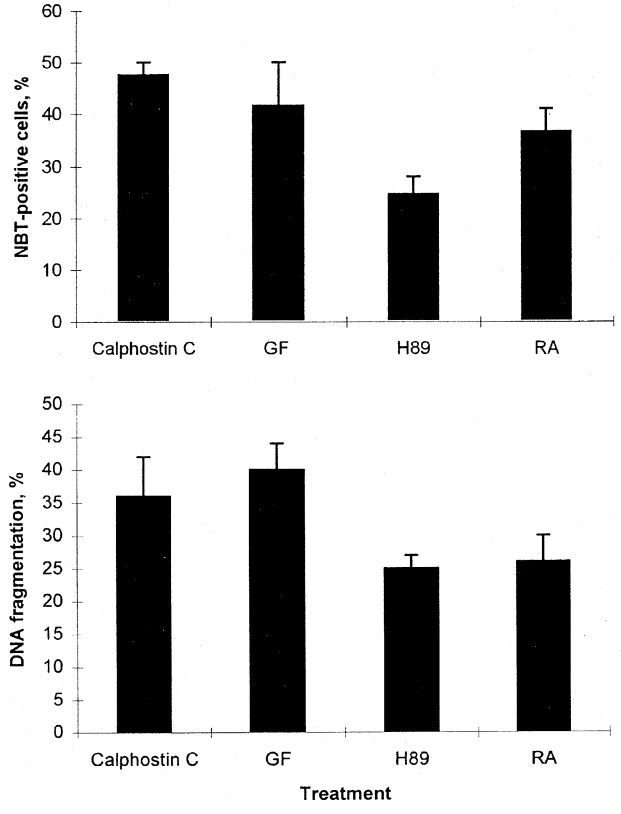

Figure 4 Effects of protein kinase inhibitors on RA-mediated HL-60 cell differentiation and induction of DNA fragmentation. Cells were incubated with 500 nM RA and protein kinase inhibitors: 100 nM calphostin C, 20 nM GF 109203X and 50 nM H89. Cell differentiation was determined on day 4 after induction by NBT reducing ability and expressed as mean percentages of the viable cell number. Samples for quantitation of DNA fragmentation (diphenylamine assay) were taken on day 3 after induction and results were expressed as the percentage of total DNA. Results represent a mean ± S.E.M. of three separate experiments

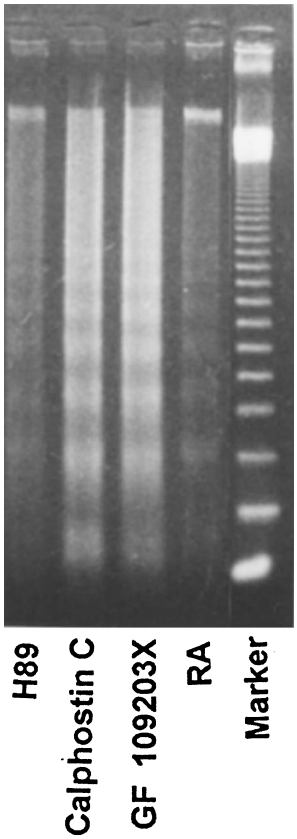

Figure 5 Induction of DNA fragmentation by protein kinase inhibitors in differentiating HL-60 cells. Cells were induced by 500 nM RA and treated with protein kinase inhibitors: 100 nM calphostin C, 20 nM GF 109203X, 50 nM H89. DNA was extracted after 72 h of co-treatment and electrophoresed in 1.5% agarose gel. Marker is the 123 bp DNA ladder

of one cell cycle only strongly potentiated RA-mediated differentiation, while the pretreatment with RA suppressed dbcAMP-mediated differentiation. The aim of the present study was to assess the effects of RA- or dbcAMP-pretreatment on modulation of apoptosis in a differentiating population of dbcAMP- or RA-induced cells, and whether

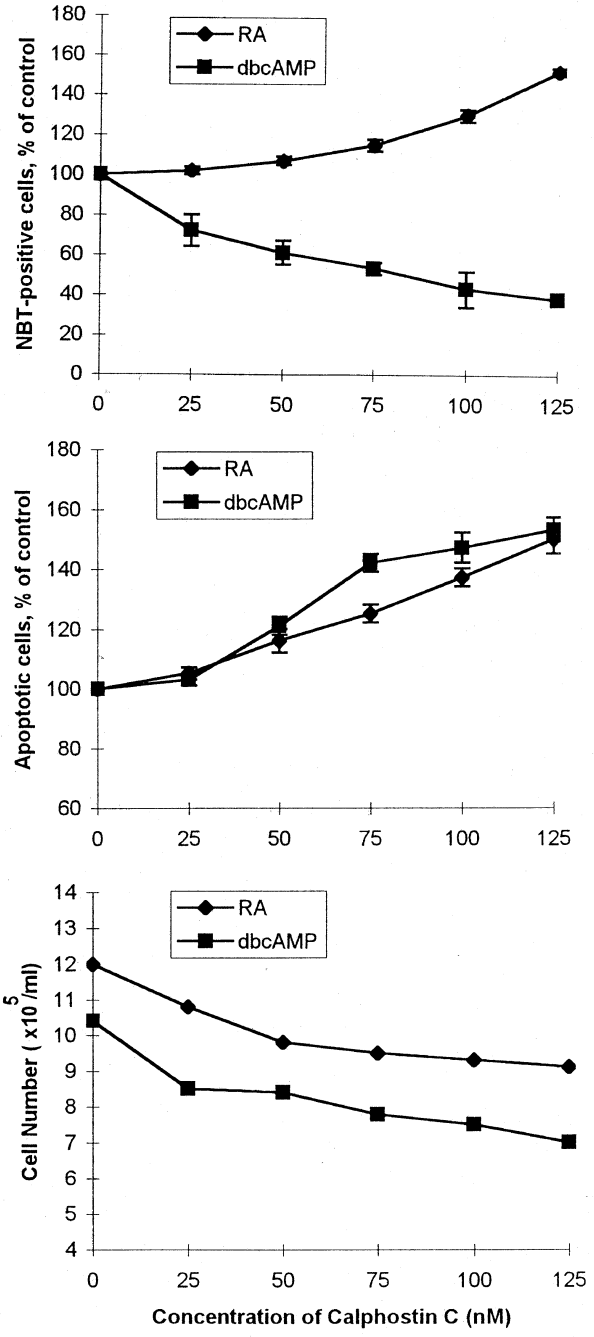

Figure 6 Effects of various concentrations of PKC inhibitor calphostin C on RA and dbcAMP-mediated HL-60 cell growth, differentiation and apoptosis. Cells (4×10^5 cells/ml) were incubated with 500 nM RA or 350 μM dbcAMP and various concentrations of calphostin C. Cell differentiation was determined on day 4 after induction by NBT reducing ability. Apoptotic cells were determined by morphological evidence on day 6. Data are expressed in relation to that of control (untreated with inhibitor) and presented as a mean $\pm$ S.E.M. of six separate experiments. Cell growth curves on day 4 are representative examples from four separate experiments in which variation of the data was less than $\pm$ 10%

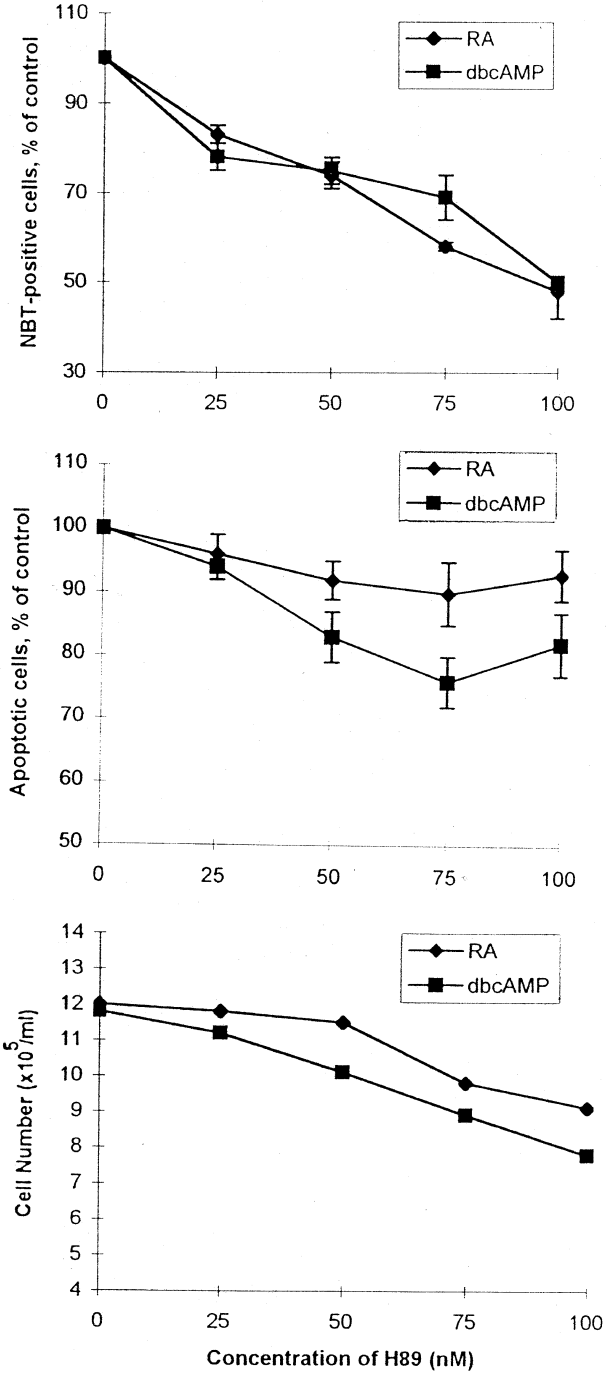

Figure 7 Effects of various concentrations of PKA inhibitor H89 on RA- and dbcAMP-mediated HL-60 cell growth, differentiation and apoptosis. Cells (4×10^5 cells/ml) were incubated with 500 nM RA and 350 μM dbcAMP and various concentrations of H89. Cell differentiation was determined on day 4 after induction by NBT reducing ability. Apoptotic cells were determined by morphological evidence on day 6. Data are expressed in relation to that of control (untreated with inhibitor) and presented as a mean $\pm$ S.E.M. of six separate experiments. Cell growth curves on day 4 are representative examples from four separate experiments in which variation of the data was less than $\pm$ 10%

apoptotic events correlate with distinct levels of differentiation. The obtained data indicate that there was no such correlation at all (Figure 9): (1) pretreatment with RA slightly altered dbcAMP-induced differentiation (by 20%), but, on the contrary, significantly promoted apoptosis depending on RA concentration (700 nM); (2) pretreatment with dbcAMP highly promoted RA-mediated differentiation (120–180%) and did not cause changes in the level of apoptosis.

Cell cycle analysis

To examine changes in the cell cycle distribution associated with cell differentiation or apoptosis, the flow cytometric analysis of HL-60 cells treated with different agents or their combinations has been performed. The data shown in Table 2 demonstrate the results of the cell cycle analysis obtained after 24, 48 and 72 h of exposure of HL-60 cells to (1) inducers of differentiation (RA or dbcAMP); (2) calphostin C and its combination with one of the inducers or (3) RA after pretreatment with dbcAMP or *vice versa*.

The data presented here revealed two general groups of cell cultures which differ in the ratio of cells arrested in G1 and G2/M phases of the cell cycle (Table 2, A and B, bold numbers). In group A obvious tendency in significant, time-dependent increase in the fraction of G1 phase during cell differentiation (from 38–44% to 65–78%) was noted. After the same period of time the number of cells in the G2/M phase was slightly decreased (by 3–8%). Our previous results reported above showed that cell differentiation was more pronounced than apoptosis in these cell cultures. On the contrary, data presented in group B (Table 2) indicates that under these experimental conditions the higher number of apoptotic cells was present in cell cultures when compared to the number of differentiated cells. In these populations the

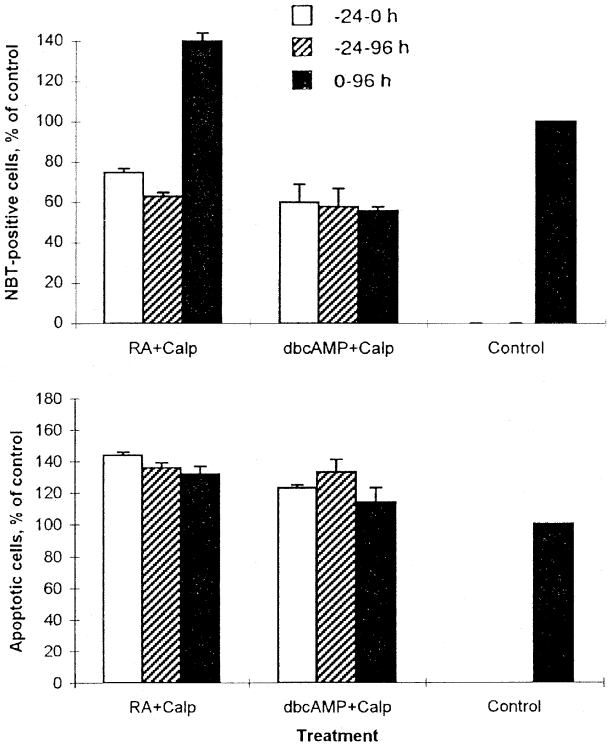

Figure 8 Effects of PKC inhibitor, calphostin C on the level of differentiation and apoptosis of HL-60 cell. Cells (4×10^5 cells/ml) were induced with 500 nM RA or 350 μM dbcAMP and cultivated with PKC inhibitor, 100 nM calphostin C, during different time shown as bars: (−24–96 h) pretreatment before induction of differentiation, (0–96 h) full time of differentiation, (−24–96 h) pretreatment and full time of differentiation. In control experiments only RA was added to the culture medium. The percentages of differentiated and apoptotic cells are given as a percentage of control cells. Results represent mean ± S.E.M. of three separate experiments

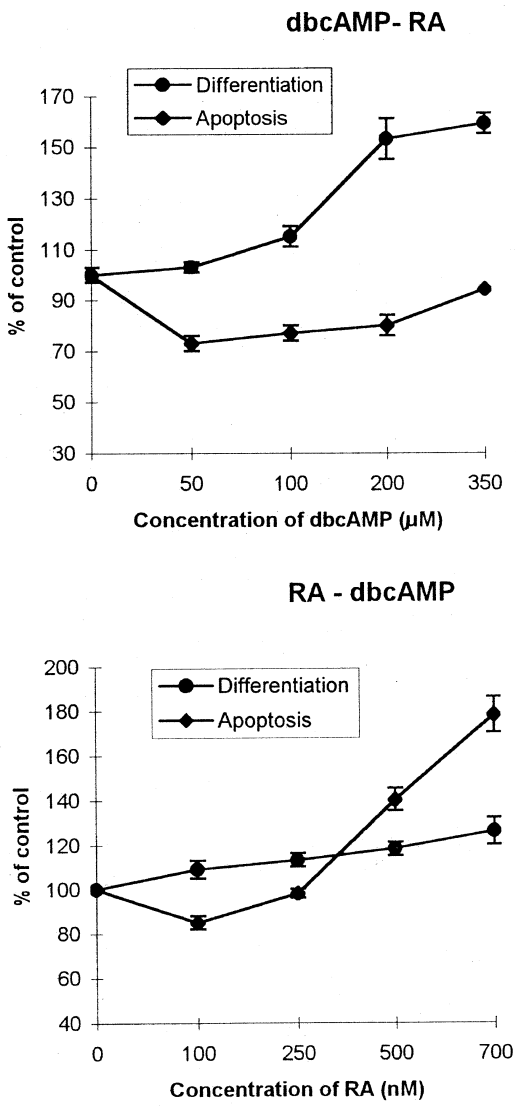

Figure 9 Effects of dbcAMP or RA pretreatment on RA-induced or dbcAMP-induced HL-60 cell differentiation and apoptosis. HL-60 cells (4×10^5 cells/ml) were pretreated with different concentrations of RA or dbcAMP for 24 h before the induction of differentiation, after the drugs were washed out and the cells were incubated with 350 μM dbcAMP or 500 nM RA, respectively for a further 96 h. Cell differentiation was determined on day 4 after induction by NBT reducing ability. Apoptotic cells were determined by morphological evidence on day 6. The number of differentiated and apoptotic cells were expressed as a percentage of control cells (without pretreatment). Results represent a mean ± S.E.M. of three separate experiments

Table 2 Flow cytometric analysis of the cell cycle distribution in HL-60 cells

	Time of treatment (h)			
	24	48	72	72
	Cell cycle distribution (%)			
Treatment	**G1/S/G2+M**	**G1/S/G2+M**	**G1/S/G2+M**	**subG1**
A				
RA	**38**/43/19	**56**/31/13	**65**/23/12	**28**
dbcAMP	**42**/38/20	**55**/20/25	**75**/13/12	**24**
RA+Clp	**50**/28/22	**66**/19/15	**78**/8/14	**48**
dbcAMP→RA	**44**/34/22	**60**/22/18	**65**/17/17	**46**
B				
Clp	42/36/**22**	38/34/**28**	38/39/**23**	**43**
dbcAMP+Clp	51/22/**27**	61/13/**26**	65/15/**20**	**45**
Clp→RA	42/38/**20**	49/32/**19**	52/26/**22**	**40**
Clp→dbcAMP	41/38/**21**	48/29/**23**	37/33/**30**	**42**
RA→dbcAMP	41/40/**19**	66/18/**16**	55/19/**26**	**64**
Control	39/51/9	42/47/10	46/45/10	12

HL-60 cells were treated: (1) with 500 nM RA or 350 μM dbcAMP; (2) with 100 nM calphostin C (Clp) alone or in combination with RA or dbcAMP (RA+Clp, dbcAMP+Clp); with RA or dbcAMP after pretreatment with calphostin C for 24 h (Clp→RA, Clp→dbcAMP); (3) with 500 nM RA after pretreatment with 350 μM dbcAMP for 24 h (dbcAMP→RA) or with 350 μM dbcAMP after pretreatment with 700 nM RA for 24 h (RA→dbcAMP). The cell cycle phase distribution of HL-60 cells during 72 h of induction was determined from DNA frequency distribution histograms of PI stained cells using CellFITT program (Becton-Dickinson). The per cent of apoptotic cells ($subG_1$) was established from respective histograms of PI stained cells following incubation in hypotonic citrate buffer using LysisII program (log scale). Each value is the average of two or more independent experiments with similar results

per cent of cells in the G2/M phase was approximately twofold increased (20–30%) compared to control cells (10%) and was higher in cell cultures from group A (12–17%) during 72 h of treatment. The per cent of cells arrested in the G1 phase in group B remained without significant changes.

As shown in Table 2, 12% of the cells in growing control population were with apoptotic characteristics (subG1 peak) after 72 h, while in differentiating cell cultures apoptotic subG1 peak was increased to 24–48% (group A) or to 40–64% (group B) after the same period of time. It should be pointed out that calphostin C-induced apoptosis resulted in appearance of cells with a significantly enhanced hypopdiploid peak (about 46%, bold numbers). Also, in RA-pretreated dbcAMP-induced cell culture subG1 peak was markedly increased (64%) in comparison to the opposite combination of the treatment (46%).

It is important to note that flow cytometric data presented in Table 2 consider very closely with the results of DNA cleavage identified by DNA fragmentation assay (Table 1, Figure 4).

Thus, the quantitative analysis of the cell cycle distribution (G1 *versus* S *versus* G2/M) clearly demonstrates that there is the correlation between the number of cells arrested in G1 or G2/M phase of the cell cycle and differentiation or apoptosis, respectively.

Discussion

In order to improve the strategy for therapy of leukemias, it is of interest to know the regulatory mechanisms gearing granulocytic differentiation and the subsequent apoptosis. It has been reported that HL-60 cells *in vitro* differentiated to granulocytes, like their normal counterparts in blood die via apoptosis.[10,12,48] However, more recent studies demonstrate that actively proliferating cells can be triggered to apoptosis by modulation of the activities of PKC and PKA[13,41,49] and the differentiating cells may die via apoptosis at distinct stages of differentiation as well.[50] Moreover, there are indirect evidences that differentiation and apoptosis are separately regulated entities.[42,51,52] In addition, some studies have shown that induction of differentiation mediates increased resistance of cells to apoptosis-inducing agents.[53–55] This phenomenon can enhance an already induced apoptotic program in leukemia cells.[13,56,57]

In this study we have shown that apoptotic events can be partially dissociated from differentiating cells in a HL-60 leukemia cell population induced to differentiate to granulocytes by RA and dbcAMP. This can be achieved by modulation of the PKA and PKC activities in proliferating cells before induction of differentiation or in differentiating cells at the early stages of differentiation as (1) when the activity of PKC is inhibited during dbcAMP-mediated differentiation (Figure 6) or the activity of PKA is suppressed during RA-mediated differentiation (Figure 7); (2) when the activity of PKC are inhibited before induction of differentiation, regardless of the inducer used or (3) when HL-60 cells are exposed for one cell cycle to dbcAMP and then differentiate in the presence of RA or *vice versa*. The latter case, in our opinion, is interesting and promising for differentiation therapy of leukemias because dbcAMP drastically potentiates RA-mediated differentiation and does not raise the number of apoptotic cells. It is of interest also that in some cases the number of apoptotic cells can exceed the number of differentiated ones in population, e.g. in both RA- and dbcAMP-induced differentiating cells pretreated with calphostin C, or dbcAMP-induced differentiating cells pretreated with calphostin C, or dbcAMP-induced differentiating cells pretreated with RA. Collectively, these data clearly demonstrate that *in vitro* differentiated mature granulocytes are not single source of apoptotic cells. Potentially there could be three sources of apoptotic cells in a differentiating promyelocytic leukemia HL-60 cell population: (1) proliferating cells resistant to inducers of differentiation but highly sensitive to all metabolic consequences caused by the inducer; (2) differentiating cells that are not capable of adjusting themselves to metabolic changes caused by inducers at distinct stages of differentiation and (3) the differentiated mature granulocytes dying due to ageing. Substantial evidence exist demonstrating that proliferating somatic cells contain an apoptotic program (for review see[58]). Short exposure of HL-60 cells to various concentrations of PKC inhibitor promotes these cells to initiate apoptosis.[13–59] In the case of RA-mediated differentiation the appearance of apoptotic cells by induction of differentiation can be explained by isomerization of RA to its isomer 9-*cis* RA. RA binds only to RAR receptors which is sufficient to induce differentiation, while 9-*cis* RA activates both classes of receptors – RAR and RXR, and ligand activation of RXR receptor is a major cause of induction of apoptosis.[14] The exposure of HL-60

cells to RA has been shown to cause an altered activity of some isoforms of PKC.[22,29] Also, the inhibition of the activity of PKC during the commitment stage markedly increases the level of differentiated HL-60 cells induced by RA.[36] This phenomenon can enhance an already induced apoptotic program.[13]

Mature neutrophils in blood have a short life span and die via apoptosis.[10] However, little is known about the circumstances of the induction of this apoptotic mechanism. The possibility exists that it can be activated from the onset of granulocytic differentiation. Alternatively, the cells possess endogenous mechanisms capable of inhibiting death signals. The product of the bcl-2 gene is known to protect different cell types from apoptosis.[60] Inhibition of some isoforms of PKC by H7 or calphostin C reduces bcl-2 gene expression and protein amount in a human erythroleukemia cell line.[61] It has also been shown that PKC phosphorylates Bcl-2 and this modification of Bcl-2 is associated with the suppression of apoptosis in murine myeloid FDC-PI/ER cell line, Jurkat cells, and human T lymphoblastoid cell line.[62,63] Recently, it has been demonstrated that suppression of apoptosis in IC.DP murine pre-mast cells correlates with the PKCβII isoform translocation to the nucleus and up-regulation of Bcl-X_L.[64] The expression of bcl-2 is down-regulated during granulocytic differentiation mediated by DMSO and RA.[65,66] Thus, the co-operation of natural down-regulation and PKC deficient-dependent hypophosphorylation of Bcl-2 in HL-60 cells induced to differentiate by RA may overcome the threshold of the apoptotic mechanism, which should be activated only in mature cells without suppression of PKC.

In the present study the cell death process in HL-60 cells treated with various combinations of calphostin C and inducers was investigated by flow cytometry. Cells treated with calphostin C displayed an increased arrest in the G2/M phase of the cell cycle at 24 h and prolonged exposure of calphostin C led to a significant increase of an apoptotic peak in the subG1 region. The initiation mechanisms of both differentiation and apoptosis deal with the blocking of the cell cycle. The data obtained in this work shows that there is correlation between cell arrest in the G1 phase and differentiation, and the G2/M phase and apoptosis. The blocking of permanently proliferating stem cells in the G1 phase is a prerequisite for cell differentiation and this undoubtedly is a natural event fastened by evolution. Most probably in some cells the initiation of apoptosis might be generated due to an impair mechanism of S-phase of the cell cycle caused by inducers of differentiation. These changes cannot be repaired, however, it allows the cells to enter apoptosis from the G2/M phase. Recently, this phenomenon has been described by.[67–71]

The fact that the pretreatment of HL-60 cells with dbcAMP strongly potentiates RA-induced differentiation and partially protects cells from apoptosis could be explained as follows. In RA-mediated differentiation the native state of PKA activity is necessary for induction and commitment for granulocytic cell differentiation.[36] Most probably, due to the ability of PKA to block the cell cycle in a variety of cancer cells[72] it may have synergistically potentiated suppression of the RA-induced progression of cells through the S-phase. Exposing of HL-60 cells to 500 μM dbcAMP results in 300-fold rapid (3 h) and sustained rise in intracellular cAMP during the commitment stage (48 h) of differentiation. This results in twofold increase in the number of cells arrested in the G0/G1 phase in comparison to the RA-treated cell population.[8] In HL-60 cells no or negligible elevations in intracellular cAMP level appeared in response to RA.[20,73] The positive role of PKA has been established in both the induction and the commitment of HL-60 cell differentiation mediated by vitamin D_3.[26] It is also known that a cAMP responsive element is involved in RA-dependent RARβ2 promoter activation.[74] Therefore, over-expression of RARβ2 in comparison to RXR can change the equilibrium between differentiation and apoptosis to favour the former.[75] Recently, it has been shown that the elevated level of cAMP markedly inhibits neutrophil apoptosis.[76] In RA-mediated differentiation besides the basal level of cAMP the activity of adenylate cyclase rises only after 96 h.[20] Therefore, very high levels (300-fold) of cAMP in dbcAMP pretreated HL-60 cells can synergistically act with cAMP levels after induction by RA. These latter events potentially may protect mature neutrophils against apoptosis in differentiating population. Finally, it should be pointed out that the agents elevating intracellular cAMP levels in HL-60 cells active nuclear factor kappa B (NF-κB).[77] This transcription regulating factor similarly as Bcl-2 mediates stress-induced cellular survival response (for review see[78,79]). However, Bcl-2 and NF-κB do not co-operate in suppression of signalling pathways of apoptosis.[80]

In summary, our results reveal the importance of stage-specific PKA and PKC activity in promotion or suppression of apoptosis and differentiation in the promyelocytic leukemia HL-60 cells. Further studies are warranted to more clearly delineate various aspects of this working hypothesis.

Materials and Methods

Reagents

RA, dbcAMP, PMA, NBT, RNAse A, proteinase K, ethidium bromide, diphenylamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calphostin C and GF 109203X were from Calbiochem. Agarose DNA grade was from AB Kemila-Preparat. The stock solutions used were: PMA (1 mg/ml), H89 (2 mM), calphostin C (1 mM) and GF 109203X (1 mM) in DMSO; RA (500 μM) in ethanol and dbcAMP (10 mM) in double distilled water. Stock solutions were stored at -20°C. In all experiments the stock solutions were diluted in culture medium to obtain the required concentration of the substance of interest.

Cell culture conditions

The human promyelocytic HL-60 cell line was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island Laboratories, NY, USA) in a 5% CO_2 humidified incubator at 37°C. For each experiment, logarithmically grown HL-60 cells were inoculated in 5 ml in 75 ml tissue culture flasks at a density of 4×10^5 cells/ml. In differentiation experiments, inducers (350 μM dbcAMP and 500 nM RA) and protein kianse inhibitors were added to the culture and the

cells were incubated for 96 h. In experiments with pretreated cells, the cells were incubated with an inhibitor of protein kinase for 24 h before induction. It was washed out from the culture medium by centrifugation at $500 \times g$ for 5 min. The pellet was rinsed once with fresh medium in the same manner and resuspended in the medium containing the inducer or both the inducer and the inhibitor of the protein kinase.

Cell viability and growth

Cell viability was assayed by exclusion of 0.2% trypan blue. Cell numbers were measured by counting cells in suspension with a haemocytometer. For dose response determination, protein kinase inhibitors were added to a final volume of 5 ml of cell culture treated with inducer, while control cultures were incubated in the absence of inhibitors (for NBT-positive cell determination). The data for cell viability and growth at 96 h after induction of differentiation were expressed in relation to untreated controls.

NBT reduction assay

The degree of differentiation was assayed by the ability of cells to reduce NBT to insoluble blue-black formazan on stimulation by PMA.[5] 100 μl of suspension from the culture medium was mixed with an equal volume of 0.2% NBT dissolved in phosphate-buffered saline containing 40 ng/ml of PMA and incubated at 37°C for 30 min. NBT-positive cells were counted using the haemocytometer. At least 200 cells were scored for each determination, and the number of cells positive for NBT reductivity were expressed as a percentage of the viable cell number.

Detection of apoptosis

Cell morphology was evaluated using fluorescence microscopy. At the end of each incubation, cells were pelleted at $500 \times g$ for 5 min and resuspended in 100 μl PBS without Ca^{2+} and Mg^{2+} (5×10^6 cells/ml). The extent of apoptosis was determined as the number of cells undergoing nuclear fragmentation and condensation with the presence of cell surface blebs as apoptotic bodies using fluorescence microscopy with 0.01% acridine orange/0.01% ethidium bromide mixture (1 : 1, v/v), 6 μl for 100 μl cell suspension.[43] At least 300 cells were scored for each determination and the number of cells positive in the test were expressed as the percentage of the total cell number.

Quantitation of DNA fragmentation

HL-60 cells treated with PK inhibitors for 72 h were pelleted by centrifugation at $500 \times g$ for 5 min and rinsed once with PBS. The aliquots of cell suspension (5×10^6 cells) were lysed by addition of 100 μl ice-cold lysis buffer containing 5 mM Tris, 20 mM EDTA and 0.5% (v/v) Triton X-100, pH 8.0, and samples were allowed to lyse for 15 min on ice before centrifugation for 15 min at $13\,000 \times g$ in a microcentrifuge to separate intact chromatin (pellet) from DNA fragments (supernatant).[44] Pellets were resuspended in 300 μl of buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0, pellet and supernatant samples were analyzed for DNA content using the diphenylamine reagent.[45]

Isolation of apoptotic DNA fragments

After harvesting, the cell samples were washed with phosphate buffered saline (PBS) and pelleted by centrifugation. The cell pellets were then treated for 10 s with lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5; 10 μl per 10^6 cells, minimum 50 μl). After centrifugation for 5 min at $1600 \times g$ the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 h with RNAse A (final concentration 2.5 μg/μl) at 56°C followed by digestion with proteinase K (final concentration 2.5 μg/μl) for at least 2 h at 37°C. After addition of 1/2 v 10 M ammonium acetate, DNA was precipitated with 2.5 vol. ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).[46]

Electrophoresis of DNA

DNA (15 μg) in 10 μl of TAE buffer containing 0.2 vol. 30% glycerol, 0.25% bromphenol blue was loaded into each well and electrophoresed on a 1.5% agarose gel containing TAE running buffer (40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) at 100 V for 2–3 h. The agarose gel was stained with ethidium bromide for 10 min and destained in H_2O. The DNA was visualised by UV illumination. The DNA marker of 123 base pairs was used as a molecular weight standard (Sigma Chemical Co. USA).

Cell cycle analysis

Untreated or drug treated cells were collected by centrifugation, suspended in PBS and fixed in ice-cold 70% ethanol (ratio 1 : 10) for 24 h at −20°C. Fixed cells were split into two aliquots. The cells from first aliquot were centrifuged at $800 \times g$ for 5 min and cell pellet (from $1-2 \times 10^6$ cells/ml) were resuspended in 40 μl of phosphate-citrate buffer, consisting of 192 parts of 0.2 M Na_2HPO_4 and eight parts of 0.1 M citric acid (pH 7.8) at room temperature for at least 30 min.[47] After centrifugation at $1000 \times$ g for 5 min, the cells were suspended in 1 ml PBS containing propidium iodide (PI) (50 mg/ml) and RNAse (0.2 mg/ml) in polypropylene tubes and incubated at room temperature for 30 min. The tubes were then placed at 4°C in the dark before the flow cytometric analysis using a FACscan flow cytometer (Becton-Dickinson, USA), LysisII, Ver 1.1 software. The data were registered on a logarithmic scale. Apoptotic cells were detected on PI histogram as a hypodiploid peak. Fixed cells from second aliquot were stained with PI, as described earlier. Samples were analyzed on a flow cytometer. The percentage of cells in G1, S and G2/M was evaluated with CellFITT software.

Acknowledgements

The study was supported by fellowships from the Swedish Institute to Jurate Savickiene and the University of Umeå to Arunas Gineitis, and Lithuanian State Science and Studies Foundation, No 98802. Authors are grateful to Drs. Dainius Characiejus and Olga Nagaeva for technical assistance in flow cytometric analysis, and Dr. Gediminas Cepinskas for critical reading of this manuscript.

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