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Phospholipase A_2 has a role in proliferation but not in differentiation of HL-60 cells

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Abstract

The role of phospholipase A_2 (PLA_2) and its metabolite arachidonic acid (AA) in the proliferation and differentiation of HL-60 cells was investigated. Addition of either 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or retinoic acid (RA) to HL-60 cells for 2 h inhibited PMA-stimulated PLA_2 activity measured by [^{3}H]AA release. The inhibitor of PLA_2 activity, *p*-bromophenacyl bromide (BPB), significantly inhibited the proliferation of HL-60 cells and of fibroblast L929 and Swiss 3T3 cells in a dose-dependent manner. The effect of BPB on proliferation is probably through its inhibitory effect on PLA_2 activity, since the same doses of BPB which inhibited proliferation also inhibited PLA_2 activity determined by [^{3}H]AA release. The importance of PLA_2 activity for cell growth was further supported by the effect of two other PLA_2 inhibitors, $AACOCF_3$ and scalaradial, which inhibited HL-60 proliferation in a dose-dependent manner. BPB, $AACOCF_3$ and scalaradial significantly increased the doubling time to 32.4 h, 34.0 h and 31.8 h, respectively, compared with 24.6 h in the control. The inhibitory effect of BPB on HL-60 proliferation was reversed by addition of exogenous free AA to HL-60 cells, indicating the importance of this metabolite for the proliferation process. This reversible effect is specific for AA since it was not achieved by other fatty acids like linolenic acid (LA) or oleic acid (OA). Addition of free AA to HL-60 cells did not induce differentiation, as expected. Although BPB, $AACOCF_3$, or scalaradial inhibited proliferation, they did not induce differentiation nor affect the differentiation induced by $1,25(OH)_2D_3$ or RA. These results implicate that PLA_2 activity has no regulatory role in differentiation of HL-60 cells. The differential effect of PLA_2 inhibitors on proliferation and differentiation of HL-60 cells suggests that these two processes function under different regulatory mechanisms.

Keywords: HL-60 cells; Phospholipase A_2; Arachidonic acid; Proliferation; Differentiation

1. Introduction

Research into the biology of human leukemia has not only led to important advances in the understanding of this disease, but has provided some useful models for the study of cellular development. Leukemia-derived human myeloid cell lines, such as the promyelocytic HL-60 cell line, are often used as models to study terminal differentiation of myelomonocytic cells. Dimethylsulfoxide (DMSO), retinoic acid (RA) and other substances induce HL-60 cells to differentiate along the myeloid lineage, whereas 12-phorbol-13-acetate (PMA), IFN-γ, and

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$1,25(OH)_2D_3$ induce them to differentiate to cells with characteristics of monocyte/macrophages [1–5]. However, the signaling pathways responsible for induction of differentiation remain unclear.

Recent studies have suggested that calcium-dependent phospholipase A_2 (PLA_2) and its metabolites participate in the differentiation process. Some groups [6–9] have shown that the activity of PLA_2 was elevated during HL-60 cell differentiation induced by RA or DMSO to the granulocyte phenotype, or by $1,25(OH)_2D_3$, PMA, or IFN-γ to the monocyte phenotype. Arachidonic acid (AA) as well as some of the AA metabolites can act as first and second messengers [10]. It has been suggested that AA metabolites are involved in the transcriptional and post-transcriptional regulation of gene expression. For instance, AA metabolites regulate *c-fms* gene expression during monocytic differentiation induced by phorbol ester [11], and gene expression of the macrophage-specific colony stimulating factor in HL-60 cells induced by TNF [12]. In previous studies, we demonstrated the requirement of calcium ions for the differentiation of HL-60 cells induced by $1,25(OH)_2D_3$ [13]. This calcium requirement may be due to calcium-dependent PLA_2 activity required for the differentiation process.

In the present study, the role of PLA_2 in inhibiting proliferation and inducing differentiation to either the monocyte or granulocyte phenotype was studied. Our results indicate that PLA_2 activity is essential for the proliferation process of both HL-60 cells and fibroblast cell lines, however, has no effect on differentiation of HL-60 cells.

2. Materials and methods

$1,25(OH)_2D_3$ was kindly provided by Dr. M. Uskokovich from Hoffmann La-Roche Inc., Nutley, NJ. [(5a,12a,17ab)-12-acetyl-oxy)4,48-trimethyl-D-homoandrost-16ene-17,17a-dicarboxaldehyde)] (scalaradial) and arachidonyltrifluoromethyl ketone ($AACOCF_3$) were purchased from Biomol Research Laboratories Inc. Plymouth Meeting, PA. These compounds were reconstituted in ethanol, stored in concentrated solutions at −20°C, and diluted in the appropriate medium before each experiment. Retinoic acid (Sigma Chemical Co., St. Louis, MO) was freshly dissolved in ethanol before each experiment. The concentration of ethanol added to the cells did not exceed 0.1%. [^{3}H]arachidonic acid (100 Ci/mmol) was purchased from New England Nuclear, Boston, MA. *b*-Bromophenacyl bromide (BPB), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H- tetrazolium hydroxide] (XTT), phenazine methosulfate (PMS), linolenic acid (LA), oleic acid (OA), RNase and propidium iodide were from Sigma Chemical Co., St. Louis, MO. Thymidine-methyl-^{3}H (51.6 Ci/mmol) was purchased from Rotem Industries, Nuclear Research Center-Negev, Beer Sheva, Israel.

2.1. Cell culture

HL-60 cells were grown in stationary suspension culture in RPMI-1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 12.5 U/ml nystatin (Biological Industries, Beth Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HL-60 cell concentration was 2×10^5 cells/ml at the time that inducers of differentiation were added to the culture.

Fibroblast L929 and Swiss 3T3 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 12.5 U/ml nystatin in an atmosphere of 5% CO_2 at 37°C.

2.2. Cell number determination

Cell number was counted by coulter counter and hemacytometer. Cell viability was determined by trypan blue exclusion [14]. In all experiments, the percentage of viable cells was $> 95\%$.

2.3. [^{3}H]Thymidine incorporation

Equal volumes (100 μl) of cell culture were placed in triplicate into 96-well plates (Nunc, Denmark) [13]. Cells were pulsed with 1.25 μCi/well [^{3}H]thymidine for 3 h at 37°C and harvested onto glass fiber filters with a Titertek Cell Harvester (Skatron Lierbyen, Norway). After incubation with

[^{3}H]thymidine for 3 h at 37°C, fibroblasts were replaced with 50 μl/well tripsin-EDTA solution for 30 min. Typsinization was terminated by adding DMEM medium. Radioactivity bound to dry filters was determined using a Bio-Imaging Analyzer (Fuji Photo Film Co., Japan).

2.4. Estimation of the number of viable cells

This was carried out by the [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) reduction assay [15] or by [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay [16]. The blue formazan produced in these assays by mitochondrial dehydrogenases is proportional to the number of viable cells.

2.5. MTT assay

The culture medium from cells grown in 96-well microplates was removed, 50 μl MTT (500 μg/ml) was added to each well and incubation was carried out for 1.5 h at 37°C. Subsequently, the MTT solution was removed and replaced with 100 μl DMSO. The plates were vigorously shaken for 20 min on a shaking plate (Pathodx rotator, Diagnostic Products Corp.) at room temperature. The optical density of each well was measured on a Thermomax Microplate Reader (Molecular Devices, Melno Park, CA) using a test wavelength of 550 nm and a reference wavelength of 630 nm.

2.6. XTT assay

25 μl of XTT solution (1 mg/ml) and PMS at final concentration of 125 μM were added to each well which contained 100 μl HL-60 cell culture. After incubation for 4 h at 37°C the plates were mixed on a shaker (Pathodx rotator, Diagnostic Products Corp.) and the optical density of each well was measured on a Thermomax Microplate Reader (Molecular Devices, Melno Park, CA) using a test wavelength of 450 nm and a reference wavelength of 650 nm.

The results of parallel estimations, under different experimental conditions, by direct counting of trypan blue excluded cells and MTT or XTT colorimetric method strongly correlated with each other ($r = 0.99$). Since the O.D. values higher than 1.0 were not linear, results were transferred to the cell numbers.

2.7. Flow cytometry

HL-60 cells were treated with or without $1,25(OH)_2D_3$, RA, BPB, AACOCF3 or scalaradial for 2 days as described above. Cells (10^6) were collected, washed with PBS and fixed in 70% ethanol for one day at −20°C. Cells were then washed with PBS, treated for 40 min with 10 mg/ml RNase at room temperature and then stained with propidium iodide at a final concentration of 150 μg/ml. Resulting DNA distributions were then analyzed for proportion of cells in G_0/G_1, S, and G_2/M of the cell cycle. Data were collected with a FACScan Flow Cytometer (Becton-Dickinson, San Jose, CA) and analyzed using the PC-Lysys program (Becton-Dickinson). All experiments were repeated at least three times, each in duplicate.

2.8. Determination of differentiation

Differentiation was measured by superoxide generation detected by cytochrome c reduction [17]. The production of superoxide anion (O_2^-) was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c by the microtiter plate technique. Cells (2.5×10^5 cells/well) were suspended in 100 μl Hanks' Balanced Salts Solution (HBSS) containing ferricytochrome c (150 μM). Superoxide production by the cells was stimulated with the addition of PMA (50 ng/ml). The reduction of ferricytochrome *c* was followed by the change of absorbance at 550 nm every 2 min on a Thermomax Microplate Reader (Molecular Devices, Melno Park, CA). The maximal rates of superoxide generation were determined and expressed as nmoles $O_2^-/10^6$ cells/min using the extinction coefficient $E_{550} = 21$ mM^{-1} cm^{-1}.

2.9. PLA_2 activity

PLA_2 activity was assayed by measuring the release of radiolabeled arachidonic acid in the presence and absence of inhibitors [18]. 10^8 cells/ml were incubated for 1 h at 37°C in Ca^{2+}/Mg^{2+}-free PBS containing 1.5 μCi [^{3}H]AA (100.00 Ci/mmol). Cells

were washed once with PBS containing 0.1% fatty acids free human serum albumin and twice with PBS. The pellet was resuspended to 10^7 cells/ml in PBS with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ containing 0.1% human serum albumin. The incorporation of [^{3}H]AA was measured. The release of [^{3}H]AA was measured after incubation with the appropriate stimulus at 37°C. The reactions were terminated by centrifugation at 4°C and samples of the supernatants were counted using a liquid scintillation spectrometer (Packard 1900CA spectrometry).

2.10. Isolation of total RNA, amplification and detection

10^7 Cultured cells were washed twice with phosphate buffered saline, placed directly into 1 ml RNAzol B (Biotecx Laboratories, Houston, TX) and stored at −70°C until further processing to isolate RNA. RNA isolation was performed in a single step method as described by Chomezynski and Sacchi [19]. Total cellular RNA was reverse transcribed in a 20-μl reaction volume with 1 × RT buffer (Gibco BRL Life Technologies), dithiothreitol 1.25 mM (Gibco BRL), 0.5 mM dNTPs (Boehringer Mannheim GmbH, Germany), 65 U ribonuclease inhibitor (Amersham Life Science, UK), 200 ng primer p(dT)$_{15}$ (Boehringer) and 200 U M-MLV reverse transcriptase (Gibco BRL). The reverse transcriptase enzyme was inactivated by heating the mixture to 65°C for 10 min. The cytosolic PLA_2 ($cPLA_2$) primer pair was constructed according to the cDNA sequence of $cPLA_2$ [20]. It amplified a 628-bp using upstream primer: 5′-CTCTTGAAGTTTGCTCATGCCCAGAC-3′; and down stream primer: 5′-GCAAACATCAGCTCTGAAACGTCAGG-3′. The secreted PLA_2 ($sPLA_2$) primer pair was constructed according to the cDNA sequence of $sPLA_2$ [21]. It amplified a 278 bp PCR product and was composed of the following sequences, upstream primer: 5′-CACAGAATGATCAAGTTGACGGGA-3′; and down stream primer: 5′-AAACAGGTGGCAGCAGCCTTATCACA-3′. The PCR conditions were found to amplify cDNA molecules in a linear fashion. The PCR amplification reaction mixture contained: 5 μl cDNA, 1.25 U Taq DNA polymerase (USA Appligene, Pleasanton, CA), 1 × Taq polymerase buffer, 0.5 mM dNTPs (Boehringer) and 1.5 μM of each primer. The PCR amplification was carried out in a microprocessor controlled incubation system, Crocodile II (Apligene). After the mixture was preheated at 95°C for 3 min, amplification was performed using a step program (94°C, 1 min; 60°C, 2 min; 72°C, 3 min) followed by a 5 min final extension at 72°C. The product were electrophoresed on a 2% agarose (International Biotechnologies, New Haven, CT) and stained with ethidium bromide.

2.11. Statistical analysis

The differences in means were analyzed by Student's *t* test. The plots were drawn as least-square regression lines and tested by analysis of variance.

3. Results

The effect of $1,25(OH)_2D_3$ or RA on PLA_2 activity measured by [^{3}H]AA release in HL-60 cells was determined in order to study the involvement of PLA_2 activity in the proliferation and differentiation processes. As shown in Fig. 1, incubation of HL-60 cells with optimal concentrations [17] of either $1,25(OH)_2D_3$ (50 nM) or RA (1 μM) for 2 h significantly ($P < 0.001$) inhibited the release of [^{3}H]AA stimulated by 100 ng/ml PMA. Shorter incubation of HL-60 cells with either $1,25(OH)_2D_3$ or RA did not

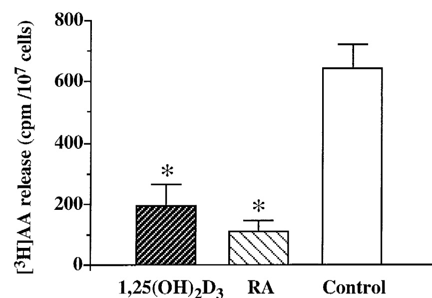

Fig. 1. The effect of $1,25(OH)_2D_3$ or RA on the release of [^{3}H]AA stimulated by PMA in undifferentiated HL-60 cells. Prelabeled HL-60 cells with [^{3}H]AA were incubated with either 50 nM $1,25(OH)_2D_3$ or 1 μM RA for 2 h before stimulation by 100 ng/ml PMA. The results show the release of [^{3}H]AA during 2 h of stimulation. There were significant differences in the release of [^{3}H]AA in the presence of $1,25(OH)_2D_3$ or RA compared with the control (* $P < 0.001$). The data are the mean ± S.E.M. from 4 experiments, each in duplicate.

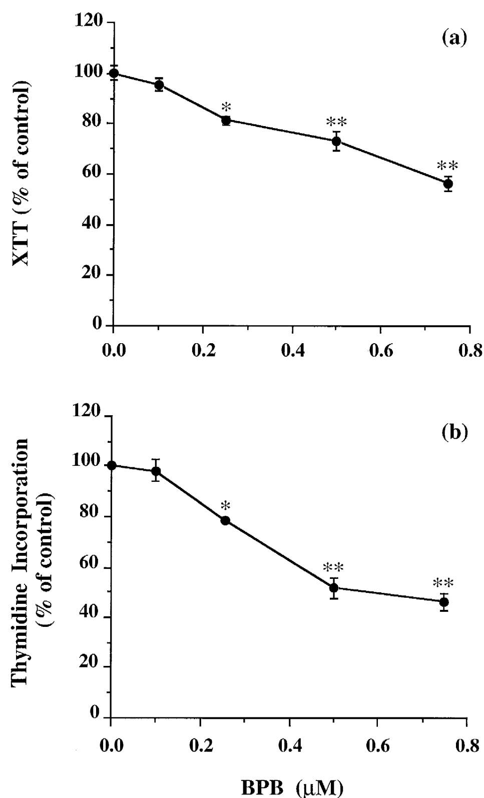

Fig. 2. The effect of BPB on HL-60 cell proliferation. Cell number was determined by XTT (a) or [^{3}H]thymidine incorporation (b). There was a significant inhibitory effect of BPB on HL-60 cell proliferation (* $P < 0.05$, ** $P < 0.005$). The results are the mean ± S.E.M. 3 days after addition of BPB in culture from 3 experiments, each in triplicate.

cause significant inhibition of [^{3}H]AA release. Either 1,25$(OH)_2D_3$ or RA alone did not affect the release of [^{3}H]AA.

The effect of the PLA_2 inhibitor, *b*-bromophenacyl bromide (BPB), on the proliferation of HL-60 cells was studied. Fig. 2 shows that BPB significantly inhibited the proliferation of HL-60 cells in a dose-dependent manner in a range of 0.1–0.75 μM. BPB (0.75 μM) caused maximum inhibition of 43.2 ± 1.7% or 46.3 ± 3.5%, measured by XTT or by thymidine incorporation, respectively. The concentration of BPB up to 0.75 μM did not affect cell viability as determined by trypan blue exclusion; higher concentrations of BPB (from 1 μM) were toxic. The inhibitory effect of BPB is time-dependent and maximum inhibition was obtained at day 3, as shown in Fig. 3.

PLA_2 activity measured by [^{3}H]AA release from prelabeled cells was examined in HL-60 cells incubated with 0.75 μM BPB. As shown in Fig. 4, [^{3}H]AA release stimulated by 100 ng/ml PMA was significantly inhibited in HL-60 cells during two days of culture with 0.75 μM BPB. Higher doses of BPB (5 μM or 10 μM) caused an immediate inhibition of the release of [^{3}H]AA stimulated by 100 ng/ml PMA in uninduced HL-60 cells (40% and 72% inhibition, respectively).

The inhibition of HL-60 cell proliferation caused by BPB could be reversed by the addition of exogenous free AA to the culture. As shown in Fig. 5, in the presence of 0.75 μM BPB HL-60 proliferation reached 74.0 ± 4.8% of the control at day 2. 24 h after addition of free AA in a range of 40 μM to 80 μM, the cell number significantly increased in a dose dependent manner. 80 μM AA caused a maximum reversible effect reaching 101.4 ± 5.1% of control compared with 56.8 ± 1.7% of control on day 3 without addition of AA. To study whether this reversible effect is specific for AA, the effect of the other fatty acids, LA or OA, was analyzed. LA or OA in a range of 40 μM to 100 μM did not reverse the inhibition induced by BPB (data not shown).

As shown in Fig. 6, the presence of two types of PLA_2; $cPLA_2$ and $sPLA_2$ could be demonstrated in

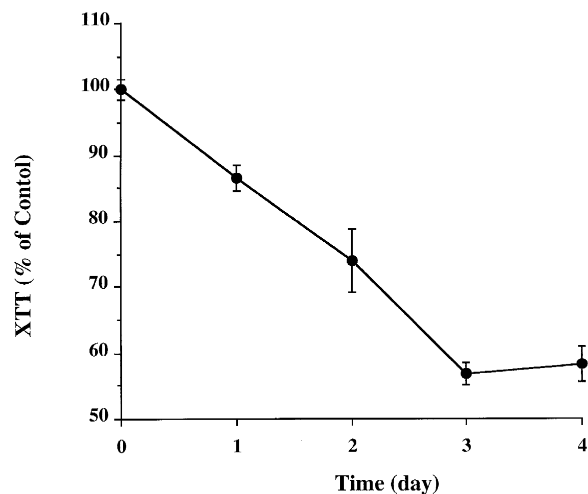

Fig. 3. The effect of BPB on HL-60 cell proliferation during 4 days. BPB at final concentration of 0.75 μM was added at day 0. Cell number was determined by XTT. The results, expressed as percentages of control, are the mean ± S.E.M. from 3 experiments, each in triplicate.

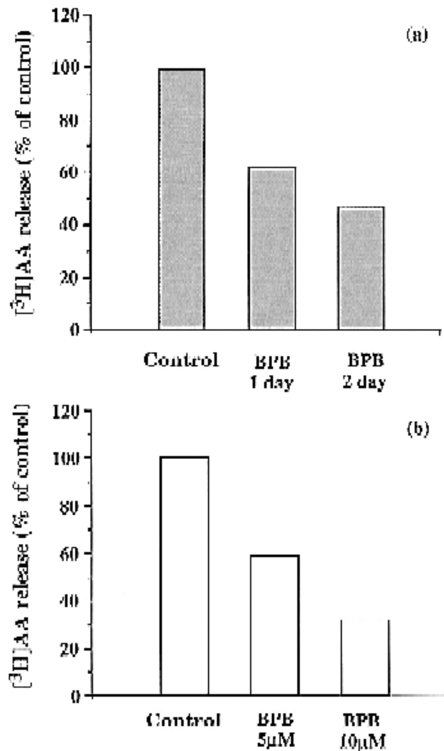

Fig. 4. The effect of BPB on $[^3H]$AA release from undifferentiated HL-60 cells stimulated by PMA. BPB at a low concentration of 0.75 μM for one or two days inhibited $[^3H]$AA release from undifferentiated HL-60 cells (a). BPB at high concentrations (5 μM, 10 μM) (b) rapidly inhibited $[^3H]$AA release from undifferentiated HL-60 cells. The results represent the release of $[^3H]$AA for 10 min and are expressed as percentage of control shown from a representative experiment of 3 independent experiments, each in duplicate.

HL-60 cells. The detection of $cPLA_2$- and $sPLA_2$-mRNA was performed by RT-PCR amplification of specific fractions of 628 bp and of 278 bp, respectively. The primers used in these reactions were constructed according to the published cDNA sequences as described in the Materials and Methods section.

The effect of two other inhibitors of PLA_2 activity, $AACOCF_3$ and scalaradial, on HL-60 cell proliferation was studied as well. As shown in Fig. 7, these two inhibitors significantly inhibited HL-60 cell proliferation in a dose dependent manner. The maximal inhibition was reached at day three: $41.8 \pm 1.9\%$ and $43.8 \pm 2.0\%$ by 30 μM $AACOCF_3$ and 7.5 μM scalaradial, respectively. Similar to the effect of BPB, $AACOCF_3$ and scalaradial suppressed HL-60 proliferation as a function of time reaching the maximum effect at day three (Fig. 8).

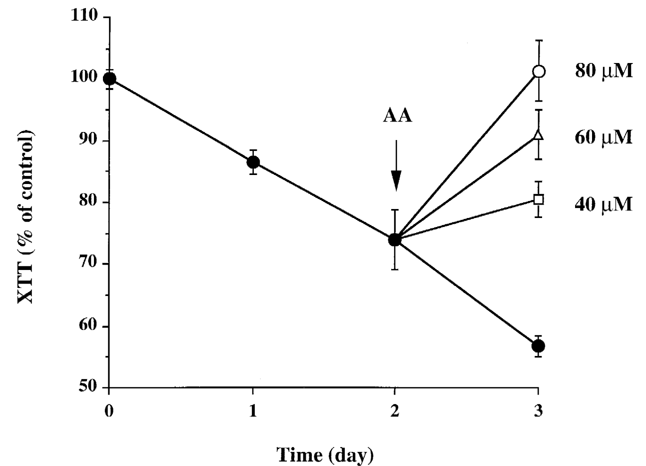

Fig. 5. The reversible effect of AA on the inhibition of HL-60 proliferation caused by BPB. HL-60 cells were cultured in the presence or absence of 0.75 μM BPB for 2 days. Then exogenous free AA (40–80 μM) was added to cell culture. The proliferation was determined by XTT during 3 days. The reversible effect of AA on HL-60 cell proliferation is significant ($P < 0.001$). The results are the mean $\pm$ S.E.M. from 3 experiments, each in triplicate.

In order to determine whether PLA_2 inhibitors cause arrest at a specific phase of the cell cycle, we studied their effect on the cell cycle by flow cytometry. Our results showed that there is no specific arrest at any phase of the cell cycle in contrast to $1,25(OH)_2D_3$ (50 nM) and RA (1 μM) which ar-

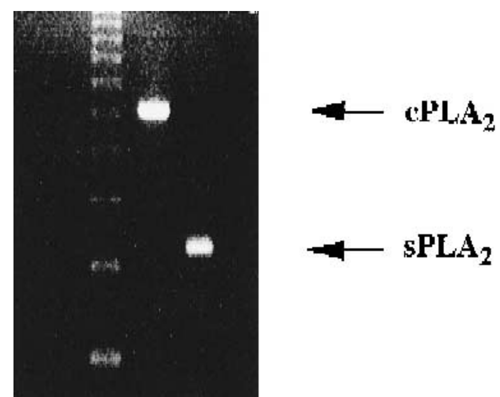

Fig. 6. RT-PCR analysis of $cPLA_2$ and $sPLA_2$ in undifferentiated HL-60 cells. The amplification of 628 bp of $cPLA_2$ and 278 bp of $sPLA_2$ are shown by arrows. The first lane shows the 123 bp size marker.

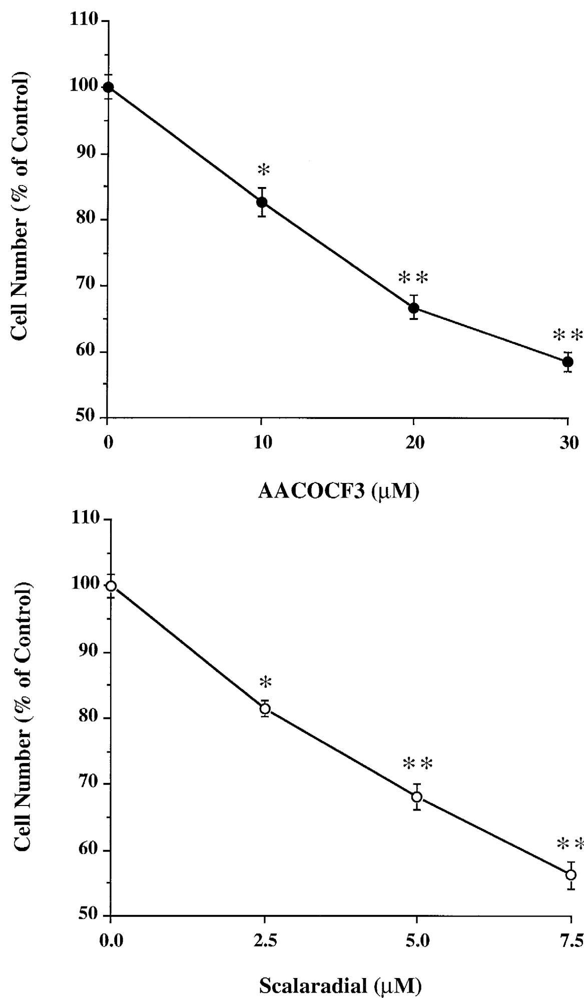

Fig. 7. The effect of PLA_2 inhibitors, $AACOCF_3$ (a) and scalaradial (b), on HL-60 cell proliferation. Cell number was counted by Coulter Counter. There was a significant inhibitory effect on HL-60 cell proliferation (* $P<0.03$, ** $P<0.005$). The results are the mean ± S.E.M. 3 days after addition of inhibitor in culture from 3 experiments, each in duplicate.

rested the HL-60 cell cycle at G_1 (Table 1). However, the three PLA_2 inhibitors decreased the proliferation rate of HL-60 cells. As shown in Fig. 9, BPB at 0.75 μM, $AACOCF_3$ at 30 μM, and scalaradial at 7.5 μM significantly increased the doubling time of HL-60 cells from 24.6 h in the control to 32.4 h, 34.0 h and 31.8 h, respectively.

In order to confirm the necessity of PLA_2 activity for the proliferation process, the role of PLA_2 on proliferation in other cell lines, fibroblast L929 and S3T3 cells, was studied. 5×10^4/ml L929 or 2×10^4/ml S3T3 were cultured in the absence or in the

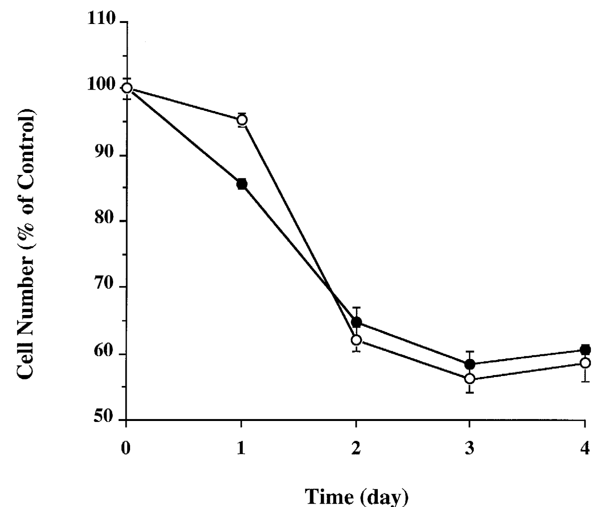

Fig. 8. The effect of $AACOCF_3$ and scalaradial on HL-60 cell proliferation during 3 days. Cell number was counted by Coulter Counter. $AACOCF_3$ (●) or scalaradial (○) was added to cell culture at a final concentration of 30 μM and 7.5 μM, respectively at day 0. The results are the mean ± S.E.M. 3 days after addition of inhibitor in culture from 3 experiments, each in duplicate.

presence of three different concentrations of BPB. BPB significantly inhibited fibroblast proliferation in a dose-dependent manner, achieving maximum effect

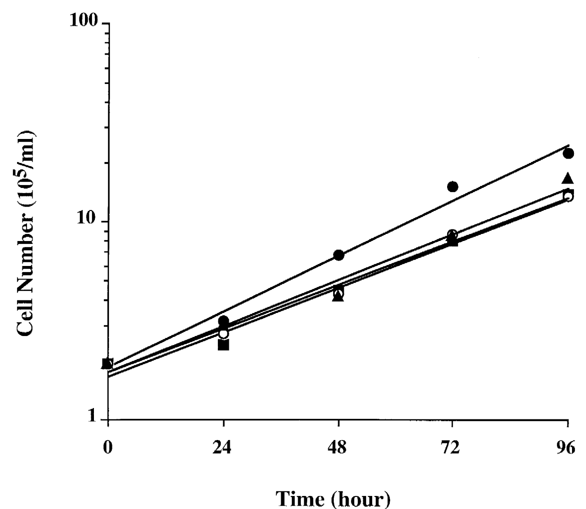

Fig. 9. Growth curves for HL-60 cells treated with or without PLA_2 inhibitors were generated from cell counts performed at approximately 24-h intervals. The doubling time was calculated from the slope of the logarithmic growth phase of the growth curve. The data are derived from three independent experiments in duplicate. Symbols: BPB (0.75 μM, ■); $AACOCF_3$ (30 μM, ○); scalaradial (7.5 μM ▲); and control (●).

Table 1
The effects of $1,25(OH)_2D_3$, RA, BPB, $AACOCF_3$, and scalaradial on cell cycle in HL-60 cells

	G_0/G_1 (%)	S (%)	G_2+M (%)
Control	42.1 ± 2.3	46.2 ± 2.2	11.7 ± 0.1
$1,25(OH)_2D_3$	68.4 ± 1.2	21.9 ± 0.8	9.7 ± 0.9
RA	59.1 ± 1.5	30.4 ± 1.0	10.5 ± 0.7
BPB	42.0 ± 0.2	44.2 ± 1.0	13.8 ± 1.1
$AACOCF_3$	44.9 ± 2.9	45.0 ± 3.6	11.1 ± 0.7
scalaradial	46.5 ± 2.2	41.6 ± 0.7	11.9 ± 1.5

Cells were exposed to 50 nM $1,25(OH)_2D_3$, 1 μM RA, 0.75 μM BPB, 30 μM $AACOCF_3$, or 7.5 μM scalaradial for 2 days before processing as described in Section 2. The results are mean ± S.D. of 3 independent experiments.

on day three. As shown in Fig. 10a, in the presence of 1 μM BPB, the growth of fibroblast L929 measured by MTT was 54.6 ± 7.4% of the control and growth of fibroblast Swiss 3T3 cell line was 37.2 ± 4.0% of the control. Similar results were obtained by thymidine incorporation (Fig. 10b). 1 μM BPB was not toxic to fibroblasts when assayed by trypan blue exclusion.

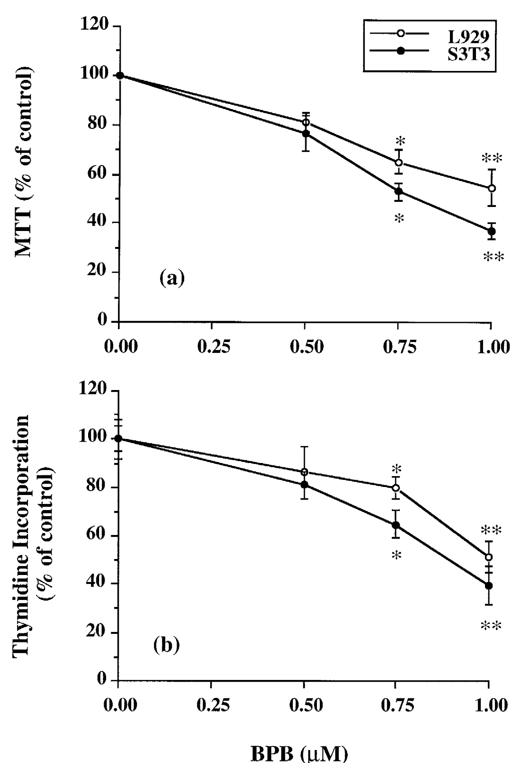

Fig. 10. The effect of BPB on fibroblast L929 and Swiss 3T3 cell proliferation. Cell number was determined by MTT (a) or [^{3}H]thymidine incorporation (b). There was a significant inhibition of BPB on proliferation of L929 and Swiss 3T3 cell lines (* $P<0.05$, ** $P<0.02$). Values represent the mean ± S.E.M. for 3 separate experiments, each in triplicate.

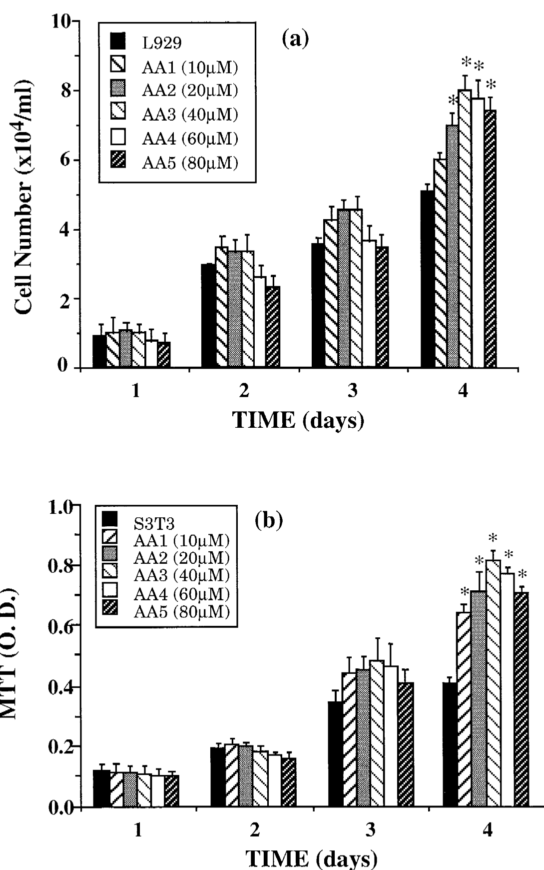

Fig. 11. The effect of AA on fibroblast proliferation. Fibroblast L929 (a) or Swiss 3T3 (b) cell number was determined by MTT in the presence or absence of AA on various concentrations. The significance of the effect of AA on fibroblast proliferation at day 4 was: a, * $P<0.05$; b, * $P<0.05$. The results are the mean ± S.E.M. from 3 to 4 experiments, each in triplicate.

Addition of free AA to HL-60 cells in a range of 0.1–80 μM, did not affect their proliferation rate and higher concentrations of AA (100–140 μM) were toxic to the cells. In the presence of 100 μM AA, the percentage of dead cells was 12.5% after two days and 45% after four days, while in the presence of 140 μM AA the percentage of dead cells was 50% after two days and 90% after four days. The effect of free AA on proliferation was also studied in fibroblast cell lines. Addition of free AA in a range of 10–60 μM to fibroblast L929 or Swiss S3T3 cell lines caused a

significant increasing biphasic effect on cell proliferation with maximum effect at 40 μM AA, observed only at day four (Fig. 11).

The effect of addition of the various PLA_2 inhibitors on HL-60 cell differentiation was studied. BPB (0.1–0.75 μM), $AACOCF_3$ (10 μM–30 μM) or scalaradial (2.5 μM–7.5 μM) did not induce differentiation either alone or in combination with optimal or suboptimal concentrations of $1,25(OH)_2D_3$ or RA (data not shown). Addition of free AA in a range of 0.1–140 μM did not induce differentiation in HL-60 cells.

4. Discussion

The present study shows that PLA_2 activity and the release of AA are essential for the proliferation process. Agents which inhibit proliferation of HL-60 cells, such as $1,25(OH)_2D_3$ or RA, caused inhibition of PLA_2 activity as measured by the release of AA stimulated by PMA. In accordance with these results, the potent inhibitor of PLA_2 activity, BPB, which covalently modify a histidine residue at the active site of PLA_2 [22,23], inhibited both the proliferation of HL-60 cells and AA release induced by PMA in a dose- and time-dependent manner. The specificity of BPB to PLA_2 is demonstrated in our recent study [18] showing that in the presence of BPB PLA_2 activity is inhibited while the phosphorylation of proteins induced by PKC activity is not affected. Similarly, two other PLA_2 inhibitors, the analog of AA, $AACOCF_3$ [24] and the potent irreversible inactivator of PLA_2, scalaradial [25], inhibited HL-60 cell proliferation in a dose- and time- dependent manner. These results suggest that PLA_2 activity plays an important role in regulating the proliferation process in HL-60 cells. The inhibitory effect of BPB on cell proliferation could be reversed by the addition of free AA in a dose dependent manner (Fig. 5). This effect was specific to AA and not to other fatty acids such OA, or LA, indicating the importance of AA metabolite for HL-60 proliferation. The three PLA_2 inhibitors used in our study did not change the cell cycle distribution, but they caused a significant increase in the doubling time of HL-60 cells. Their effect is in contrast to that of $1,25(OH)_2D_3$ or RA which inhibit proliferation of HL-60 by arresting the cell cycle in G_1 phase (Table 1 and [26]). This discrepancy may be attributed to the fact that $1,25(OH)_2D_3$ or RA inhibit proliferation and induce differentiation, while the PLA_2 inhibitors only inhibit proliferation and do not induce differentiation.

The type of PLA_2 that catalyzes the release of AA during proliferation and is inhibited by the different PLA_2 inhibitors used in our study is not yet known. Over the past decade a number of distinct types of PLA_2 have been isolated and characterized. The best known of these are a family of 14-kDa calcium dependent secreted enzymes ($sPLA_2$) [21], an 85-kDa cytosolic calcium dependent enzyme ($cPLA_2$) [20] and an intracellular calcium independent enzyme [27]. In the present study we used both scalaradial, which is known to be more specific to $sPLA_2$ [21], and $AACOCF_3$ which is known to be more specific to $cPLA_2$ [25]. Both inhibitors inhibited HL-60 cell proliferation to about 40% after 3 days, similar to the effect of BPB. These results suggest that both $sPLA_2$ and $cPLA_2$ participate in the proliferation process. These two types of PLA_2 are present in HL-60 cells as shown by the presence of mRNA (Fig. 6) and by the presence of the $cPLA_2$ protein [28]. However, there is a limitation to using inhibitors, since although the different inhibitors are more specific to certain types of PLA_2 they may inhibit other types as well [25,29,30]. Other experiments are being conducted in our laboratory to define the exact type of PLA_2 that plays a role in the proliferation process.

The role of PLA_2 was further emphasized in other cell lines. Inhibition of PLA_2 activity by BPB suppressed fibroblast cell line proliferation (Fig. 10), indicating that this phenomena is not restricted to HL-60 cells only, but may have more general implications. The importance of PLA_2 activity for the proliferation process shown in our study is supported by other investigations. The PLA_2 inhibitors, BPB, quinacrine and 7,7-dimethyleicosadienoic, have been shown to significantly inhibit colony formation of erythroid progenitor cells in the presence or absence of erythropoietin (Epo) which controls the proliferation and differentiation of erythroid progenitor cells [31]. In another study [32] when quiescent cells were incubated with PLA_2 type I for 24 h, DNA synthesis of fibroblast Swiss 3T3 cells was stimulated in a dose-dependent manner. Furthermore, the exogenous addition of PLA_2 type II to fibroblast Swiss 3T3 and

BALB/3T3 cell lines was found to stimulate DNA synthesis [33].

Addition of exogenous free AA did not affect the proliferation rate of HL-60 cells. Similar to our results it has been reported [34] that addition of free AA to U937 did not affect the rate of proliferation. Another study [35] has shown that 10 μM AA could decrease growth of HL-60 cells by 50% as early as 18 h, but this growth inhibition was transient, and cells were able to recover and grow to control levels 72 h following AA treatment. In contrast, a recent study [36] reported the inhibitory effect of AA on HL-60 cell proliferation, but only with concentrations above >30 μM. In this study BSA alone, which was used to present AA to the cells, inhibited cell growth by 30%. At higher concentrations of AA (120 μM), the inhibition of proliferation was due to apoptosis and necrosis, in accordance with the toxic effect of high concentrations of AA on HL-60 cells shown in our study. In contrast to HL-60 cells, addition of free AA increased fibroblast cell growth, but the effect was significant only after 96 h, suggesting differences in sensitivity to AA in various cell lines. It is possible that HL-60 cells proliferate at an optimal rate so that addition of AA would be inefficient in increasing the extent of proliferation. In accordance with our results, two other studies have shown that AA does not dramatically affect Swiss 3T3 fibroblast proliferation during the first few days. The first study [37] showed that addition of free AA caused a weak and non significant stimulatory effect on proliferation in a dose-dependent manner within 24 h. The second one [38] reported that there was no effect of AA on cell proliferation 40 h after addition of AA. In another cell line, rat messangial cells [39], exogenous AA caused increased proliferation.

The effect of the addition of free AA on differentiation of HL-60 cells was studied. As anticipated by the effect of the PLA_2 inhibitors on proliferation, the addition of AA to HL-60 cells did not induce differentiation.

In contrast to the significant effect of the three PLA_2 inhibitors on the proliferation of HL-60 cells, they did not affect the differentiation process, either alone or together with $1,25(OH)_2D_3$ or RA. Although the differentiation process is usually accompanied by inhibition of proliferation, we demonstrate in the present study that inhibition of PLA_2 activity suppressed the proliferation process but did not induce differentiation. These observations suggest that the two processes may be differentially regulated in HL-60 cells.

In conclusion, our study suggests that PLA_2 activity plays an important role in HL-60 and fibroblast L929 and Swiss 3T3 cell proliferation. While the inhibition of PLA_2 suppresses proliferation, it is not sufficient to induce differentiation of HL-60 cells. The type of PLA_2 participating in the proliferation process has to be determined and is presently being investigated.

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