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Cell cycle phase-dependent effect of retinoic acid on the induction of granulocytic differentiation in HL-60 promyelocytic leukemia cells

Evidence for sphinganine potentiation of retinoic acid-induced differentiation

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The efficiency of retinoic acid (RA)-induced differentiation was dependent on the position of HL-60 cells in the cell cycle. Our results demonstrated that cells at the G_1/S border were more efficiently induced to differentiate by short exposure to RA than cells at other phases of the cell cycle. Synchronization of cells in G_1/S phase by aphidicolin (APH) or mimosine (MIMO) increased the sensitivity of cells to RA short exposure treatment. Pretreatment with sphinganine (SP), a protein kinase C (PKC) inhibitor, potentiated RA-induced cell differentiation. By cell cycle analysis, SP was found to block the cell progression through the G_1/S phase. Consequently, cells accumulated in the G_1/S phase of the cell cycle. The present data therefore suggest a possible mechanism of action of SP to enhance RA-induced differentiation.

Cell cycle; Granulocytic differentiation; Retinoic acid; Sphinganine

1. INTRODUCTION

The retinoids, and in particular, retinoic acid (RA), exert a wide range of biological effects related to cell proliferation and differentiation (see [1] for review). Human promyelocytic leukemia HL-60 cells can be induced to differentiate along the granulocytic pathway by RA [2]. Although the mechanism of RA action remains unclear, there is some evidence suggesting that RA acts at the cell surface [3] or interacts with a nuclear RA receptor (RAR) family [4–6]. In general, the antiproliferative activity of RA is a reversible phenomenon [7,8]. Therefore, in the management of cancer disease, RA is usually used in combination with other inducers [9–13] to facilitate the RA-induced differentiation. Previously, Stevens et al. [13] have shown that sphinganine (SP), a potent inhibitor of protein kinase C (PKC) in vitro [14], could potentiate RA-induced differentiation. In our previous studies we have also demonstrated that pretreatment with SP can modulate the reversibility of the effects induced by brief RA treatment [15]. Recently, we have shown that the differentiation of HL-60 promyelocytic cells toward mature granulocytic cells induced by RA was accompanied by a decrease in PKC activity [16]. The enhancement of RA-induced differentiation and the potentiation of the decrease of PKC activity by SP seemed to correlate with each other [16]. However, the precise mechanism of action of this sphingolipid long-chain base in the potentiation of RA-induced differentiation is still presently unclear.

Gezer et al. have demonstrated that the growth fraction of HL-60 cells is decreased and the total cell cycle time is prolonged during cellular differentiation in response to RA [17]. This is a result of the lengthening of both G_1 and S phases in the cells [17]. Moreover, induced differentiation of transformed cells has been shown to be associated with modulation of expression of a number of gene products such as some protooncogenes c-*myb*, c-*myc*, c-*fos* and p53 [18–23], that appear to be involved in the control of cell cycle progression and induction of differentiation. Hence, we believe that the cell cycle events could be involved in the RA-induced differentiation. The present study was therefore undertaken to elucidate the cell cycle-dependent effect of RA. In this report, we also present studies that explore the possible mechanism of action of SP in the potentiation of RA-induced maturation of promyelocytic leukemia cells. Our results showed that cells in G_1/S border were maximally sensitive to brief RA treatment while cells in other phases of the cell cycle were less

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Abbreviations: APH, aphidicolin; CAM, camptothecin; DMSO, dimethyl sulfoxide; EC, embryonal carcinoma; FBS, fetal bovine serum; MIMO, mimosine; ND, nocodazole; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; RA, retinoic acid; RAR, retinoic acid receptor; R point, restriction point; SP, sphinganine; ST, staurosporine; TCA, trichloroacetic acid; VIN, vinblastine.

sensitive. Our results also showed that the potentiation of the RA-induced differentiation by SP pretreatment could be rationalized with cell cycle-dependent effect of brief RA treatment. SP pretreatment blocked the cell progression through G_1/S phase. SP-pretreated cells being blocked at G_1/S phase were highly responsive to brief RA treatment.

2. MATERIALS AND METHODS

2.1. *Drugs*

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aphidicolin (APH) was prepared as 1 mg/ml stock solution in 70% ethanol. The stock solutions of camptothecin (CAM) and nocodazole (ND) were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and 5 mg/ml, respectively. Mimosine (MIMO) was dissolved in phosphate-buffered saline (PBS; 8.5 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 0.145 M NaCl, pH 7.2) at a 10 mM concentration. RA, SP and staurosporine (ST) were dissolved in ethanol (Merck, Darmstadt, Germany) at 0.002 M, 0.01 M and 50 μM, respectively. Vinblastine (VIN) was obtained from David Bull Laboratories (DBL, Victoria, Australia) and was dissolved in diluent solution (from DBL) at a 1 mg/ml concentration.

2.2. *Radiochemicals*

[^{3}H]RA (49.3 Ci/mmol) was obtained from DuPont New England Nuclear (NEN) Co. (Boston, MA, USA). [^{3}H]Thymidine (25 Ci/mmol) and [^{3}H]uridine (15–30 Ci/mmol) were purchased from Amersham (Buckinghamshire, England).

2.3. *Cell culture*

The HL-60 promyelocytic leukemic cells were provided by Dr. Zee Fen Chang (Department of Biochemistry, Chang Gung Medical College) and maintained in continuous cultures in our laboratory for three years. Cells were grown in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences; Lenexa, KS, USA), 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco BRL; Gaithersburg, MD, USA) in a 5% CO_2 humidified incubator at 37°C.

2.4. *Cell synchronization by serum deprivation*

72 h after the seeding of cells, the medium was removed, cells were washed three times with serum-free medium, and HL-60 cells were incubated with serum-free medium for 24 h. Under these conditions, cells become arrested in a quiescent state. The serum-free medium was then removed and replaced with fresh serum-supplied medium.

2.5. *Cell synchronization by chemical method*

Exponentially growing cultures containing approximately 1×10^6 cells/ml were treated with 300 μM MIMO or 2 μg/ml APH for 16 h. Such treatments resulted in synchronization of over 80% of the cells in late G_1 and G_1/S phases, respectively [24]. Treatment of cells (3×10^5 cells/ml) with 0.1 μg/ml CAM for 4 h, resulted in having majority of the cells arrested in G_1 phase [25]. Stathmokinetic experiments for M phase arrest were done by treating the cultures (2×10^5 cells/ml) with 0.05 μg/ml of VIN for 6 h [25] or with 0.5 μg/ml of ND for 12 h.

2.6. *Induction of differentiation*

In the induction of differentiation, HL-60 cells (2×10^5 cells/ml) were incubated with 1 μM RA. After 2 h of incubation, RA was removed (washed three times with PBS) and the differentiation was assessed by morphological change (as described below) at day 4 after the removal of RA.

2.7. *Wright-Giemsa stain*

Morphological assessment of the induced cells was performed using the Wright-Giemsa staining method. Cells (4×10^4 cells) were prepared on slides by Cytospin (Shandon Southern, UK) and stained with Wright-Giemsa stain. The morphology of cells was examined under a light microscope (× 1,000). The criteria for the morphological judging was according to those previously described [26]. Triplicate 200-cell counts were performed.

2.8. *DNA and RNA synthesis rate determination*

The synthesis rate of DNA was determined by a modification of a previously described method [27]. Briefly, cultures were pulsed with 0.5 μCi/ml of [^{3}H]thymidine 1 h prior to the end of the incubation time. Then the cells were harvested and washed three times with 10% trichloroacetic acid (TCA; Merck) and dissolved in 0.5 ml of 0.25 N NaOH and further shaken for 30 min. Aliquots of 300 μl were dissolved in 4 ml of scintillation fluid (Merck) and the radioactivity was measured by scintillation counter (Beckman LS5000 TD; Beckman Instruments Inc.; Palo Alto, CA, USA).

The synthesis rate of RNA determination was similar to the DNA synthesis rate determination described above. Cells were pulsed with 0.5 μCi/ml of [^{3}H]uridine 1 h prior to the end of the incubation time. The cells were then washed with 10% TCA and dissolved in 1 N NaOH. Aliquots of 300 μl were dissolved in scintillation fluid and counted for radioactivity.

2.9. *Cell cycle analysis*

To estimate the proportions of cells in different phases of the cell cycle, cellular DNA contents were measured by flow cytometry as described by Ormerod [28]. Briefly, cells (2×10^6 cells) were fixed by 70% ethanol (in PBS) in ice for 30 min and then resuspended in PBS containing 40 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase (Boehringer, Germany). After 30 min at 37°C, 2×10^4 cells were analyzed on a FACStar cytofluorometer (Becton-Dickinson; San Jose, CA, USA) equipped with an argon-ion laser at 488 nm.

2.10. *Fractionation of HL-60 cells*

Fractionation of cells was performed as described previously [29]. Briefly, the cells were incubated with [^{3}H]RA for various times before

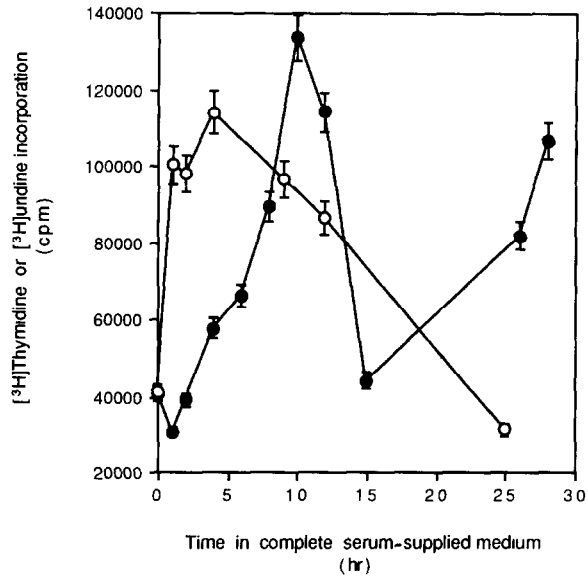

Fig. 1. Cell cycle synchronization – [^{3}H]thymidine and [^{3}H]uridine incorporation. HL-60 cells were cultured in 25-cm^2 plastic tissue flasks with serum-free medium for 24 h and then transferred to complete serum-supplied medium. One hour prior to the end of the incubation period, the cells were pulsed with 0.5 μCi/ml of [^{3}H]thymidine (●——●) or [^{3}H]uridine (○——○) for 1 h. Incorporation of these radioactive materials into DNA or RNA were assayed as described in section 2.8. Each point is the mean ± S.D. (n = 3).

they were suspended in cold TM buffer (0.01 M Tris-HCl, pH 7.6, containing 1 mM $MgCl_2$) and were homogenized 18 strokes with all-glass homogenizer (Wheaton, Millville, NJ, USA). The nuclei were then collected by centrifugation at 500 × *g* for 5 min. The supernatant was further centrifuged at 10,420 × *g* for 45 min. The final supernatant (cytosol fraction) and the pellet (membrane fraction) were collected separately. The nuclei, cytosol and membrane fractions were resuspended in TM buffer. Each fraction (100 μl) was then added into 5 ml scintillation fluor (Merck), and were counted for radioactivity (Beckman LS5000 TD).

3. RESULTS

3.1. *G_1/S phase border cells are maximally sensitive to brief RA treatment*

To ensure that the cells were capable of synchronously re-entering the cell cycle after 24-h serum starvation, the cells' abilities of [^{3}H]thymidine and [^{3}H]uridine incorporation were determined (Fig. 1). RNA synthesis increased immediately and reached its maximum 5 h after the cells were transferred to complete serum-supplied medium. At the same time, the DNA synthesis increased slowly and reached its maximum within 10 h. The rate of RNA and DNA synthesis declined soon after they reached their maximums. The G_1 phase can be confined to the first 2 h. The S phase can then be between 4 and 14 h while the G_2/M phase is between 15 to 24 h. The effects of RA on cellular differentiation in cells of various phases of cell cycle were then examined.

As illustrated in Table I, brief RA treatment (1 μM, 2 h) had little effect on induction of differentiation; less than 40% of the cells resembled mature granulocytes at day 4 after the removal of RA (1 μM; 2 h). Similar results were obtained in cells that were briefly treated with RA at G_0/G_1, middle S (mid-S), late S or G_2/M phases. Interestingly, cells within the G_1/S phase border were maximally responsive to RA short exposure (1 μM; 2 h). Higher percentage of the cells (> 60%) resembled mature granulocytes at day 4 after the removal of RA. This degree of differentiation was similar to the one under continuous treatment with 1 μM RA (Table I).

3.2. *APH or MIMO-treated cells are highly responsive to RA brief treatment*

Furthermore, different cell cycle blockers were applied to synchronize cells. The effects of brief RA treatment on induction of differentiation were determined in

Table I

RA-induced differentiation of HL-60 cells in various phases of cell cycle

Time after transferred to serum-supplied medium	Cell cycle phases	RA treatment	Cell suspension (% of total cells)					
					Mature cells[b]			
			Blast[a]	Pro[a]	Myelo[a]	Granulocytes		
						Meta[a]	Band[a]	Seg[a]
–	–[c]	–[d]	2	87	8	3	0	0
–	–[c]	con[e]	0	42	46	10	2	0
–	–[c]	2 h[f]	0	61	26	9	3	1
0	G_0	2 h[f]	2	66	20	9	3	0
1	G_1	2 h[f]	3	56	30	10	1	0
2	G_1*	2 h[f]	0	48	44	8	0	0
4	early S*	2 h[f]	1	38	46	12	2	1
6	early S*	2 h[f]	1	50	33	14	2	0
8	early S	2 h[f]	3	57	28	9	3	0
10	mid-S	2 h[f]	1	62	30	5	1	1
12	late S	2 h[f]	0	62	29	4	5	0
24	G_2 + M	2 h[f]	3	68	24	3	1	1

HL-60 cells were cultured in 25-cm^2 plastic tissue flasks in serum-free medium. Cells (2 × 10^5 cells/ml) were then transferred to the complete serum-supplied medium after 24 h. At various times (as indicated) of incubation in serum-supplied medium, 1 μM RA was added. After 2 h, the drug was removed (washed 3 times with PBS). Differentiation was assessed by morphological changes, as described in section 2.7 at day 4 after the removal of RA.

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Viability of the cells was over 90% under these conditions.

[b] Assessment of differentiation (myelo, meta, band and seg).

[c] Unsynchronized cells.

[d] Control without RA.

[e] Continuous RA treatment.

[f] 2 h brief RA treatment.

*Significantly different from values of 2 h brief RA treatment of unsynchronized cells at $P < 0.05$ (χ^2-test).

such drug-treated cells. As shown in Table II, cells after treatment with APH (2 μg/ml; 16 h) or MIMO (300 μM; 16 h) were highly responsive to RA short exposure. As compared to only 30–40% of CAM, ND or VIN-treated cells being differentiated by brief RA treatment, 55–66% of MIMO or APH-treated cells became mature granulocytes at day 4 after removal of RA (1 μM; 2 h). These results further confirmed that G_1/S border cells (arrested by MIMO or APH) were highly responsive to brief RA treatment while cells in the early G_1 phase (arrested by CAM) or M phase (arrested by VIN or ND) were less sensitive to RA short exposure.

3.3. *SP blocks cells in G_1 and S phases*

As shown previously, the SP pretreatment (2.5 μM, 24 h) enhanced the RA-induced differentiation [15,16]. Higher percentage (> 60%) of SP-pretreated cells resembled mature granulocytes at day 4 after the removal of RA (1 μM; 2 h) as compared with non-SP-pretreated cells (< 40%) (Table II). In order to understand the biological action of SP in potentiation of RA-induced differentiation, the effect of SP on cell cycle regulation was determined. As shown in the DNA histogram (Fig. 2), APH- or MIMO-treated cells were blocked in G_1/S phase and G_1 phase (preferentially late G_1), respectively (Fig. 2B and C). Similarly, exposure of cells to 2.5 μM SP for 24 h resulted in the accumulation of high percentage of cells (> 90%; Fig. 2D) in G_1/S phase. Our results showed that SP synchronized cells in G_1/S phase as APH or MIMO did. Being synchronized at G_1/S phase, SP-pretreated cells were highly sensitive to brief RA treatment. Staurosporine (ST), another PKC inhibitor, being able to potentiate RA-induced differentiation had similar ability to synchronize cells in G_1/S phase as SP did (data not shown).

3.4. *[^{3}H]RA accumulation and distribution*

To further understand the potentiation of RA-induced differentiation by G_1/S blockers, we examined the effects of pretreatment with SP, MIMO or APH on the accumulation and distribution of [^{3}H]RA in cell nuclei, cytosol and membrane during and after brief RA exposure. Our results showed that there were virtually no differences in [^{3}H]RA accumulation nor excretion during and after brief RA exposure in cells treated with or without SP, MIMO or APH pretreatment (Fig. 3A). Furthermore, [^{3}H]RA that were accumulating or remaining during and after brief RA exposure were in majority (> 75%) found in nuclei of cells that had or had not been pretreated with SP, MIMO or APH (Fig. 3B and C).

Table II

Effect of various cell cycle inhibitors on RA-induced differentiation of HL-60 cells

Cell cycle inhibitor	Blocked at various cell cycle phases	RA treatment	Cell suspension (% of total cells)					
					Mature cells[b]			
			Blast[a]	Pro[a]	Myelo[a]	Granulocytes		
						Meta[a]	Band[a]	Seg[a]
–	–	–[c]	2	80	17	1	0	0
–	–	2 h[d]	0	61	26	9	3	1
CAM	early G_1	2 h[e]	2	69	18	9	1	1
MIMO	late G_1*	2 h[e]	0	45	32	19	3	1
APH	G_1/S	2 h[e]	0	34	33	31	1	1
ND	M	2 h[e]	1	70	14	14	1	0
VIN	M	2 h[e]	2	68	20	9	1	0
SP	nd*	2 h[e]	0	36	36	19	4	5

HL-60 cells were cultured in 25-cm^2 plastic tissue culture flasks. Cells (2×10^5 cells/ml) were pretreated with cell cycle blocker (as in section 2.5) before 1 μM RA was added to the medium. After 2 h incubation, the drugs were removed (washed 3 times with PBS). Differentiation was assessed by morphological change, as described in section 2.7 at day 4 after the removal of drugs.

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Viability of the cells was over 90% under these conditions.

[b] Assessment of differentiation (myelo, meta, band, and seg).

[c] Control without RA nor SP.

[d] Control with 2 h brief RA treatment, without other drugs.

[e] 2 h brief RA treatment.

nd, not defined yet.

*Significantly different from values of control with 2 h brief RA treatment without other drugs at $P < 0.05$ (χ^2-test).

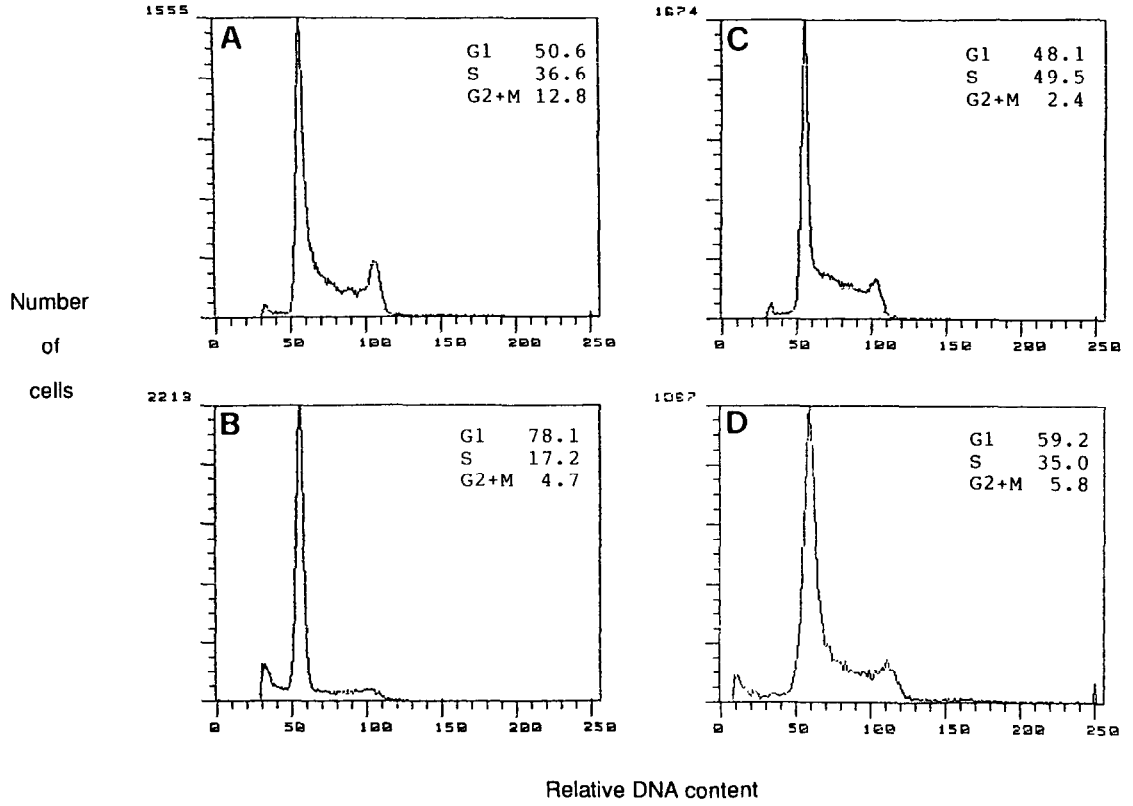

Fig. 2. Treatment of HL-60 cells with APH, MIMO or SP caused cell cycle arrest at G_1/S phase. This figure describes the cell cycle distribution of untreated control cells (A), or cells treated with APH (2 μg/ml; 16 h) (B), MIMO (300 μM; 16 h) (C), or SP (2.5 μM; 24 h) (D). Cells were washed free of drugs before aliquots of cells were sampled for flow cytometric analysis.

4. DISCUSSION

Three principal controls or transition points are associated with the eukaryotic cell division cycle [30]. These are G_0 to G_1, G_1 to S, and G_2 to M transitions. The elucidation of how these transitions are regulated is of fundamental importance in the study of cellular proliferation and differentiation. The processes in late G_1 prior to S phase (G_1 to S transition) is called restriction (R) point [31]. The R point may contain the 'switch' by which a variety of signals are turned on. Once past this point, cells become committed to the mitotic cell cycle, and the alternative developmental fate of conjugation cannot take place until the present cell cycle has been completed (see [32,33] for review). In this regard, the R point (within the G_1 body) is equivalent to the START point (might be at the G_1/S border) in yeast cells [32]. It is possible that there may be a growth/differentiation control signal at G_1/S border which orders cells to grow or to become committed to terminal differentiation. Cell cycle prolongation in both G_1 and S phases were demonstrated after RA treatment [17]. In this report, we have demonstrated that the G_1/S border phase is critical for RA-induced differentiation. It is suggested that the maintenance of G_1 and S phases might be necessary for HL-60 granulocytic differentiation. In parallel, previous studies by others have shown that cultured rat and mouse myoblasts become committed to myogenesis only in the G_1 phase of the cell cycle [34,35]. Furthermore, embryonal carcinoma (EC) P19 cells are induced to differentiate by RA only if the cells are in G_1 phase during drug exposure [36]. A recent report by Berg and McBurney has stated that the density of P19 cells in the culture is critical in determining the efficiency of differentiation induction [37]. Our present finding that RA-induced granulocytic differentiation is cell cycle phase-dependent provides an important information in understanding the regulatory control in cellular growth and differentiation.

Sphingolipids participate in fundamental biological processes, such as cell–cell interaction, proliferation, differentiation, and oncogene transformation (see [38] for reviews). The discovery that sphingolipid-derived molecules, such as sphingosine and lysosphingolipids are potent and reversible inhibitors of PKC have spurred the studies of these molecules' biological roles [39]. Until recently, it was demonstrated that SP, the saturated form of sphingosine, dramatically enhances RA- and other antileukemia chemotherapeutic agent-induced differentiation [13,40]. In this report, we have further shown that SP exhibits an effect on blocking the cell progression through G_1/S phase. The data explain

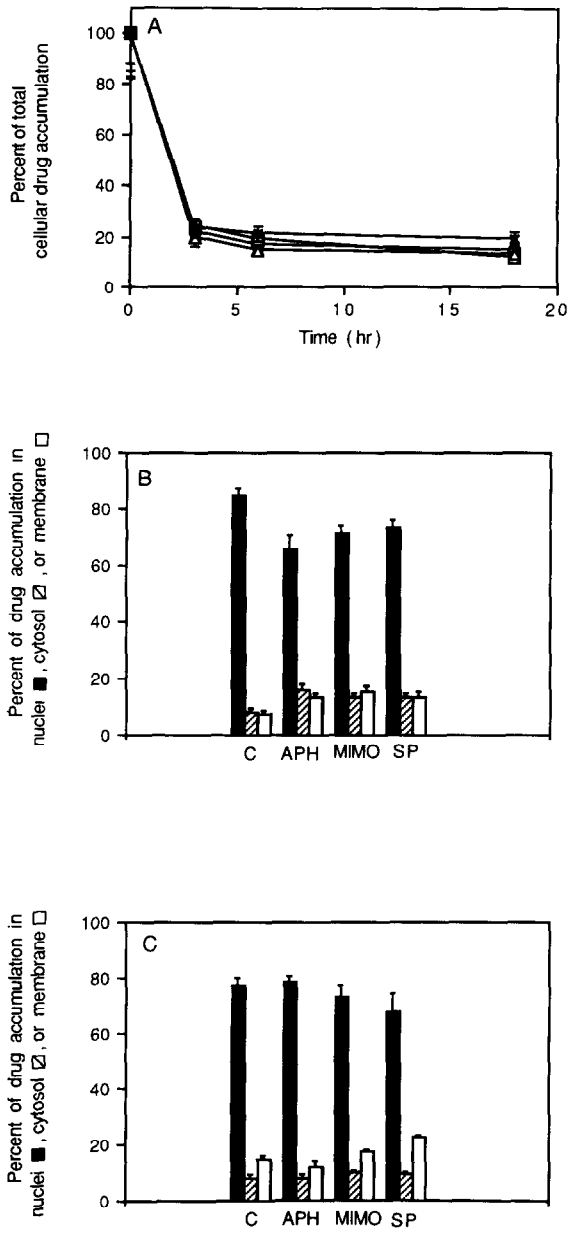

Fig. 3. Intracellular distribution of [^{3}H]RA. HL-60 cells were cultured in 25-cm^2 plastic tissue flasks. Cells were cultured without any drugs in the medium (control, C) (○) or pretreated with APH (□), MIMO (△) or SP (×) as described in Fig. 2 before [^{3}H]RA (final concentration = 1 μM) was added. After 2 h of RA incubation, the drugs were removed (washed three times with PBS). At various times after the washing procedure the uptake of [^{3}H]RA into the cells (A) was determined. At 0 h (B) or 18 h (C) after washing procedure, cell fractionation was carried out, the distribution of [^{3}H]RA was determined as described in section 2.10. Points = mean; bars = S.D. (n = 3).

why SP can potentiate the RA-induced differentiation. SP regulates the induction of differentiation by allowing cells to accumulate in an extended G_1/S phase. The G_1/S phase is the period in the cell cycle at which RA has the greatest effect on induction of differentiation. Furthermore, the present study of [^{3}H]RA accumulation and distribution has indicated that the potentiation of RA-induced differentiation by G_1/S blocker cannot be simply explained by the extent of accumulation and differential distribution of RA in cells that had or had not been pretreated with SP, MIMO or APH. Some other important factors probably exist in G_1/S phase, playing a critical role in the regulation of the induction of differentiation.

The molecular events involved in this cell cycle phase-dependent effect of RA are not understood. These types of studies are under current investigation. Expression of some cell cycle specific genes and enzymatic activity (such as PKC) during the induction of differentiation may provide instructive clues. Compounds that perturb the cell cycle at or near the G_1/S phase boundary may allow the identification of the key transcriptional and posttranscriptional processes that regulate the initiation of DNA synthesis and cellular differentiation in cells.

Our present study may have important therapeutic implications. Following drug administration, the circulating and plasma concentrations decline as the excretion processes take place. Exposure of the tumor to RA for a short period of time may not be effective enough to control the leukemia growth. Our finding that pretreatment with SP can increase the percentage of cells differentiated by brief RA treatment and the finding of the cell cycle phase-dependent effect of RA suggests that RA therapy can be largely improved by co- or pretreatment with appropriate modulator of cell cycle progression.

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