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Differential Cellular Distribution of Retinoic Acid during Staurosporine Potentiation of Retinoic Acid-Induced Granulocytic Differentiation in Human Leukemia HL-60 Cells

Key Words
Retinoic acid distribution
Staurosporine potentiation
Induction of differentiation

Abstract
Pretreatment of cells with staurosporine, a protein kinase C (PKC) inhibitor, was found to potentiate the granulocytic differentiation induced by a brief (2 h) retinoic acid treatment. By cell cycle analysis, staurosporine was found to have little effect on the cell cycle. Retinoic acid was distributed equally in the nuclei (40%) and in the plasma membrane (40%) of staurosporine-pretreated cells while less than 20% of retinoic acid was found in the membrane of control non-staurosporine-pretreated cells during the retinoic acid-induced differentiation. These results indicate that the enhancing effect of staurosporine may be somehow associated with the localization of retinoic acid in the plasma membrane of the cell.

Introduction

Tumors are usually recognized by the fact that their cells show abnormal growth. Tumor cells differ from normal cells in that they are no longer responsive to normal growth-controlling mechanisms. Current chemotherapeutic drugs act for the most part by killing cancer cells directly. Their use in patients, however, is limited due to high toxicity. Much attention is paid to averting their toxicity and increasing their therapeutic efficacy. Researchers have also begun to focus their studies on agents that act instead by changing the biological properties of cancer cells so that they lose one of their major characteristics, namely, the ability to divide continuously. Recent work on tumor differentiation aims to shift the balance back again, thereby removing the potential for uncontrolled growth of the tumor cells.

The human promyelocytic leukemia cell line HL-60 differentiates to become morphologically and functionally mature granulocytes in the presence of retinoic acid [2]. Clinical trials and case reports thus far show variable success of therapy using retinoic acid in leukemia. In general, the antiproliferative activity of retinoic acid is a reversible phenomenon [5]. Therefore, much attention has been focused on the interaction of retinoic acid with other agents that can facilitate retinoic acid-induced differentiation.

Okazaki et al. [20] have shown that staurosporine, a microbial alkaloid produced by *Streptomyces actuosus*, can potentiate retinoic acid-induced differentiation. Orig-

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inally described as an inhibitor of phospholipid- and Ca^{2+}-dependent protein kinase (protein kinase C; PKC) [9], staurosporine is found to bind to the ATP-binding domain (catalytic site) of PKC [8]. It has also been reported that staurosporine inhibits a number of other protein kinases in vitro, including $pp60^{src}$ tyrosine kinase [17], insulin receptor tyrosine kinase [6], cGMP-dependent protein kinase (protein kinase G) [18], and $p34^{cdc2}$ kinase and $p34^{cdc2}$-like kinase [7].

Sphinganine, another inhibitor of PKC [16], which interacts with the regulatory domain of PKC [10], has a similar potentiation effect on retinoic acid-induced differentiation [22]. We have earlier demonstrated that pretreatment of cells with sphinganine could potentiate granulocytic differentiation induced by a brief (2 h) retinoic acid treatment [11–13]. Most of the retinoic acid (>70%) was found to be accumulated in the nuclei and only a minor portion (<20%) in the plasma membrane in control or sphinganine-pretreated cells during the induction of differentiation [12]. Sphinganine therefore regulates the induction of differentiation not by changing the extent of accumulation and differential distribution of retinoic acid in cells, but 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 retinoic acid has the greatest effect on the induction of differentiation. In this paper, we present results showing that pretreatment of cells with staurosporine can also potentiate brief retinoic acid-induced differentiation. Whereas sphinganine blocks the cells' progress through G_1/S phase, pretreatment with staurosporine exerts little effect on the cell cycle. In contrast to the control or sphinganine-pretreated cells, retinoic acid is found to be distributed equally in the nuclei (40%) and in the plasma membrane (40%) of staurosporine-pretreated cells. Our results suggest the possible involvement of a membrane signal transduction mechanism during retinoic acid-induced differentiation.

Methods

Drugs

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, Mo., USA). All *trans*-retinoic acid and staurosporine were dissolved in ethanol (Merck; Darmstadt, Germany) at 2 and 0.5 m*M*, respectively. Stock solutions were stored at -20°C. Before use, each stock solution was diluted with culture medium to the required concentration. The final concentration of solvent was no more than 0.5%, which had no effects on HL-60 cell proliferation and differentiation.

Radiochemicals

[^{3}H]retinoic acid (50.7 Ci/mmol) was obtained from Du Pont New England Nuclear (NEN) Company (Boston, Mass., USA).

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 [21]. Briefly, cells (2×10^6 cells) were fixed by 70% ethanol in phosphate-buffered saline (PBS) buffer in ice for 30 min and then resuspended in PBS containing 40 µg/ml propidium iodide 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, Calif., USA) equipped with an argon-ion laser at 488 nm.

Fractionation of HL-60 Cells

Cells were incubated with [^{3}H]retinoic acid for various times before they were suspended in chilled TM buffer (0.01 *M* Tris-HCl, pH 7.6, containing 1 m*M* $MgCl_2$) and were homogenized for 18 strokes with an all-glass homogenizer (Wheaton; Millville, N.J., 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 was counted for radioactivity (Beckman LS5000 TF; Beckman Instruments, Palo Alto, Calif., USA).

Results

Staurosporine Pretreatment Potentiated Retinoic Acid-Induced Differentiation

A short exposure (2 h) to retinoic acid induced little effect on cell growth inhibition and induction of differentiation in HL-60 cells. Cell growth on day 4 after the removal of retinoic acid (1 µ*M*; 2 h) was only reduced to 85% of that in control cells (no drug treatment) and less than 40% of the cells resembled mature cells (table 1). In contrast, a short exposure to retinoic acid induced differentiation and cell growth inhibition to a greater extent in HL-60 cells that had been pretreated with staurosporine (5 n*M*; 24 h pretreatment) indicating that staurosporine pretreatment potentiated the retinoic acid-induced (2 h short exposure) differentiation. As shown in table 1, there were a higher percentage of mature cells and a greater decrease in the rate of cell growth in staurosporine-pretreated cells during the brief retinoic acid-induced differentiation: as compared to 35% of cells without staurosporine pretreatment which differentiated, more than 60% of the staurosporine-pretreated cells became mature cells as assessed by either morphological of functional methods. The cell growth of staurosporine-pretreated cells was found to be reduced to 69% of the control on day 4 after

Table 1. Effect of staurosporine on retinoic acid (RA)-induced differentiation of HL-60 cells

	1 μ*M* RA treatment	5 n*M* staurosporine treatment	Cell growth % of control[a]	Differentiation, %	
				morphological assessment[b]	functional assessment[c]
1	–	–	100	5±1	4±1
2	–	+	100	8±2	8±2
3	+[d]	–	59±3*	81±7*	82±3*
4	2 h[e]	–	85±4	35±4	32±2
5	2 h[e]	before[f]	69±6*	65±5*	60±6*
6	2 h[e]	after[g]	86±7	36±3	30±5

HL-60 cells (2×10^5 cells/ml) were not treated, pretreated (24 h) or posttreated with 5 n*M* staurosporine. RA (1 μ*M*) was added to the medium. After 2 h incubation, the drugs were removed (washed 3 times with PBS). Cell growth inhibition and differentiation (morphological and functional assessment) were assessed on day 4 after the removal of RA. For posttreatment, staurosporine was supplied after the removal of RA. * $p < 0.05$ (χ^2 test), vs. control (2 h brief RA exposure without staurosporine).

[a] Cultures from each set were harvested and monitored for cell number by counting cells with hemocytometer. Cell growth was expressed as the percentage relative to control (no drug treatment).

[b] Morphological assessment of the induced cells was performed using the Wright-Giemsa staining method. The percentage of mature cells (myelocytes, metamyelocytes, banded and segmented neutrophil) was presented here.

[c] Functional assessment of the induced cells was performed by nitroblue tetrazolium reduction assay.

[d] Continuous treatment.

[e] 2 h treatment.

[f] Treated before RA short exposure.

[g] Treated after RA short exposure.

retinoic acid removal. When staurosporine was added after the retinoic acid removal, however, no similar potentiating effect was observed (line 6, table 1). The posttreatment with staurosporine did neither increase the percentage of mature cells nor the inhibition of cell growth. These results, therefore, suggest that the effects of a combination treatment of retinoic acid with staurosporine depend on the time of addition of staurosporine, i.e. before or after treatment with retinoic acid.

Staurosporine Had Little Effect on Cell Cycle

Stevens et al. [22] have shown that sphinganine can potentiate retinoic acid-induced differentiation. Our previous studies have also demonstrated that pretreatment with sphinganine can modulate the reversibility of the effects induced by brief retinoic acid treatment [11, 13]. More recently, we have shown that the effect of retinoic acid on the induction of granulocytic differentiation is cell cycle phase-dependent [12]. Sphinganine pretreatment accumulates HL-60 cells in the G_1/S phase, and in this phase, cells are highly responsive to retinoic acid. To obtain information on the approximate stage of the cell cycle after the staurosporine treatment, flow cytometry on DNA distribution was therefore performed (fig. 1). Exposure of cells to 5 n*M* staurosporine for 24 h did not cause HL-60 cells to be arrested in any specific cell cycle phase (fig. 1b). This was consistent with a previous report indicating that low concentrations of staurosporine had little effect on the cell cycle [3]. It can, therefore, be reasonably assumed that the mechanism of action of staurosporine in potentiating retinoic acid-induced differentiation may be different from that on sphinganine, whose effect on retinoic acid-induced differentiation is to block the cell cycle at G_1/S phase.

Retinoic Acid Distributed Equally in Nuclear and Membrane Fractions

To elucidate the possible mechanism of action of staurosporine in the potentiation of retinoic acid-induced differentiation, we examined the effect of pretreatment with staurosporine on the accumulation and distribution of [^{3}H]retinoic acid in the cell nuclei, cytosol and plasma membrane during and after the brief retinoic acid exposure. The results showed that there were no differences in [^{3}H]retinoic acid accumulation or excretion during and after the brief retinoic acid exposure in cells with or without staurosporine pretreatment (fig. 2a). However, the majority (>70%) of [^{3}H]retinoic acid was found in the nuclei in control (fig. 2b), whereas in staurosporine-pretreated cells, [^{3}H]retinoic acid was found distributed equally in the nuclei (40%) and in the plasma membrane (40%) at the early time (<24 h) after the retinoic acid exposure (fig. 2c). This result differed from that obtained from sphinganine-pretreated cells, in which the majority of retinoic acid (>70%) was found in the nuclei of the cells [12]. Furthermore, it is interesting to note that in staurosporine-pretreated cells, retinoic acid was translocated from the membrane to the nuclei after 24 h of retinoic acid exposure (fig. 2c), at which time over 70% of retinoic acid was then found in the nuclei. These results indicate that the enhancing effect of staurosporine may be somehow associated with the localization of retinoic acid in the plasma membrane at the early time (<24 h) of retinoic acid treatment. It is possible that the early initial stage of retinoic acid-induced cellular differentiation may involve membrane signal transduction.

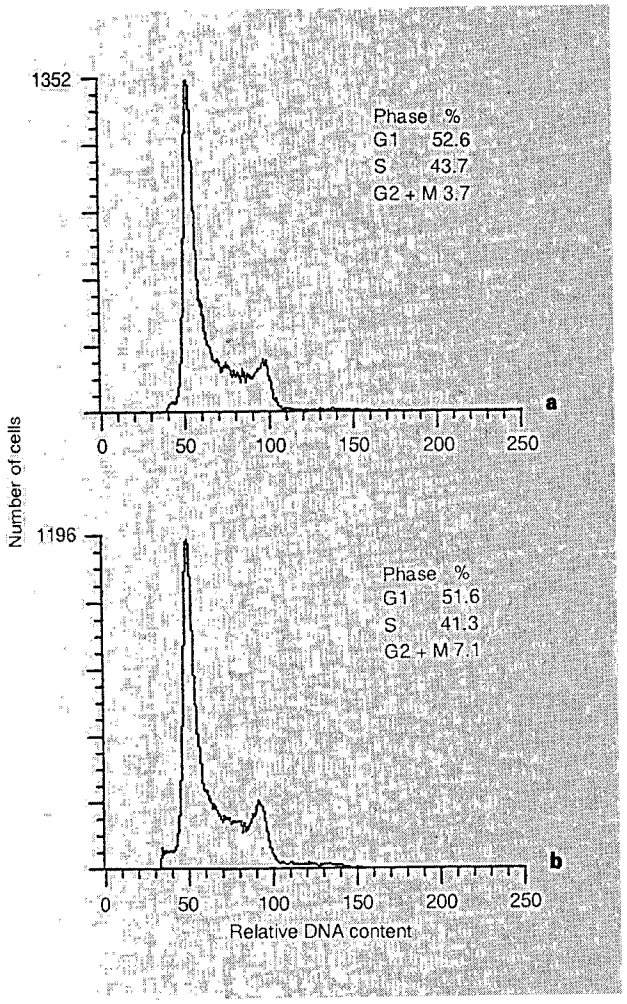

Fig. 1. Cell cycle distribution of untreated control cells (**a**) or cells treated with staurosporine (**b**) is described. HL-60 cells were incubated with or without 5 n*M* staurosporine for 24 h and then were washed free of drug (washed 3 times with PBS) before aliquots of cells were sampled for flow-cytometric analysis.

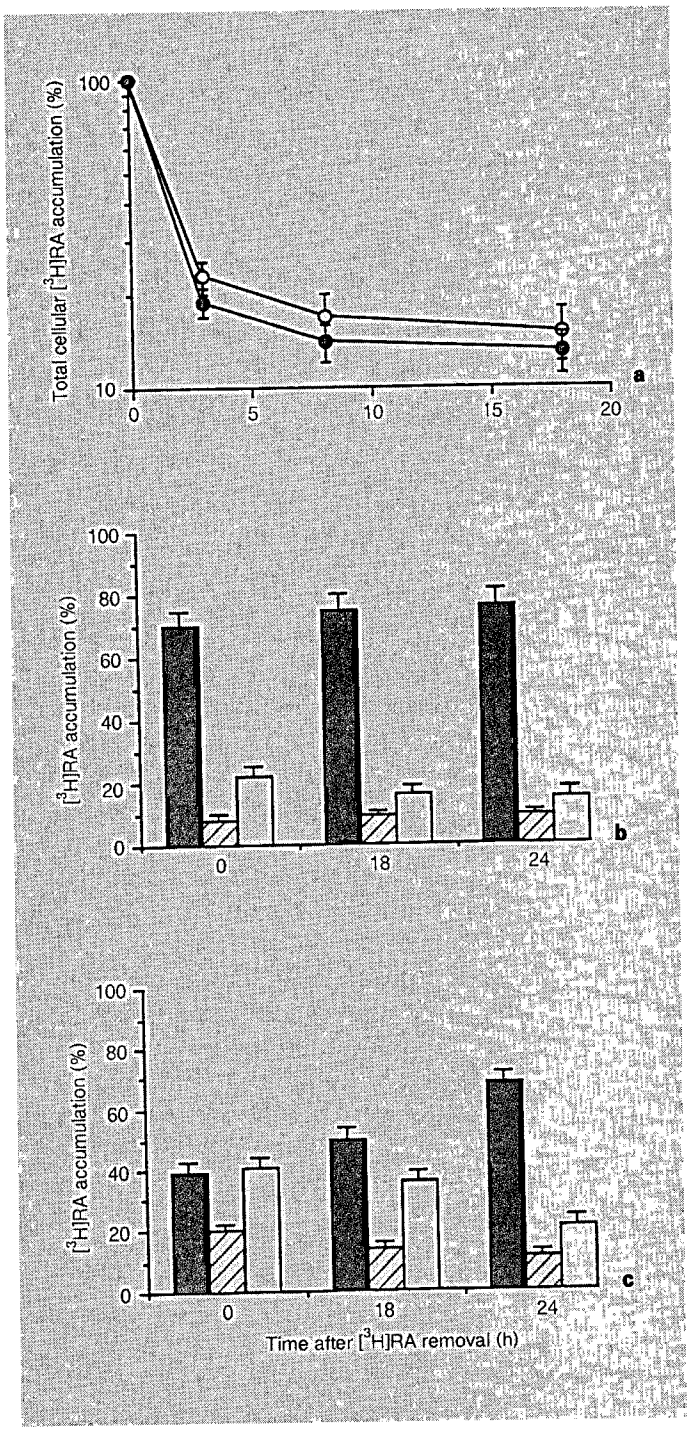

Fig. 2. Intracellular accumulation and distribution of [^{3}H]retinoic acid (RA). Cells were cultured without any drugs in the medium (○) or pretreated with 5 n*M* staurosporine (●) before 1 μ*M* retinoic acid and 2 μCi [^{3}H]retinoic acid were added. After 2 h of [^{3}H]retinoic acid incubation, the drugs were removed (washed 3 times with PBS). At various times (indicated) after the washing procedure, the intracellular levels of [^{3}H]retinoic acid were determined. Each point is the mean ± SD (n = 4). At 0, 18 or 24 h after washing procedure, cell fractionations were carried out and the distributions of [^{3}H]retinoic acid were determined. Bars represent SD (n = 4). **a** Intracellular accumulation of [^{3}H]retinoic acid at various times after washing procedure. **b** Intracellular distribution of [^{3}H]retinoic acid in control cells at 0, 18 and 24 h after washing procedure. **c** Intracellular distribution of [^{3}H]retinoic acid in staurosporine-pretreated cells at 0, 18 and 24 h after washing procedure. At 0 h after washing procedure, the total accumulation of retinoic acid was 250 fmol/10^6 cells. At 18 or 24 h after washing procedure, the total accumulation was about 50 fmol/10^6 cells. ▩ = Nuclei; ▨ = cytosol; □ = membrane.

Discussion

Some discrepancies exist in the literature regarding the details of the interactions between retinoic acid and the nuclear retinoic acid receptor family [14]. Retinoids have also been known to affect the properties of lipid bilayers and membranes [4, 24]. Some investigators have reported the location of retinoids within bilayers and the interactions of these ligands with phospholipids [4, 19, 24]. In our present study, 40% of retinoic acid in the cells was found in the plasma membrane fraction of staurosporine-pretreated cells: the amount is twice that of control cells (fig. 2c). In parallel to the 2-fold increase in retinoic acid distribution in the membrane, a 2-fold higher level of induction of differentiation in staurosporine-pretreated cells than in control cells was seen (table 1).

Much evidence has so far suggested the presence of retinoic acid receptors in the nuclei. Our present study has demonstrated that the plasma membrane may be another important target for retinoic acid in the induction of granulocytic differentiation. Previously, Yen et al. [25] have shown that a drug interaction that is limited to the cell surface is sufficient for the induction of differentiation. Recently, Almagor and Bar-Tana [1] have demonstrated that retinoic acid inhibits the myristoylation of a 25-kDa membrane protein in HL-60 cells. They suggest that the nonmyristoylated state of this membrane protein could perhaps signal the differentiation of HL-60 cells induced by retinoic acid. The present data offer another insight into the mechanism of action of retinoic acid, i.e., the signal transduction for retinoic acid-induced differentiation may be initiated and mediated through the membrane.

In addition, it has been suggested that although the initial process of differentiation induced by retinoic in HL-60 cells is different from that induced by dimethylsulfoxide (DMSO), these two processes converge at a certain point in the signaling pathway for differentiation [15, 23]. These findings suggest that the differentiation program initiated by each inducer is composed of both common and inducer-specific processes. Our present results raise the possibility that there may exist a differentiation initiation program that involves the membrane. Whether the membrane signaling mechanism can be executed by other agents is not clear at present. These lines of investigation may contribute to understanding the role of retinoic acid in the signal transduction pathway for the terminal differentiation of HL-60 cells.

The studies described in this report also hint at the potential use of staurosporine in retinoic acid-induced differentiation therapy. Our results indicate that there might be considerable variations in the level of the activities induced by a combination treatment with retinoic acid and staurosporine, depending on the sequence of administration. Staurosporine pretreatment followed by the retinoic acid schedule could be the most favorable growth-inhibitory combination and may be utilized clinically in the treatment of patients with certain leukemias.

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