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RETINOIC ACID INHIBITS PHOSPHATIDYLINOSITOL TURNOVER ONLY IN RA-SENSITIVE WHILE NOT IN RA-RESISTANT HUMAN NEUROBLASTOMA CELLS

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Phosphatidylinositol (PI) turnover has recently been implicated in the regulation of cell proliferation and transformation. We have investigated its role in differentiation using LAN-1 cells, a human neuroblastoma cell line which can be induced to differentiate along the neuronal pathway by retinoic acid (RA), and a derivated RA-resistant subline of it (LAN-1-res). We have found that treatment of LAN-1 cells with RA is followed by a rapid decrease of inositol phospholipid metabolism, using myo-[1,2-^{3}H] inositol or [1,(3)-^{3}H] glycerol. Analysis of labelled phosphatidylinositol metabolites from prelabelled LAN-1 cells indicated a rapid decrease of inositol (1,4,5)-trisphosphate and (1,2) diacylglycerol within 1 min. of induction of differentiation by RA, while no changes were observed in RA-treated LAN-1-res cells. These findings indicate that phosphoinositides-derived metabolites may be directly implicated in the induction processes of RA-triggered NB cell differentiation.

In response to extracellular signals, the phosphatidylinositols (PI) are degraded rapidly to produce a host of products. The initial reaction involves a phospholipase C which catalyses the formation of the cellular second messengers, inositol 1,4,5- trisphosphate (IP_3) and diacylglycerol (DG) from phosphatidylinositol bisphosphate [1-3]. IP_3 apparently mobilizes Ca^{2+} from endoplasmic reticulum [4,5], wheras DG acts, in concert with Ca^{2+}, as the endogenous activator of protein kinase C [6,7].

It is accepted that the cellular levels of PI metabolites play key roles in the control of proliferation, differentiation, and transformation [8,9].

Neuroblastoma (NB) cells provide a model system for studying tumor cell differentiation and neuronal development. Cultured mouse and human NB cells can be induced to differentiate by RA [10,11]. Binding of RA to a citoplasmic RA-binding protein

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Abbreviations: PI, phosphatidylinositol; RA, retinoic acid; IP_3, inositol trisphosphate; DG, diacylglycerol; NB, neuroblastoma.

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(CRABP) has been documented [12]. In addition more recent studies [13,14] indicate that RA somehow affects genomic expression by its indirect or direct interaction with the cell nucleus. However information is lacking with respect to the mechanism whereby the signal is transduced across the membrane. We previously shown [15] that in vitro induction of differentiation of LAN-1 cells by RA is associated with a rapid decrease of PI turnover, while gamma-interferon (γIFN) treatment does not alter the levels of PI-derived metabolites. We then examined the relationship between RA-induced growth inhibition, induction of differentiation, and decrease of PI turnover, taking advantage of RA resistant NB cells.

MATERIALS AND METHODS

Reagents.

Myo-[1,2-^{3}H] inositol (specific activity 19 Ci/mmole) and [1(3)^{3}H] glycerol (specific activity 3 Ci/mmole) were from Amersham (Buckinghamshire, England.). Lipid standards, carbachol (CB), and all-trans-RA were from Sigma (St. Louis, MO, USA.). The purchase of all other materials has been described [16].

Cell line.

Human neuroblastoma cell line LAN-1 was maintained in culture in RPMI 1640 medium containing 15% FCS. A variant of LAN-1 (designed LAN-1-res) were developed by culturing LAN-1 cells in the continuous presence of 10^{-6} M RA for 6 months. Both cells were grown, subcultured, and treated with RA as described elsewere [17].

Assay for [^{3}H] Thymidine incorporation and morphological differentiation.

The effects of RA on cell growth and morphological differentiation of NB cells have been previously described [18].

Cell labelling procedure.

Generation of radiolabelled inositol phosphates was studied as previously described [15]. Briefly, inositol-depleted cells (2 x 10^6/ each point) were labelled for 24 hours in inositol-free RPMI 1640 medium containing 10 μCi/ml myo-[1,2-^{3}H] inositol, and the incubation continued at 37°C in the presence or absence of 10^{-5}M RA.

DG levels were evaluated with the same protocol detailed above for inositol phosphates using [1(3)-^{3}H] glycerol.

Extraction procedures.

Lipids were extracted using a method previously described [16].

Thin layer chromatography of (1,2) diacylglycerol.

The pattern of distribution of the chloroform-soluble radioactivity into neutral lipids was determinated by thin layer chromatography (TLC) using LK6DF pre-lined Silica Gel G plates (Whatman Inc.,Clifton, N.J., USA) developed with hexane / diethylether / acetic acid, 89:59.5:1.5 (v/v/v). Lipid standards were visualized by exposure to iodine vapors. Spot corresponding to the location of authentic standards were scraped and counted for radioactivity in a Packard Tri-Carb 4530 beta counter.

Separation of water-soluble inositol phosphates.

Anion exchange High Performance Liquid Chromatography (HPLC) was used to separate [^{3}H] inositol phosphates according to a method described by Dean [19]. The column was calibrated using authentic labelled standards obtained commercially or prepared according to published methods [19,20].

RESULTS

When the LAN-1 NB cell line is treated with RA, the cells are induced to differentiate resulting in markedely reduced rates of proliferation which are reflected by reduction of [^{3}H] thymidine incorporation and morphological changes [17] (Tab. 1). Levels of inositol phosphates were measured over the first hour of treatment with RA. Cells were labelled to equilibrium with myo- [1,2-^{3}H] inositol to ensure that any observed changes resulted from changes in mass and not of specific activities. After addition of 10^{-5} M RA to the cells the level of [^{3}H]-I(1,4,5)P_3 fell to approximately 20 % of the control value within 1 min. (Tab. 1). This level remained 35-50 % lower than control values, up to 1 hour. Measurements of [^{3}H] inositol monophosphate (IP) and [^{3}H] inositol bisphosphate (IP_2) gave results similar but smaller to those obtained for IP_3, when compared (data not shown).

To distinguish between the possibilities that the early decrease we observed in IP_3 after induction of LAN-1 cell differentiation by RA was a result of decreased PI turnover or increased IP_3 phosphatase activity, we measured the steady-state levels of DG. Tab. 1 shows that levels of DG decreased after RA induction of LAN-1 cells to 45 % of control within 1 min. by RA induction of differentiation.

To further examine the relationship between RA-induced growth inibition, induction of differentiation and decreased levels of PI-metabolites, we tested the ability of RA to influence PI- turnover in a RA-resistant variant of LAN-1. This resistant cells, designed LAN-1-res cells, are markedly less sensitive than the parental line to the antiproliferative effects of RA and do not show morphological differentiation (Tab. 2). Culturing LAN-

Table 1 Effect of RA on [^{3}H] thymidine incorporation and IP_3 and DG levels in LAN-1 cells

Treatment	I (1,4,5)P_3level (fmole/ 10^6 cells)	DG level (fmole/ 10^6 cells)	[^{3}H]Thymidine incorporation (cpm)	Morphological differentiation (%)
Control	190 ± 4[a]	205 ± 7[a]	125,900 ± 4,920[a]	< 10
RA(10^{-5}M)	38 ± 7	95 ± 8	29,500 ± 2,620	> 90

[a]Mean ±SEM of quadruplicate experiments

LAN-1 cells were treated for 1 min or 4 days for assessing IP_3 and DG levels or [^{3}H]-thymidine incorporation and morphological changes, respectively, as described in Materials and Methods .

Table 2 Effect of RA on [^{3}H] thymidine incorporation and IP_3 and DG levels in LAN-1-res cells

Treatment	I (1,4,5)P_3level (fmole/ 10^6 cells)	DG level (fmole/ 10^6 cells)	[^{3}H]Thymidine incorporation (cpm)	Morphological differentiation (%)
Control	169 ± 9[a]	181 ± 8[a]	110,650 ± 7,510[a]	< 10
RA(10^{-5}M)	165 ±10	186 ± 9	99,800 ± 9,300	< 10
CB(10^{-3}M)	422 ±15	375 ±10	nd	nd

[a]Mean ± SEM of triplicate experiments
nd, not detected

LAN-1-res cells were treated for 1 min. or 4 days for assessing IP_3 and DG levels or [^{3}H] thymidine incorporation and morphological changes, respectively, as described in "Materials and Methods".

1- res cells with 10^{-5} M RA did not affect the levels of IP_3 and DG. In contrast, after addition of 10^{-3} M CB to the cells the levels of I(1,4,5)P_3 and DG increased to approximately 250 % and 200 %, respectivily, of the control value within 1 min. (Tab. 2).

DISCUSSION

In this study we demonstrated that retinoic acid rapidly decrease PI-turnover in differentiating NB cells, while RA treatment of RA-resistant NB cells does not alter the levels of the second messengers, IP_3 and DG. Retinoic acid, a biologically active metabolite of vitamin A, has been shown to have multiple effects on cells. In neuroblastoma tissue cultures, RA appears to be a potent compound for promoting differentiation, inhibiting cell growth, and perhaps reducing tumorigenicity [11,17,21,22]. This diversity may preclude the existence of a single mechanism operating at the molecular level. Although recent studies indicate that RA somehow affect genomic expression by its direct or indirect interaction with the cell nucleus [13,14], we suggest that possible the primary site of action of RA takes place on the plasma membrane. Through such interaction " second messengers" are formed which in turn make possible the interaction between RA and specific nuclear RA receptor(s), later affecting gene expression.

Based on the hypothesis that the cellular levels of certain PI metabolites play key roles in the control of proliferation, transformation, and differentiation [3-9] and on our previous results [15] that PI-turnover is not a general regulator of NB cell differentiation, we were interested to specifically evaluate the role that PI-turnover metabolism may be playing in the RA- induced differentiation of NB cells.

The results presented in this paper confermed that in vitro induction of differentiation of LAN-1 neuroblastoma cells by RA is associated with a rapid decrease of PI turnover. Decrease in inositol (1,4,5) trisphosphate and diacylglycerol were detectable within 1 min. after the addition of RA. The possibility that the very early decreases in concentrations of PI metabolites after induction by RA of LAN-1 cell differentiation were due to a decrease in inositol or glycerol uptake was eliminated, as there was observed no decrease in myo-[1,2-^{3}H] inositol or [1(3)^{3}H] glycerol uptake in RA treated cultures, compared to control cells (data not shown). The inhibition of PI-turnover in differentiating NB cells is strikingly correlated to the capacity of RA to trigger the differentiation process. In a RA-resistant variant of LAN-1 cell line, that is markedly less sensitive to the antiproliferative and/or differentiative effects of RA, this agent does not signal cells by rapid alterations in phosphoinositides-metabolites levels.

The possibility that LAN-1-res cells were not able to response to extracellular signals by using common signal transduction mechanisms has been ruled out since treatment of these cells with 10^{-3} M carbachol increased the levels of IP_3 and DG of about 250 % of control value.

Serum starvation of LAN-1 cells does not decrease cell viability nor induce morphological differentiation [23], while markedly delay cell growth. In such a condition (manuscript in preparation) PI turnover is not inhibited, suggesting that alterations in PI derived metabolites play a direct role in triggering NB differentiation by RA.

We conclude that down regulation of poliphosphoinositides metabolites (i.e. IP_3 and DG) is part of the coordinated series of molecular events responsible for RA- mediated commitment of LAN-1 human NB cells to terminal differentiation.

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