[CANCER RESEARCH 42, 4918-4920, December 1982]
0008-5472/82/0042-0000$02.00

Correlation between Activation of Quiescent 3T3 Cells by Retinoic Acid and Increases in Uridine Phosphorylation and Cellular RNA Synthesis

Eliezer Rapaport,[1] Edward W. Schroder,[2] Alisa Kastan Kabcenell, and Paul H. Black[3]

Department of Microbiology, Boston University School of Medicine, and The Hubert H. Humphrey Cancer Research Center at Boston University, Boston, Massachusetts 02118

ABSTRACT

Short exposure of cultured quiescent cells to micromolar quantities of β-all-*trans*-retinoic acid (RA) has been reported to potentiate the effects of phorbol myristate acetate in promoting the transition from the resting to growing states of these cells. Longer periods of exposure to RA result in substantial inhibition of cellular proliferation. We now show that short-term treatment of quiescent Swiss 3T3 cells with RA yields marked increases in uridine phosphorylation and total cellular RNA synthesis as well as 2-deoxyglucose uptake. Upon subsequent treatment of the cells with phorbol myristate acetate, a direct correlation between the comitogenic activity of RA and its stimulation of uridine phosphorylation and RNA synthesis is apparent. The increases in 2-deoxyglucose uptake persist after long-term exposure of the cells to RA when the growth-inhibitory effects of this agent are observed.

INTRODUCTION

In view of the recent interest in vitamin A and its analogs (retinoids) as potential cancer chemopreventive agents in animals (8, 10, 17, 21), their growth-inhibitory effects on cultured mammalian cells have been widely studied (3, 4, 9, 19). Retinoids, however, were also shown to exhibit cell-activating effects when allowed to act synergistically with the tumor promotor PMA[4] (1, 7, 14, 18, 19, 23). We have recently suggested that both the cell-activating and antiproliferative activities of RA on murine Swiss 3T3 cells are mediated by cellular acid-soluble nucleotide pools (14). We now report that short-term treatment of quiescent 3T3 cells with RA leads to the stimulation of events which are associated with the transition from the resting to growing states of these cells, namely, uridine phosphorylation, RNA synthesis, and 2-deoxyglucose uptake. The direct relationship between increases in cellular proliferation and enhancement of these metabolic events is well established (2, 5, 11, 15, 16, 22, 24). In this communication, however, we demonstrate a system where the increases in uridine phosphorylation, RNA synthesis, and 2-deoxyglucose uptake are independent of, and well separated from, a subsequent mitogenic stimulus. A tight correlation is observed between RA-promoted increases in the rates of uridine phosphorylation and RNA synthesis in quiescent 3T3 cells and the subsequent enhancement of the mitogenic stimulation of these cells by PMA.

MATERIALS AND METHODS

Cells were cultured in 35-mm Petri dishes, rendered quiescent, and treated with RA as reported previously (14, 19). Cells were labeled with [^{3}H]uridine (0.5 μCi/ml; 37.6 Ci/mmol) for 1 hr before extraction of acid-soluble nucleotides. Acid-soluble nucleotides were extracted and analyzed by high-pressure liquid chromatography on ion-exchange columns as described (13, 19). Specific radioactivity of [^{3}H]UTP was determined by collecting the UTP peak after its chromatographic separation. Incorporation of [^{3}H]uridine into acid-insoluble material was determined in the same dishes after extraction of acid-soluble nucleotides. Determination of the effects of the duration of RA pretreatment on its enhancement of the mitogenic response of 3T3 cells to PMA as determined by pulse labeling with [^{3}H]thymidine has been reported (19). Swiss 3T3 cells were plated at 4×10^4/35-mm-diameter tissue culture dish in DME-10% serum. After a 4-hr attachment period, cells were changed to DME-0.25% serum and incubated for 72 hr. This protocol yielded 0.8 to 1.0×10^5 quiescent cells/35-mm dish. Quiescent cells were treated with 10 μM of RA in serum-free DME for the period of time indicated, washed once with DME, and subjected to mitogenic stimuli with PMA (20 ng/ml) in DME without serum. DNA synthesis was assayed 18 hr after stimulation with PMA. RA treatment alone without stimulation with PMA did not produce any significant increases in DNA synthesis over the unstimulated controls (no RA or PMA treatment). Stimulation by PMA without pretreatment with RA is reported as time zero of the length of retinoic acid treatment (Chart 1). Quantitatively, stimulation of DNA synthesis in quiescent Swiss 3T3 cells with PMA alone for 18 hr yielded 11.6 ± 0.6% (S.E.) of the stimulation achieved by treatment with 10% serum under the same conditions. Unstimulated controls produced 2.0 ± 0.3% and quiescent cells pretreated with RA for 4, 7, or 18 hr followed by 18-hr incubation with DME not containing PMA yielded 5.2 ± 3.3% of the stimulation of DNA synthesis obtained after 18-hr treatment with 10% serum. The data in Chart 1 are presented as specific radioactivity of total cellular UTP pools in UTP (cpm/nmol), the level of RNA synthesis in cpm incorporated into acid-insoluble material/cpm/nmol of [^{3}H]UTP precursor, and the enhancement of the cellular mitogenic response to PMA in percentage of cellular response to 10% serum, all plotted against the duration of RA treatment. Determinations made after stimulation of cells with 10% serum are reported with the corresponding solid symbols. Each point is the mean of 4 determinations (±S.E.). The time course of [^{3}H]uridine incorporation into acid-soluble and acid-insoluble material was linear for 60 min.

Determinations of 2-[^{3}H]deoxyglucose uptake were performed according to standard procedures (20). Control cultures were treated with 0.1% ethanol for the same period of time since RA is added in this manner. The uptake of 2-[^{3}H]deoxyglucose was linear for 60 min. Data are presented as the mean of 4 determinations (±S.E.).

[1] Supported by Research Grant CA-28803. To whom requests for reprints should be addressed.
[2] Supported by Research Grant IN-97F from the American Cancer Society.
[3] Supported by Research Grant CA-28107 from the National Cancer Institute.
[4] The abbreviations used are: PMA, phorbol myristate acetate; RA, β-all-*trans*-retinoic acid; DME, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenol.
Received June 7, 1982; accepted August 13, 1982.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/42/12/4918/2412423/cr0420124918.pdf by guest on 27 January 2023

RESULTS AND DISCUSSION

Chart 1 demonstrates dramatic increases in [^{3}H]uridine incorporation into total cellular UTP pools after treatment of G_0-G_1-arrested Swiss 3T3 cells with 10 μM RA. Stimulation of uridine phosphorylation is evident after a 1-hr exposure to RA and reaches a maximum at 5 hr. The maximal effects of 10 μM RA on uridine phosphorylation are comparable to the magnitude of stimulation achieved by a 2-hr exposure to 10% fetal calf serum. A 2-fold enhancement of total cellular RNA synthesis, indicated by the increases in the ratio of [^{3}H]uridine incorporation into acid-insoluble material to the total [^{3}H]UTP precursor pool, is observed after 4 hr of treatment with RA. High-specific-activity [^{3}H]uridine is utilized, and incorporation is performed for 60 min (at which point it is still linear). Therefore, the possibility of compartmentalization of the precursor [^{3}H]UTP pools for either ribosomal or heterogeneous RNA synthesis is eliminated due to equilibration of the radioactively labeled precursor pools under these conditions (6). Thus, the values presented for total cellular RNA synthesis take into account the total cellular [^{3}H]UTP precursor pools. We studied the relationship between the duration of RA pretreatment and the subsequent enhancement of the cellular mitogenic response to PMA (19). Data suggesting a direct correlation between 3T3 cell activation by RA and its stimulation of uridine phosphorylation and RNA synthesis are presented (Chart 1). Uptake of 2-deoxyglucose by quiescent Swiss 3T3 cells is markedly enhanced after treatment with 10 μM RA (Chart 2). The increases in 2-deoxyglucose uptake, however, do not correlate with the comitogenic activity of RA (Charts 1 and 2).

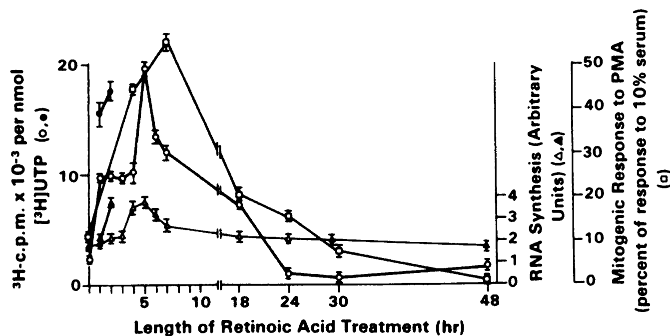

Chart 1. Stimulation of [^{3}H]uridine uptake into total cellular [^{3}H]UTP pools and enhancement of RNA synthesis in quiescent Swiss 3T3 cells after treatment with 10 μM RA or 10% serum. Correlation with the comitogenic activity of RA. ●, ▲: determinations made after treatment with 10% serum for the indicated amount of time.

The uptake of uridine by mammalian cells has been studied extensively (5, 11, 24). It has been shown that the stimulation of uridine uptake by quiescent Swiss 3T3 and other cultured mammalian cells after short-term exposure to serum or other growth factors proceeds by enhancement of uridine phosphorylation, while the rate of uridine transport, which is independently regulated, remains unchanged (15, 16). The uptake of 2-deoxyglucose by mammalian cells is regulated at the level of transport (22). However, due to the efficient transport of 2-deoxyglucose into mammalian cells, a high intracellular concentration of the free sugar can be achieved which will in turn make the phosphorylation of 2-deoxyglucose rate limiting (25).

We have previously shown that the increase in cellular responsiveness to mitogens and the antiproliferative effects of RA treatment depend upon the length of exposure of the cells to RA (Chart 1) (16). We have also suggested that these effects are mediated by RA-promoted effects on cellular ATP pools (19). Small expansions of cellular ATP pools have been linked to increases in the rate of rRNA synthesis during the G_1 phase of the 3T3 cell cycle and have been termed necessary but not sufficient for the onset of DNA replication in these cells (2). Prolonged treatment (over 48 hr) of 3T3 cells with RA yielded marked increases in total cellular ATP pools. These increases directly correlated with the growth-inhibitory activity of RA (14). The data reported here (Table 1) indicate that exposure of quiescent 3T3 cells to 0.2 mM DNP for the last 2 hr of RA

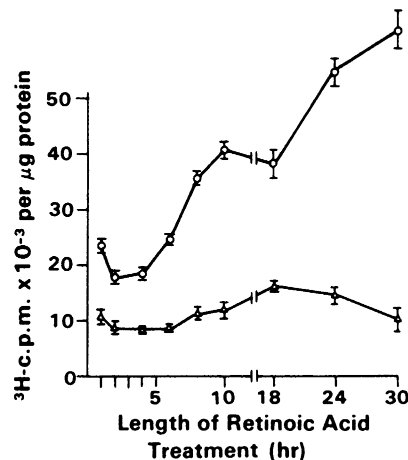

Chart 2. Stimulation of 2-[^{3}H]deoxyglucose uptake by quiescent Swiss 3T3 cells after treatment with 10 μM RA. The data are reported as cpm/μg protein of 2-[^{3}H]deoxyglucose plotted against the duration of RA treatment for RA-treated (○) and control (△) cells.

Table 1

Effects of RA treatment (10 μM) and exposure to DNP (0.2 mM for the last 2 hr) on total cellular ATP and UTP pools, [^{3}H]uridine incorporation into total cellular [^{3}H]UTP, and total cellular RNA synthesis

Experimental procedures are described in the text.

Length of RA treatment (hr)	DNP treatment	nmol/10^6 cells			[^{3}H]UTP (cpm/nmol)	RNA synthesis (% of control)
		ADP	ATP	UTP		
	−	1.8 ± 0.4[a]	11.9 ± 0.6	1.8 ± 0.1	2,362 ± 271	100
	+	3.6 ± 0.2	11.1 ± 1.0	0.8 ± 0.2	8,318 ± 498	103
4	−	1.8 ± 0.4	12.9 ± 0.4	1.6 ± 0.2	11,764 ± 1,025	199
4	+	2.8 ± 0.3	11.3 ± 0.6	0.9 ± 0.1	8,513 ± 661	89
7	−	2.1 ± 0.4	12.1 ± 1.2	1.6 ± 0.1	12,100 ± 699	150
7	+	3.7 ± 0.1	11.7 ± 0.2	1.0 ± 0.1	10,095 ± 234	106
24	−	2.3 ± 0.4	13.5 ± 0.6	4.8 ± 0.2	998 ± 122	121
24	+	3.7 ± 0.9	12.0 ± 1.1	3.1 ± 0.5	1,061 ± 239	101

[a] Mean ± S.E.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/42/12/4918/2412423/cr0420124918.pdf by guest on 27 January 2023

treatment produces marked decreases in total cellular UTP pools and RNA synthesis for RA-treated cells. Total cellular ATP pools are not significantly altered, while the rate of uridine phosphorylation is only slightly reduced.

It has been shown that DNP alters cellular ATP pools by uncoupling oxidative phosphorylation and enhancing mitochondrial ATPase activity (12). The resulting increased availability of ADP (Table 1) stimulates glycolysis (12) and thus the maintenance of ATP pools in DNP-treated cells (Table 1). The data in Table 1 indicate that a constant portion of the total cellular pools of UTP is susceptible to DNP treatment. This fraction of the total cellular UTP pools affected by DNP treatment is not dependent on the length of RA treatment despite the time-dependent increase in total cellular UTP pools induced by RA after 24 hr. It is thus suggested that ATP pools which participate in the biosynthesis of UTP exist as 2 functionally distinct pools, one of which is sensitive to DNP. Considered together, these findings suggest that total cellular UTP pools and RNA synthesis, but not the rate of uridine phosphorylation, are regulated by a functionally compartmentalized ATP pool which is sensitive to DNP treatment.

Thus, although the growth-inhibitory properties of RA are clearly observed after long-term treatment (Chart 1) (14, 19), this analog of vitamin A also possesses the ability of potentiating the effects of mitogens on mammalian cells via mechanisms related to its stimulation of rRNA synthesis which must occur prior to initiation of DNA replication.

REFERENCES

1. Dicker, P., and Rozengurt, E. Retinoids enhance mitogenesis by tumor promotor and polypeptide growth factors. Biochem. Biophys. Res. Commum., *91:* 1203–1210, 1979.
2. Grummt, F., Paul, D., and Grummt, I. Regulation of ATP pools, rRNA and DNA synthesis in 3T3 cells in response to serum or hypoxanthine. Eur. J. Biochem., *76:* 7–12, 1977.
3. Haddox, M. K., Scott, K. F., and Russell, D. H. Retinol inhibition of ornithine decarboxylase induction and G_1 progression in Chinese hamster ovary cells. Cancer Res., *39:* 4930–4938, 1979.
4. Jetten, A. M., Jetten, M. E. R., Shapiro, S. S., and Poon, J. P. Characterization of the action of retinoids on mouse fibroblast cell lines. Exp. Cell Res., *119:* 289–299, 1979.
5. Jimenez de Asua, L., and Rozengurt, E. Multiple control mechanisms underlie initiation of growth in animal cells. Nature (Lond.), *251:* 624–626, 1974.
6. Khym, J. X., Jones, M. X., Lee, W. H., Regan, J. D., and Volkin, E. On the question of compartmentalization of the nucleotide pool. J. Biol. Chem., *253:* 8741–8746, 1978.
7. Levine, L., and Ohuchi, K. Retinoids as well as tumor promoters enhance deacylation of cellular lipids and prostaglandin production in MDCK cells. Nature (Lond.), *276:* 274–275, 1978.
8. Levine, N., and Meyskens, F. L. Topical vitamin A-acid therapy for cutaneous metastatic melanoma. Lancet, *2:* 224–226, 1980.
9. Lotan, R., Giotta, G., Nork, E., and Nicolson, G. L. Characterization of the inhibitory effects of retinoids on the *in vitro* growth of two malignant murine melanomas. J. Natl. Cancer Inst., *60:* 1035–1041, 1978.
10. Mayer, H., Bollag, W., Hanni, R., and Ruegg, R. Retinoids, a new class of compounds with prophylactic and therapeutic activities in oncology and dermatology. Experientia (Basel), *34:* 1105–1119, 1978.
11. Plagemann, P. G. W., and Richey, D. Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture. Biochim. Biophys. Acta, *344:* 263–305, 1974.
12. Racker, E. Why do tumor cells have a high aerobic glycolysis. J. Cell. Physiol., *89:* 697–700, 1976.
13. Rapaport, E., Christopher, C. W., Ullrey, D., and Kalckar, H. M. Selective high metabolic lability of uridine triphosphate in response to glucosamine feeding of untransformed and polyoma virus-transformed hamster fibroblasts. J. Cell. Physiol., *104:* 253–259, 1980.
14. Rapaport, E., Schroder, W. E., and Black, P. H. Retinoic acid-promoted expansion of total cellular ATP pools in 3T3 cells can mediate its stimulatory and growth inhibitory effects. J. Cell Physiol., *110:* 318–322, 1982.
15. Rozengurt, E., Mierzejewski, K., and Wrigglesworth, N. M. Uridine transport and phosphorylation in mouse cells in culture: effect of growth-promoting factors, cell cycle transit and oncogenic transformation. J. Cell. Physiol., *97:* 241–252, 1978.
16. Rozengurt, E., Stein., W. E., and Wrigglesworth, N. M. Uptake of nucleosides in density inhibited cultures of 3T3 cells. Nature (Lond.), *267:* 442–444, 1977.
17. Schroder, E. W., and Black, P. H. Retinoids: tumor preventers or tumor enhancers? J. Natl. Cancer Inst., *65:* 671–674, 1980.
18. Schroder, E. W., Chou, I.-N., and Black, P. H. Effects of retinoic acid on plasminogen activator and mitogenic responses of cultured mouse cells. Cancer Res., *40:* 3080–3094, 1980.
19. Schroder, E. W., Rapaport, E., Kabcenell, A. K., and Black, P. H. Growth inhibitory and stimulatory effects of retinoic acid for murine 3T3 cells. Proc. Natl. Acad. Sci. U. S. A., *79:* 1549–1552, 1982.
20. Sefton B. M. and Rubin, H. Stimulation of glucose transport in cultures of density-inhibited chick embryo cells. Proc. Natl. Acad. Sci. U. S. A., *68:* 3154–3157, 1971.
21. Sporn, M. B., and Newton, D. L. Chemoprevention of cancer with retinoids. Fed. Proc., *38:* 2528–2534, 1979.
22. Weber, M. J., Hale, A. H., Yau, T. M., Buckman, T., Johnson, M., Brady, T. M., and Larossa, D. D. Transport changes associated with growth control and malignant transformation. J. Cell. Physiol., *89:* 711–722, 1976.
23. Wilson, E. L., and Reich, E. Plasminogen activator in chick fibroblasts: induction of synthesis by retinoic acid: synergism with viral transformation and phorbol ester. Cell, *15:* 385–392, 1978.
24. Wohlheuter, R. M., Marx, R., Graff, J. C., and Plagemann, P. G. W. The application of rapid kinetic techniques to the transport of thymidine and 3-O-methylglucose into mammalian cells in suspension culture. J. Cell. Physiol., *89:* 605–612, 1976.
25. Wohlheuter, R. M., and Plagemann, P. G. W. The roles of transport and phosphorylation in nutrient uptake in cultured animal cells. Int. Rev. Cytol., *64:* 171–240, 1980.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/42/12/4918/2412423/cr0420124918.pdf by guest on 27 January 2023