Retinoic Acid Increases Cyclic AMP-dependent Protein Kinase Activity in Murine Melanoma Cells*

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The influence of all trans-retinoic acid on cyclic AMP metabolism was examined in B16-F1 mouse melanoma cells. Treatment of these cells with retinoic acid resulted in a dose-dependent inhibition of cell growth, which was accompanied by a concentration-dependent increase in both basal and cyclic AMP-stimulated protein kinase activity. Intracellular levels of cyclic AMP, however, were not altered by retinoid treatment. A protein kinase-deficient variant of B16-F1 (MR-4) did not exhibit decreased growth or increased protein kinase activity in response to retinoic acid treatment. At least 24 h of incubation was required before increased protein kinase activity could be detected in treated B16-F1 cells. Retinoic acid treatment increased the Vmax of protein kinase, but the Km for cyclic AMP activation was not altered. These findings suggest that in B16 mouse melanoma cells, cyclic AMP-dependent protein kinase may be a target for the growth inhibitory effects of the retinoid.

Vitamin A has been demonstrated to be required for maintenance of normal epithelial cell differentiation (1). Analogues of vitamin A (retinoids) were found to affect glycosylation reactions (2), increase iodosinatinal fibronectin (3), increase cell-to-substratum adhesiveness (4), inhibit growth (5, 6), increase tyrosinase and melanin biosynthesis in mouse melanoma cells (7), and reverse the tumor promoter induction of ornithine decarboxylase activity in mouse epidermal cells (8). Although cytoplasmic binding proteins for retinol and retinoic acid have been found in many cell types, it is not understood how the interaction of retinoids with specific receptor proteins ultimately results in a cellular response.

The cyclic AMP system has also been implicated in regulating the differentiated functions of many cell types (9-12). In particular, cyclic AMP has been shown to inhibit growth and stimulate melanin production in murine melanoma cells (11, 13). Thus, its action in these cells mimics that of retinoids (7). Therefore, we undertook a study to determine whether there was any interrelationship between the actions of retinoids and the cyclic AMP system.

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MATERIALS AND METHODS

B16-F1 cells were obtained through the courtesy of Dr. J. I. Fidler, Frederick Cancer Research Center, Frederick, Md. The protein kinase-deficient variant of F1 (MR-4) was obtained in our laboratory through selective cloning procedures as previously described (14). Both cell lines were routinely maintained in minimal essential medium plus Earle's salt supplemented with nonessential amino acids L-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics (50 µg/ml of streptomycin sulfate, 50 units/ml of penicillin G), and 10% fetal bovine serum (heat-inactivated, Gibco) and incubated in a 37°C, 95% air, 5% CO2 humidified atmosphere. Growth inhibition in both cell lines was determined by seeding 2.0 × 10⁴ cells onto 90-mm tissue culture dishes (Falcon, Oxnard, Calif.). Twenty-four hours after seeding, triplicate plates were used to determine cell number through the use of a hemocytometer. Also, at this time, one-half of the dishes were refed with medium supplemented with either 10⁻⁵ M all trans-retinoic acid (Sigma) or various concentrations of retinoic acid (10⁻¹⁰ to 10⁻³ M). Control plates received medium plus solubilization vehicle (0.1% ethanol). At 24, 48, 72, and 96 h of incubation with or without retinoid, triplicate plates were analyzed for cell number. Also, after 48 h of incubation, control and treated plates were refed with the appropriate medium. All manipulations involving cells and retinoic acid were performed under indirect light.

Protein kinase activity in control and retinoid-treated cells was assayed by the method of Corbin et al. (15), using a 10-min incubation time. Briefly, this involved washing the cells three times with 4 ml of 50 mM phosphate buffer (pH 6.8), scraping the cells from the dishes with a rubber policeman and sonicating the resultant suspension (1.5 ml) for 30 s at setting No. 5 in a model W185 sonicator (Heat Systems, Plainview, N. Y.). The reaction mixture consisted of 50 mM phosphate buffer (pH 6.8), 0.2 mM [γ-³²P]ATP (180 cpm/µmol), 0.5 mg of histone (type II-A) ± various concentrations of cyclic AMP, and 20 µl of cell homogenate. The reaction was terminated by pipetting 50 µl of the reaction mixture onto Whatman 3MM filter paper discs (2.3 mm diameter; Whatman, Inc., Clifton, N. J.). The discs were dropped into ice-cold 10% trichloroacetic acid and washed in sequence with 10% trichloroacetic acid, 95% ethanol, and ether. After drying, the filters were placed in scintillation vials containing Omnifluor (New England Nuclear, Boston, Mass.) and counted. Activity was assessed in the absence or presence of 10⁻⁵ to 5 × 10⁻³ M cyclic AMP. The samples were corrected for endogenous protein phosphorylation in the absence of histone and cyclic AMP. Under our assay conditions, activity was linear with respect to protein concentration.

All chemical reagents were obtained from Sigma Chemical Co., St. Louis, Mo. [γ-³²P]ATP specific activity, 10 Ci/µmol, was obtained from New England Nuclear, Boston, Mass.

RESULTS

Fig. 1 illustrates the effect of 10⁻⁵ M retinoic acid on B16-F1 cell growth. Within 24 h after the addition of retinoic acid to the culture medium, growth inhibition was evident. The relative growth inhibition increased with time and, at 4 h of retinoic acid treatment, reached 71%. In contrast, there was little, if any, growth inhibition in MR-4 cells treated in the same manner at any time point examined (Fig. 2).

The growth inhibition of B16-F1 cells induced by retinoic acid was concentration-dependent (Fig. 3) with significant inhibition occurring at 10⁻⁷ M. Also, it should be noted that the degree of inhibition obtained by 10⁻⁵ M retinoic acid did vary somewhat between separate experiments. For example, after 48 h of treatment (Fig. 1), 54% inhibition was achieved, while in a separate experiment (Fig. 3), 70 to 75% inhibition was achieved. It appeared that the condition of the cells (subconfluent versus confluent) used to set up the experiment influenced the degree of retinoic acid-induced growth inhibi-
Retinoic Acid Stimulates Protein Kinase

**Fig. 1 (left).** Effect of retinoic acid (R.A.) on the growth of B16-F1 mouse melanoma cells. B16-F1 cells were seeded at a density of 2 x 10^5/90 mm tissue culture dish (Falcon; Oxnard, Calif.). Following a 24-h cell attachment period, the cells were refed with medium containing either 0.1% ethanol (control) or 10 μM retinoic acid (all trans β) dissolved in a final concentration of 0.1% ethanol. At the indicated time points, triplicate dishes were used to determine cell number. The data are represented as the mean ± S.E. The numbers in parentheses represent the percentage of inhibition of growth by retinoic acid relative to control cells at each time point.

**Fig. 2 (right).** Effect of retinoic acid (R.A.) on the growth of MR-4 mouse melanoma cells. This experiment was conducted exactly as described in the legend to Fig. 1. The data are represented as the mean ± S.E.

We next determined whether retinoic acid might have caused growth inhibition by increasing intracellular cyclic AMP levels since we have shown (16) that the latter inhibit B16-F1 cell growth. However, in agreement with the results reported by Lotan for S91 murine melanoma (7), we were unable to observe an increase in B16-F1 cyclic AMP levels at any concentration of retinoic acid tested (data not shown).

To examine the possible involvement of the cyclic AMP system further, we determined the activity of cyclic AMP-dependent protein kinase, the only known mediator of cyclic AMP action, in control and retinoic acid-treated cells. The data from these experiments (Fig. 4A) clearly show that retinoic acid significantly increased the activity of protein kinase in B16-F1 cells. Increased enzyme activity was dependent upon the concentration of retinoic acid and the activity was increased in both the absence and presence of 10^-6 M cyclic AMP. In contrast to B16-F1 cells, retinoic acid had relatively little effect on either basal or cyclic AMP-stimulated protein kinase activity in MR-4 cells at any concentration of retinoic acid tested (Fig. 4B). In other experiments (data not shown), we found that treating a B16-F1 cell sonicate directly with 10^-5 M retinoic acid for as long as 6 h at 37°C did not alter protein kinase activity.

Retinoic acid stimulation of B16-F1 protein kinase activity does not occur immediately but, rather, requires more than 6 h of incubation (Fig. 5). Significant stimulation of protein kinase is obtained by 24 h of treatment and this level is more or less maintained through 48 h of treatment. In some experiments where cells were refed after 48 h of incubation with fresh medium containing retinoic acid, a further stimulation of protein kinase was observed at 72 h.

**Fig. 3.** Effect of various concentrations of retinoic acid on mouse melanoma cell growth. B16-F1, and MR-4 mouse melanoma cells were seeded at 5 x 10^5/90 mm plastic tissue culture dish (Falcon; Oxnard, Calif.) in minimal essential medium with Earle’s salts, plus 10% heat-inactivated fetal bovine serum (GIBCO). Following a 24-h cell attachment period, the cells were refed with medium containing either 0.1% ethanol (control) or various concentrations of retinoic acid (all trans β, Sigma) dissolved in 0.1% ethanol. After 48 h of incubation, all the plates were harvested and the cells were counted. Retinoic acid was freshly prepared before each experiment. During the incubation with retinoic acid, the dishes were placed on a stainless steel tray which was then wrapped with aluminum foil to prevent light from entering the dishes. A, B16-F1 cells; B, MR-4 cells.

**Fig. 4.** Effect of various concentrations of retinoic acid on cyclic AMP-dependent protein kinase activity in mouse melanoma cells. B16-F1, and MR-4 cells were prepared as described in the legend to Fig. 3. After 48 h of incubation with 10 μM retinoic acid, protein kinase activity was assayed as described in the text. The data was corrected for the activity of kinase in the absence of both cyclic AMP and histone and in the presence of cyclic AMP, but the absence of histone. Under our assay conditions, enzyme activity was proportional to protein concentration. A: B16-F1 protein kinase activity; ○, no cyclic AMP; □, +1 μM cyclic AMP. B: MR-4 protein kinase activity; □, no cyclic AMP; □, +1 μM cyclic AMP.
follows. (a) Retinoic acid inhibits growth and increases tyrosine activity at least some of the actions of retinoic acid in the F13 murine melanoma cell line. The evidence for this is as follows. Cyclic AMP duplicates both of these effects that cyclic AMP-dependent protein kinase is involved in mediating the actions of retinoic acid in the F13 cells. (b) Retinoic acid significantly increases the activity of cyclic AMP-dependent protein kinase which is the major if not the only receptor responsible for the effects of this cyclic nucleotide. (c) Retinoic acid does not inhibit growth or enhance protein kinase activity in a variant of B16-F1 cells (MR-4) which is resistant to the growth-inhibiting effects of melanocyte-stimulating hormone and has deficient protein kinase activity.

The mechanism by which retinoic acid increases protein kinase activity in B16-F1 cells is not clear. There are specific cytoplasmic receptor proteins for retinoic acid (17). It is thought that the retinoic acid-receptor protein complex is transported to the nucleus where it modifies gene transcription analogous to the action of steroid hormones (18, 19). Our data would fit this model in that there is a long lag period subsequent to retinoic acid treatment before increased protein kinase activity is observed. However, we do not yet have evidence suggesting that increased protein kinase activity after retinoic acid treatment is due to de novo enzyme synthesis. In regard to the model proposed for retinoid action and its similarity to steroid hormone action, it is interesting that Fuller et al. (20) have found a specific regulation of the amount of type I cyclic AMP-dependent protein kinase by testosterone especially in the prostate.

Lotan et al. (5, 6) have reported that retinoids inhibit the growth of many, but not all, cells in tissue culture. In particular, they demonstrated that B16 mouse melanoma cells were inhibited by retinoic acid in a concentration-dependent fashion. However, at a retinoic acid concentration of 10^{-5} M, they could not observe growth inhibition until 3 days of treatment, whereas we found significant growth inhibition at 24 h of treatment. This discrepancy may be due to the fact that we used a different growth medium and different treatment protocol than Lotan et al. (6). Whether the ability of retinoids to inhibit the growth of cells in culture has any relationship to their ability to inhibit in vivo chemical carcinogenesis (21-23) remains to be determined.

A variety of reports have provided evidence that cyclic AMP-dependent protein kinase is a mediator of growth inhibition. These include: the increase in cyclic AMP binding protein and protein kinase activity in mammary tumors regressing due to ovariectomy (24), the inability of cyclic AMP to inhibit cell growth in a protein kinase-deficient variant of S49 lymphoma cells (25), the inability of adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH) to inhibit growth in protein kinase-defective variants of Y1 adrenal cells (26) and B16 mouse melanoma cells (14), respectively, and the correlation between the ability of various cyclic AMP analogs to inhibit growth in human carcinoma cell cultures and their ability to activate cyclic AMP-dependent protein kinase from the same cell line (27). There are, however, some reports which have correlated increased cyclic AMP-dependent protein kinase activity with stimulation of growth. These include the activation of protein kinase by growth hormone in the liver and the adrenal (28), an increase in protein kinase during mitosis in Chinese hamster ovary (CHO) cells (29), and an early activation of protein kinase in mitogen-stimulated human lymphocytes (30). However, for the most part, these increases in protein kinase activity were restricted to type I protein kinase and it was shown that treatment of human lymphocytes with analogs of cyclic AMP which blocked the mitogenic response resulted in activation of both types I and II protein kinase (30). Whether the increase in protein kinase activity in retinoic acid-treated B16-F1 cells is restricted to type I or type II protein kinase remains to be determined. Also, future studies should be directed to other cell lines where retinoids have been shown to affect
growth and differentiated functions (7, 31, 32) in order to
determine whether stimulation of cyclic AMP-dependent pro-
tein kinase activity is a common feature of retinoid action.

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