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ANTICARCINOGENIC EFFECT OF RETINOIDS ON 7,12-DIMETHYLBENZ(a)ANTHRACENE-INDUCED MAMMARY TUMOR INDUCTION, AND ITS RELATIONSHIP TO CYCLIC AMP-DEPENDENT PROTEIN KINASE

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SUMMARY: Administration of 13-cis retinoic acid and N-(4-hydroxyphenyl) retinamide daily in the diet to female Sprague-Dawley rats beginning one day after intubation with 7,12-dimethylbenz(a)anthracene (DMBA) prolonged the latency periods and inhibited the percentage incidence of mammary tumors. A significant reduction in the total number of tumors was also evident. The inhibition of mammary tumor growth by retinoids was associated with a significant increase (3-fold) in cytosolic cAMP-binding and histone kinase activities. The increase of histone kinase activity was almost totally in the cAMP-dependent protein kinase Type II. Retinoic acid increased the amount of the regulatory subunit (R_{II}) rather than altering its cAMP binding affinity. These results suggest that cAMP-dependent protein kinase Type II may be involved in mediating the retinoid action in the inhibition of mammary tumor growth in vivo.

INTRODUCTION: Studies have shown that retinoids can suppress the process of carcinogenesis in vivo in many experimental systems (1-3), and inhibit the growth of several cell types in culture (4,5). It has also been suggested that retinoids play an important role in early development. Wolbach and Howe (6) reported that retinoic acid is required for normal epithelial cell differentation. Relative to the present study, both natural and synthetic retinoids markedly inhibit mammary carcinogenesis in vivo (7-12) as well as in vitro (13). Recently there has been a considerable interest in elucidating the mechanism by which retinoids mediate cell growth and differentiation. In addition, a concern has been raised as to whether the ability of retinoids to inhibit the growth of cells in culture has any relationship to their ability to inhibit in vivo

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Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; RA, 13-cis retinoic acid; HPR, N(4-hydroxyphenyl) retinamide; cAMP, cyclic adenosine 3',5' monophosphate.

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chemical carcinogenesis (14,15). Although several hypotheses have been suggested to explain the mechanism of action of retinoids, none of them proved to be sufficient to explain the diverse effects of retinoids (4).

The cAMP system has been implicated in the regulation of cell growth and differentiation (16,17). In particular, cAMP-dependent protein kinase has been shown to be involved in the inhibiton of mammary tumor growth (18). Thus, its action mimics that of retinoids. Therefore, we have undertaken this study to determine whether there is any interrelationship between the actions of retinoids in vivo and the cAMP system.

MATERIALS AND METHODS: DMBA, cAMP, ATP, calf thymus histone (Type II) and DEAE-cellulose were purchased from Sigma Chemical Co., St. Louis, MO. (G-^{3}H)cAMP (27 Ci/mmol) and (γ-^{32}P) ATP (200 Ci/mmol) were obtained from ICN Pharmaceuticals, Inc., Irvine, CA. 13-cis retinoic acid and N-(4-hydroxyphenyl) retinamide were generously provided by Roche Pharmaceutical Co., Rahway, NJ., and the National Cancer Institute, Bethesda, MD., respectively.

Tumor Induction and Retinoid Treatment: Primary mammary tumors were induced into 50-day old virgin female Sprague-Dawley rats (Harlan Indust., Indianapolis, IN) by one single intubation of 20 mg DMBA dissolved in 1 ml sesame oil/rat. Beginning one day after DMBA intubation a group of rats received 13-cis retinoic acid and another group received 4,hydroxyphenyl retinamide. Retinoids were blended with ground Purina chow at a concentration of 1.5 mmol of RA (450 mg/kg), or 1.5 mmol of 4 HPR (586 mg/kg). Each of the retinoid-treated rats received 30 g of this retinoid diet daily. The retinoids were prepared fresh every week and were dissolved in a vehicle containing 6% α-tocopherol as an antioxidant as previously described (19). A control group of DMBA-treated rats received the ground Purina chow blended with only the vehicle in which the retinoids were dissolved (30 g/rat/day). The rats were observed daily, palpated twice each week for tumor appearance, and weighed biweekly. Tumors were measured 3 times weekly by a Vernier caliper in 2 perpendicular dimensions to ascertain active tumor growth. Animals were killed by decapitation when moribund or 180 days after DMBA administration.

Preparation of Tissue Extracts: All procedures were performed at 0-4°C. Tissues were weighed, minced and homogenized in Teflon glass homogenizer with 5 volumes of buffer A (0.25M sucrose, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM KCl, 20 mM Tris-HCl pH 7.5). The homogenates were centrifuged at 105,000 X g for 60 min and the resulting supernatants were used as cytosols.

cAMP-Binding Assay: (^{3}H)cAMP-binding activity was determined according to the method of Gilman (20) at cAMP exchange conditions (21); all results were corrected for nonspecific binding determined from the amount of radioactivity retained in the presence of 1mM unlabeled cAMP.

Protein Kinase Assay: Protein kinase activity was determined by measuring ^{32}P incorporation from (γ-^{32}P)ATP into histone as previously described (22). The incubation mixtures contained in a final volume of 0.2 ml 100mM potassium phosphate buffer pH 7.5, 10mM $MgCl_2$, 1mM theophylline, 0.6 mg of calf thymus histone, 0.5mM ATP together with (γ^{32}-P)ATP, and 100-200 mg of cytosol protein ±1 mM cAMP. All incubations were carried at 30°C for 5-10 min in a shaking water bath. All data were corrected for endogenous phosphorylation in tubes contain-

ing no histone. A unit of enzyme activity was defined as that amount of enzyme which transferred 1 pmol of ^{32}P from (γ-^{32}P)ATP to protein in 5 min at 30°C.

Protein concentration was measured by the method of Lowry et al (23) using bovine serum albumin as a standard.

RESULTS: The administration of RA or HPR greatly prolonged the latency period of mammary cancer. A comparison of the time of appearance of the first palpable tumors in rats that received DMBA only, and those that received DMBA plus retinoids is shown in Fig. 1. In rats that received DMBA intubation only, the first tumors appeared at 50 days post intubation, and almost 80% of the rats had mammary tumors by 110 days post intubation. On the other hand, in the retinoid-treated rats, tumor induction was markedly delayed and reduced. The first tumors were observed at 90 days and 110 days post DMBA intubation in the RA- or HPR-treated animals, respectively. After 150 days, only 40% and 30% as many tumors appeared in the RA- or HPR-treated rats, as compared to the control rats (DMBA only). In addition to reducing the incidence of mammary cancer, RA and HPR also caused a marked reduction in the total number of cancers. At 150 days post intubation, the control rats had an average of 1.8 tumors/rat, whereas rats receiving RA or HPR had an average of 0.6 and 0.4 tumors/rat, respectively (data not shown).

In order to examine the possible involvement of the cAMP system in the retinoid-mediated inhibition of tumor induction, we determined the activity of cAMP-dependent protein kinase, the only known mediator of cAMP action, in RA-

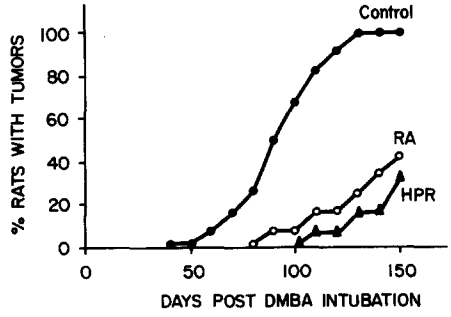

Fig. 1: Effect of retinoids (RA and HPR) administered daily in the diet on the rate of tumor induction by DMBA. All rats received a single dose DMBA (20 mg in 1.0 ml sesame oil administered i.g. per rat). Specific dosages of retinoids are given in Materials and Methods.

TABLE I. Effect of Retinoid Treatment on Cytosolic (^{3}H)cAMP Binding and Protein Kinase Activities of DMBA-Induced Mammary Tumors

Treatment	(^{3}H)cAMP Bound (pmoles/mg protein)	Protein Kinase Activity (units/mg protein)	
		(-cAMP)	(+cAMP)
DMBA Only	3.3 ± 0.66	245 ± 26	670 ± 56
DMBA + RA	7.1 ± 0.67	282 ± 64	1288 ± 119
DMBA + HPR	10.8 ± 1.75	337 ± 55	1544 ± 140

Results represent the mean of five tumors, each assayed in duplicate ± S.E.M.

and HPR-treated tumors as compared to the control tumors. Table 1 shows that total histone kinase activity was increased 2 to 3-fold in the retinoid-treated tumors. The increase of the histone kinase activity was almost totally in the cAMP-dependent component of the enzyme. This increase in cAMP-dependent protein kinase activity was also evidenced by a marked increase (2 to 3-fold) in cytosolic cAMP-binding activity (Table I).

The increase of cAMP-dependent protein kinase in retinoid-treated tumors was further examined by chromatographing cytosol preparations over DEAE-cellulose columns to resolve the Type I or Type II cAMP-dependent protein kinases. Elution profiles of histone kinase activity assayed in the absence and presence of 5 uM cAMP, as well as the (^{3}H)cAMP-binding activity of control and RA-treated tumors are shown in Fig. 2. Two peaks of cAMP-dependent protein kinase activity, referred to as Type I and Type II protein kinases according to their order of elution from the DEAE-cellulose column with increasing salt concentrations (24), were observed. Retinoid treatment caused a preferential increase (3-fold) of the Type II cAMP-dependent protein kinase peak eluted from the DEAE-cellulose column. A minor increase (approximately 20%) in the Type I cAMP-dependent protein kinase peak was also observed. The increase in peak II of the cAMP-dependent protein kinase activity was also evidenced by a marked increase (approximately 3-fold) in the cAMP-binding activity of this kinase fraction. As shown in Fig. 2, the elution profile of (^{3}H)cAMP-binding activity coincided with that of the histone kinase activity.

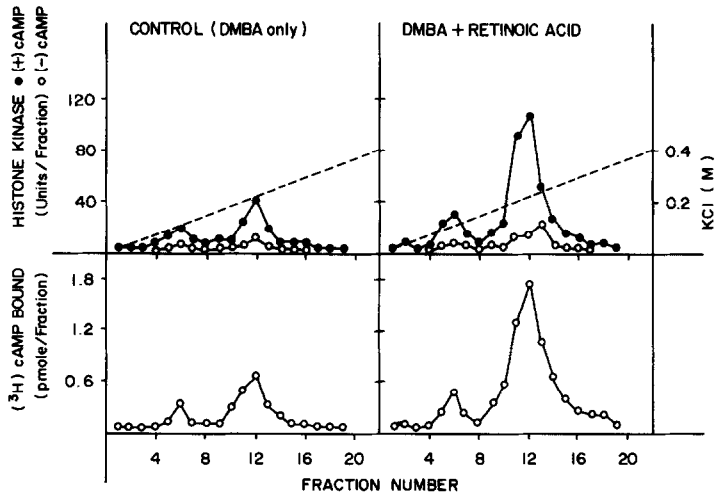

Fig. 2: DEAE-cellulose column profiles of protein kinase (Type I and Type II), and cAMP-binding activities in cytosols of control and retinoic acid-treated DMBA-induced mammary tumors. 1.5 ml of cytosol preparations obtained from pooled control tumors or RA-treated tumors, containing 10 mg protein, were loaded onto two identical columns (1 X 10 cm), and eluted at the same flow rate with a linear KCl gradient (0-0.4M) in 0.01M Tris-HCl, pH 7.4. Fractions of approximately 2 ml were collected. Protein kinase activity in the absence (o-o) or presence (•--•) of 5 μM cAMP, using 100 ul aliquots of corresponding fractions was measured as described under Materials and Methods. cAMP-binding activity was also measured using 300 ul of each fraction. Results are expressed as activity/column fraction.

To determine whether the increased binding of cAMP was due to an increase in the amount of the protein, or an increase in the affinity of the protein for cAMP, the concentration-dependent binding of (^{3}H)cAMP into cytosol proteins of the retinoid-treated and control tumors was studied. Results are shown in the form of Scatchard plots in Fig. 3. The apparent dissociation constants (K_d) for the high and low affinity binding of (^{3}H)cAMP into cytosol proteins of the RA-treated tumors (1.4×10^{-8} and 1.1×10^{-7}M) were similar to that of the control tumors (1.2×10^{-8} and 1.0×10^{-7}M). At saturating concentration of (^{3}H)cAMP (1 uM), however, the amount of (^{3}H)cAMP bound in the retinoid-treated tumors (15.4 pmol/mg protein) was 2.5-fold greater than that of the control tumors (6.2 pmol/mg protein). These results indicate that RA increased the amount of R_{II} subunit rather than altering its binding properties.

DISCUSSION: There has been considerable interest in elucidating the mechanism by which retinoids mediate the control of cell growth and differentiation. Very

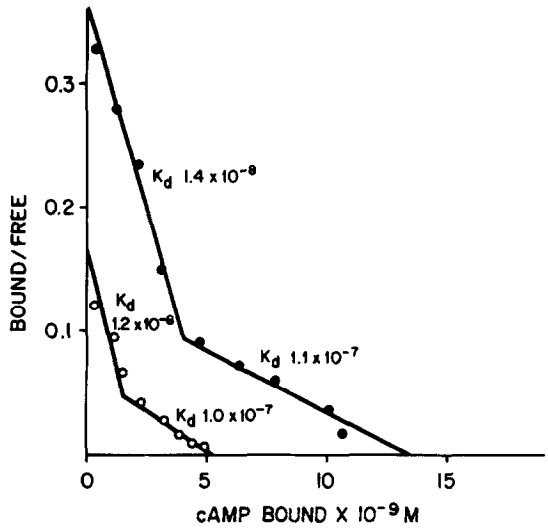

Fig. 3: Scatchard plots for the specific binding of (^{3}H)cAMP to cytosol proteins of tumors from control rats (DMBA only 0-0) and retinoic acid-treated rats (•--•). Each point represents the mean of duplicate assays for 3 tumors.

recently, an intriguing hypothesis involving the cAMP system in mediating some of the actions of retinoids has been proposed in B_{16} melanoma cells (25) which are highly sensitive to their antiproliferative effects (4), as well as in F_9 teratocarcinoma cells which are induced to differentiate by retinoic acid (26). This hypothesis was derived from the fact that cAMP has been implicated in the regulation of cellular growth and differentiation (16,17). Our results provide evidence in support of this hypothesis, extending this concept to mammary gland carcinomas. The inhibiton of mammary tumor induction by two retinoids (RA and HPR) was associated with 2 to 3-fold increase in cAMP-binding and histone protein kinase activities. The increased activities of both cAMP-binding and protein kinase, due to retinoid treatment, could be attributed to new protein synthesis. Thus, these results *in vivo* as well as the *in vitro* results (25,26) indicate that the stimulation of cAMP-dependent protein kinase activity may be a common feature of retinoid action.

Several studies have provided evidence that cAMP-dependent protein kinase may be involved as a mediator of growth inhibition. Our results (27) as well as those of Cho-Chung et al. (28) have shown an increase in cAMP-dependent protein

kinase activity in mammary tumors regressing due to ovariectomy or dibutyryl cAMP treatment of the hosts. Coffino and Gray (29) also reported that cAMP was unable to inhibit cell growth in a protein kinase-deficient variant of S_{49} lymphoma cells. Furthermore, retinoic acid did not inhibit growth in a protein kinase-deficient variant of B_{16}-F_1 cells (MR-4) (25). Therefore, it appears likely that modulation of the type and amount of intracellular cAMP-dependent protein kinase may have a pivotal function in the retinoid-induced inhibition of mammary tumor growth.

The significance of the marked increase of the Type II protein kinase, relative to Type I, during retinoid treatment is not yet clear. Both holoenzymes (Type I and Type II) are identical in their catalytic subunits, but differ from each other only with respect to regulatory subunits (30). Our results are different from those of Plet et al. (26) who showed that the increase of both isozymes in the cytosol of RA-treated F_9 embryonal carcinoma cells was of similar magnitude. In the plasma membrane, however, there was a preferential increase in Type II. Our results are in agreement with others (18) who showed that an increase in Type II cAMP-dependent protein kinase activity is an early event in dibutyryl cAMP- or ovariectomy-induced arrest of growth in rat mammary carcinomas. Thus, the Type II protein kinase may also be involved in the late phase inhibition of mammary carcinogenesis. Further studies on the subcellular distribution of cAMP-dependent protein kinase in response to retinoid treatment are under investigation.

This study represents the first evidence that cAMP-dependent protein kinase activity is increased in vivo in response to retinoid treatment. Whether or not the retinoid is affecting epithelial differentiation or is affecting a reversal of carcinogen-induced proliferation and anaplasia, is not readily apparent from this study. Further studies along these lines should prove most informative on the mechanism of action of retinoid in mammary carcinogenesis.

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