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Antagonistic Action between Cyclic Adenosine 3′:5′-Monophosphate and Estrogen in Rat Mammary Tumor Growth Control[1]

Yoon Sang Cho-Chung

Laboratory of Pathophysiology, National Cancer Institute, NIH, Bethesda, Maryland 20014

Abstract

Estrogen- and cyclic adenosine 3′:5′-monophosphate (cAMP)-binding activities were measured in the biopsy specimens of 35 carcinogen-induced or transplanted rat mammary carcinomas, and the tumor responses to host ovariectomy were followed. The results showed that all of the tumors with high estrogen-binding and low cAMP-binding activities regressed, whereas tumors with either low or high estrogen-binding and high cAMP-binding activites grew following ovariectomy. When 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumors undergo growth arrest following either ovariectomy or N^6,$O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate (DBcAMP) treatment of the host, a change in the estrogen- and cAMP-binding activities occurred. Three days after either ovariectomy or DBcAMP treatment, cAMP binding increased 5- and 2-fold in the tumor nuclei and cytosol, respectively, whereas nuclear and cytoplasmic estrogen binding decreased by 70 and 25%, respectively. These changes in cAMP- and estrogen-binding activities were detectable within 1 day after either ovariectomy or DBcAMP treatment, and the changes were reversed when resumption of tumor growth was induced by the injection of estradiol valerate or cessation of DBcAMP treatment. When DMBA tumors failed to regress after either ovariectomy or DBcAMP treatment, the change in estrogen- and cAMP-binding activities did not occur. Concomitant with the increase of cAMP-binding activity in regressing tumors were increases in protein kinase activity and cAMP content of the tumors. New phosphorylation of a nonhistone nuclear protein also occurred in regressing DMBA tumors but ceased when tumor growth was resumed after injection of estradiol valerate or cessation of DBcAMP treatment. Phosphorylation of the regression-associated protein was induced *in vitro* by preincubation of growing DMBA tumor slices with 10^{-5} M cAMP and was inhibited by the simultaneous addition of 10^{-7} M 17β-estradiol with cAMP. These data suggest an interaction between a steroid hormone and cAMP in the growth control of a hormone-dependent mammary tumor.

Introduction

It has long been assumed that estrogens may be essential for the growth of mammary tumors since deprivation or antagonists of estrogens produce regression of certain mammary carcinomas. A relationship between the presence of estrogen receptors and ovarian-dependent tumor growth has been known (18, 27). The presence of estrogen receptors *per se*, however, is not an absolute indication of hormone dependency (2). Clinical studies have shown that only 50% of patients whose tumors contained estrogen receptors responded favorably to hormone therapy (25). Recently, we obtained evidence that the regulation of hormone-dependent tumor growth may depend on the antagonistic action between estrogen and cAMP[1] (5, 6). The observations supporting this relationship will be described.

Materials and Methods

Theophylline, cAMP, DBcAMP, and [^{3}H]cAMP (27 Ci/mmol) were obtained from Schwarz/Mann, Orangeburg, N. Y.; 17β-[^{3}H]estradiol (17β-[2,4,6,7-^{3}H]estradiol, 91.3 Ci/mmol) and Econofluor were from New England Nuclear, Boston, Mass.; [γ-^{33}P]ATP (19.2 Ci/mmol) was from ICN Pharmaceuticals, Inc., Irvine, Calif.

Tumors. Primary DMBA-induced mammary carcinoma (17) in Sprague-Dawley females was obtained from Hazleton Laboratories America, Inc. (Vienna, Va.), and *N*-nitrosomethylurea-induced mammary carcinoma (16) in Buffalo-N inbred females was obtained from K & K Laboratories, Plainview, N. Y. DMBA #1 (tumor received from Dr. W. F. Dunning in 1967, carried in the Laboratory of Pathophysiology by s.c. transplant for 172 generations, and then stored at −80°) and MT13762 (29) mammary carcinomas were transplanted in F-344 females, and MTW9A [a subline of MTW9 (19, 24) growing in untreated hosts] mammary carcinomas were transplanted in Wistar-Furth females. All tumors were used when their size was 2 to 4 g. Tumor volumes were calculated from daily tumor measurements with vernier calipers (4). Bilateral ovariectomy was performed under ether anesthesia. DBcAMP treatment was 10 mg DBcAMP in 0.1 ml 0.85% NaCl solution per day per rat s.c. (4).

Preparation of Tumor Cytosol and Nuclear Extract. All procedures were performed at 0–4° following the methods described previously (6). Tumors were homogenized in a Teflon-glass homogenizer with 5 volumes of Buffer A (0.25 M sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, and 20 mM Tris-HCl, pH 7.5). The homogenates were centrifuged at 755 × *g* for 10 min. Supernatants were then centrifuged at 105,000 × *g* for 60 min, and the resulting supernatants were used as cytosols. Pellets from the centrifugation at 755 × *g* were rehomogenized in the original volume of Buffer A and then passed through 3 layers of gauze. Crude nuclear

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[1] The abbreviations used are: cAMP, cyclic adenosine 3′:5′-monophosphate; DBcAMP, N^6,$O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate; DMBA, 7,12-dimethylbenz(α)anthracene.

pellets were obtained after 2 centrifugations at 755 × *g* (6). These crude nuclear pellets were suspended in 2.2 M sucrose (3) in Buffer A (6 ml/g original tissue) and centrifuged at 2° in a Beckman SW25.1 rotor at 24,000 rpm for 45 min. The purified nuclear pellets[2] thus obtained were suspended in 1 M KCl in 10 mM Tris-HCl, pH 7.5 (1.0 ml/g nuclear pellet), and extracted at 0° for 90 min. The suspensions were centrifuged at 105,000 × *g* for 45 min, and the clear supernatants were used as nuclear extracts.

cAMP-binding Assay. cAMP binding to proteins was measured by the modification of the membrane filtration method of Gilman (14) at cAMP exchange conditions (10). The assay mixtures [0.2 ml final volume; containing 50 mM potassium phosphate buffer, pH 6.5; 10 mM theophylline, 10^{-6} M [^{3}H]cAMP (27 Ci/mmol), ±1 mM nonradioactive cAMP, and tumor cytosol or nuclear extract] were incubated at 23° for 3 hr, and then the reactions were stopped by the addition of cold 50 mM potassium phosphate buffer, pH 6.5.The reaction mixtures were immediately drawn by suction through Millipore filters (0.45-μm pore size); the filters were washed and dried; and the radioactivity was counted in a liquid scintillation spectrometer. The binding was expressed as the specific binding (10, 28) which was proportional to the protein concentration over a range of 30 to 200 μg/0.2 ml reaction mixture. The binding reaction was specific for cAMP (6).

Estrogen Binding Assay. Estrogen binding was measured by the modification (26) of the charcoal adsorption assay originally described by Korenman (20). Cytosol or nuclear extract, 200 μl (approximately 1 mg protein), was added to 50 μl of 10 mM Tris-HCl-1 mM EDTA buffer, pH 7.5, containing 1.25 pmol (final concentration, 5 M) of 17β-[^{3}H]estradiol (91.3 Ci/mmol) ± 250 pmol of unlabeled 17β-estradiol. The mixtures were incubated at 0–3° for 16 to 18 hr and at 23° for 2 hr, respectively; then unbound estradiol was removed by the dextran-coated charcoal method (20). The radioactivity was counted in a liquid scintillation spectrometer. The binding was expressed as the specific binding (26) which was proportional to the protein concentration over a range of 0.3 to 1.2 mg/0.25 ml reaction mixture.

Protein concentrations were measured by the method of Lowry *et al.* (23) with bovine serum albumin as a standard. Glucose-6-phosphate dehydrogenase was assayed according to the method of Glock and McLean (15).

Endogenous Phosphorylation of Nuclear Proteins. Nuclear pellets (from 0.2 g tumor) were added to reaction mixtures (0.3 ml) containing 100 mM potassium phosphate buffer, pH 7.5; 1 mM theophylline; 20 mM magnesium acetate; 12.6 μM [γ-^{33}P]ATP (19.2 Ci/mmol); and ±1 μM cAMP. The mixture was incubated at 30° for 15 min, immediately cooled to 4°, and centrifuged; supernatants were discarded. The nuclear proteins were dissolved into polypeptide chains (30) and subjected to electrophoresis.

Electrophoresis in Sodium Dodecyl Sulfate-Polyacrylamide Gels. Samples containing 40 μg of protein were subjected to electrophoresis in 0.1% sodium dodecyl sulfate-10% polyacrylamide gels as described by Weber and Osborn (30). Parallel gels were stained with Coomassie blue (30) and scanned for protein at 550 nm (13). ^{33}P incorporation into nuclear proteins was measured in 1-mm gel slices as previously described (11).

Results

Estrogen- and cAMP-binding Capacities and Tumor Response to Ovariectomy. Estrogen- and cAMP-binding activities were measured in the biopsy specimens of 35 rat mammary tumors, and the response of these tumors to host ovariectomy was followed. The results in Chart 1 clearly show that all of the tumors with high estrogen-binding (>1 pmol/g tissue) and low cAMP-binding (<80 pmol/g tissue) activities regressed following ovariectomy, whereas tumors with low estrogen-binding and high cAMP-binding activities grew following ovariectomy. Tumors with a high or low binding capacity for both estrogen and cAMP also grew during ovariectomy. Thus the binding capacities of estrogen and cAMP are closely related to the response of tumors to ovariectomy.

Estrogen- and cAMP-binding Activities in Regressing DMBA Tumors. When hormone-dependent DMBA mammary tumors undergo growth arrest following either ovariectomy or DBcAMP treatment of the host (4), a change in the specific binding of estrogen and cAMP occurs in the tumors (Chart 2). Three days after either ovariectomy or DBcAMP treatment, cAMP binding increased 5- and 2-fold in the tumor nuclei and cytosols, respectively, whereas nuclear and cytoplasmic estrogen binding decreased by 70 and 25%, respectively. cAMP binding increased initially in the cytosol, where it later decreased when the nuclei-associated cAMP binding reached peak activity. On the contrary, estrogen binding decreased initially in the nuclei and later in the cytosol. These reciprocal changes in cAMP- and estrogen-binding activities were detectable within 1 day after either ovariectomy or DBcAMP treatment, when there was no appreciable change in tumor size. The changes were reversed, however, when tumor growth was resumed fol-

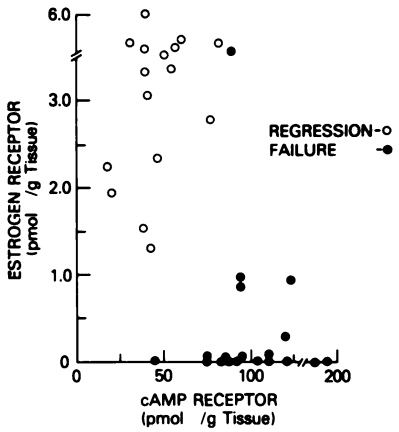

Chart 1. Correlation of estrogen receptor and cAMP receptor concentrations with tumor response to ovariectomy. Estrogen- and cAMP-binding activities in tumor cytosols were measured on biopsy specimens before ovariectomy as described under "Materials and Methods." Tumor responses to ovariectomy are: regression (>50% decrease in tumor volume by Day 6); failure (>60% increase in tumor volume by Day 6). Tumors used are DMBA, DMBA #1, MT13762, and MTW9A.

[2] Nuclei were judged to be free of cytoplasmic contamination by the absence of glucose-6-phosphate dehydrogenase activity. The nuclear preparations obtained from growing and regressing tumors were indistinguishable by phase-contrast microscopy.

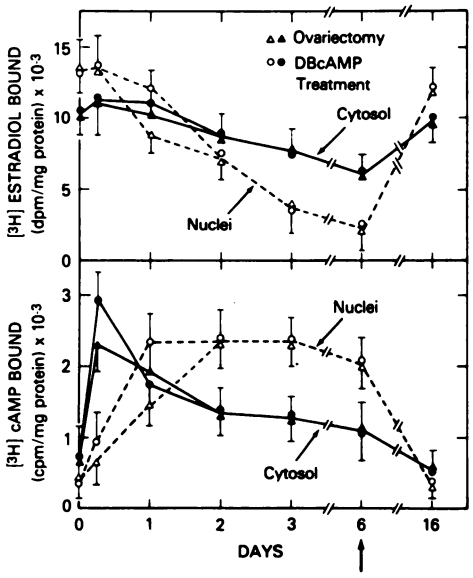

Chart 2. Specific binding of estrogen and cAMP by cytosol and nuclear extract of DMBA tumors during growth and regression. DBcAMP (10 mg per 0.1 ml 0.85% NaCl solution per 200 g rat s.c.) was injected daily as described previously (4). *Arrows*, cessation of DBcAMP treatment or start of estradiol valerate injection (Delestrogen, 33 μg per 0.1 ml sesame oil per 200 g rat s.c., every 6 days) (4). Tumors, growing or regressing (at times indicated following ovariectomy or DBcAMP treatment), respectively, were homogenized immediately after removal, and cytosols and nuclear extracts were prepared as described under "Materials and Methods." Estrogen- and cAMP-binding activities were assayed in triplicate as described under "Materials and Methods." *Points*, mean of 10 tumors; *bars*, S.E.

lowing the injection of estradiol valerate or cessation of DBcAMP treatment.

Concomitant with the increase of cAMP-binding activity in the regressing tumors was the increase in protein kinase activity. The identity between cAMP-dependent protein kinase and cAMP-binding protein (6), as found in DEAE-cellulose column chromatography profiles and sucrose density gradient sedimentation patterns, suggested a similar relationship between these proteins in DMBA tumors as has been shown in other tissues (21, 22). The increased activities of both cAMP binding and protein kinase due to ovariectomy could be attributed to new protein synthesis, since cycloheximide was found to block these activities (5).

Ovariectomy and DBcAMP treatment of DMBA tumor-bearing hosts resulted in an immediate increase in the cAMP content of the respective tumors (5). A peak level (approximately 3-fold that of the growing tumor) was reached 1 day after DBcAMP treatment and 2 days after ovariectomy. The increase in cAMP content of the tumors followed a sharp increase in adenylate cyclase activity (5, 6). Three days after either treatment, the cAMP content decreased simultaneously as the cAMP phosphodiesterase activity increased (5, 6). The increases in cAMP level and in adenylate cyclase and phosphodiesterase activities subsided in those tumors that resumed growth following the injection of estradiol valerate or cessation of DBcAMP.

Nuclear Protein Phosphorylation in Regressing DMBA Tumors. A change in protein kinase-dependent phosphate incorporation in isolated nuclei of DMBA tumors was also observed following either ovariectomy or DBcAMP treatment (11). As shown in Chart 3, protein species IV was found to be the major endogenous substrate for nuclei-associated protein kinase in growing tumors, whereas in nuclei of regressing tumors, the radioactivity peaks associated with protein species IV decreased by approximately 40% and new radioactivity peaks coincident with protein species I appeared. Phosphorylation of this regression-associated protein began within 1 day after either ovariectomy or DBcAMP treatment and stopped when tumor growth was resumed following the injection of estradiol valerate or cessation of DBcAMP treatment.

Whether phosphorylation of regression-associated protein is, indeed, related to the effect of cAMP was examined under more clearly defined conditions *in vitro*. As shown in Chart 4, when slices from growing DMBA tumors were preincubated with 10^{-5} M cAMP at 30° for 20 min, phosphorylation of regression-associated protein was induced, whereas this phosphorylation was inhibited when 10^{-7} M 17β-estradiol was added simultaneously with cAMP to the incubation medium. Thus phosphorylation of regression-associated protein appears to be related to the specific action of cAMP that counteracts the effect of estrogen in the nuclei of hormone-dependent mammary tumors.

Discussion

This study presents evidence of an antagonistic action between cAMP and a steroid hormone in the growth control of hormone-dependent mammary tumors.

During growth, high estrogen-binding and low cAMP-binding activities are measured in DMBA tumors. Following ovariectomy or DBcAMP treatment of the host, estrogen binding decreases while cAMP binding increases in the regressing DMBA tumors. These changes in estrogen- and cAMP-binding activities are detectable within 1 day after either ovariectomy or DBcAMP treatment, suggesting that the changes are early events in the regression process rather than the result of tumor regression. Concomitant with the increase of cAMP-binding activity, protein kinase activity also increases in the regressing tumor following either ovariectomy or DBcAMP treatment. These increases in cAMP binding and protein kinase activities may be attributed to new protein synthesis since the increases are blocked by cycloheximide. The decrease of estrogen-binding activity was shown to be due to the decrease in total binding sites without any modification of either binding affinity or sedimentation characteristics (1).

A possible counteraction between cAMP and estrogen at the nuclear level is suggested by the observation that in the nuclei of regressing tumors both cAMP-binding and protein kinase activities increase whereas estrogen-binding activity decreases. In growing tumors a reversal of these events takes place. The differential effect of ionic salts and non-ionic detergents on the release of cAMP- and estrogen-binding components from tumor nuclei suggests that the localization and/or binding of these macromolecules in the nuclei may differ (6). Further evidence of an interrelationship between cAMP and estrogen at the nuclear level, both *in vivo* and *in vitro*, has been shown by the stimulation and prevention of endogenous phosphorylation of nuclear proteins, identified as regression-associated proteins, by cAMP and estrogen, respectively.

It is important that the inverse relationship between

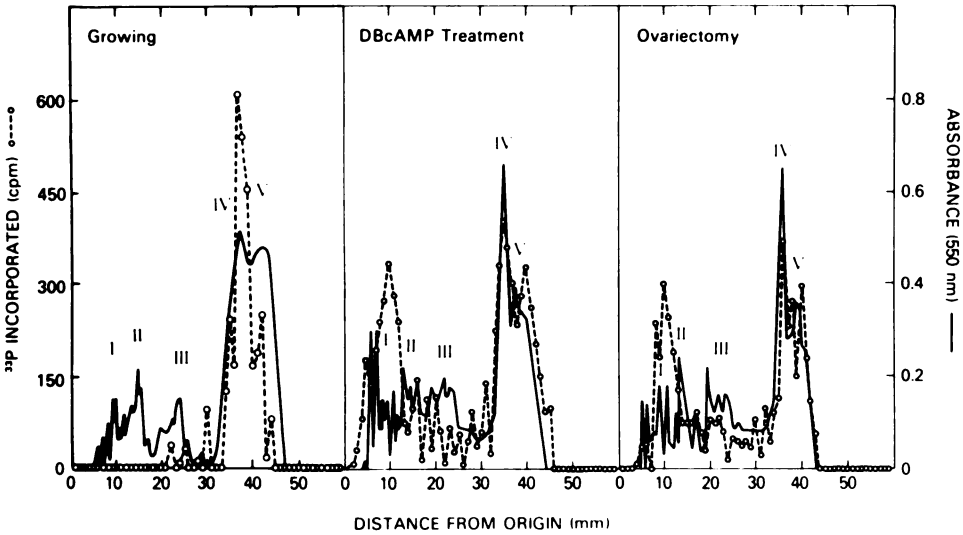

Chart 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of phosphorylated nuclear proteins from growing, regressing, and growth-arrested DMBA tumors. *Growing*, tumors increased in volume >30% in 3 days; *DBcAMP treatment*, growth-arrested tumors, 3 days after treatment (4); *ovariectomy*, regressing tumors (regressed by 20% of the original size) 3 days after ovariectomy. Isolated nuclei from each tumor were incubated with [γ-^{32}P]ATP to phosphorylate the nuclear proteins via intrinsic protein kinase; nuclear proteins were then treated with 1% sodium dodecyl sulfate and 1% mercaptoethanol and subjected to electrophoresis. Reprinted from Cho-Chung and Redler (11).

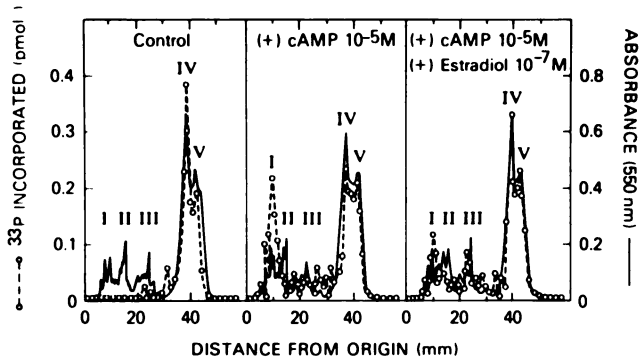

Chart 4. Effect of cAMP and 17β-estradiol on endogenous nuclear protein phosphorylation of DMBA mammary tumor slices *in vitro*. Tumor slices (0.2 g) were incubated in 5 volumes of 10 mM Tris-HCl, pH 7.5, in the absence or presence of 10^{-3} M cAMP ± 17β-estradiol (10^{-7} M) at 30° for 20 min, and the reactions were stopped by diluting the medium 2.5-fold with cold Tris-HCl. Tumor slices were immediately centrifuged and washed once with cold Tris-HCl. The nuclei were then isolated, and the nuclear protein phosphorylation was measured by intrinsic protein kinase (see legend for Chart 3).

cAMP- and estrogen-binding activities is closely related to the hormone dependency of tumor growth. Hormone-independent mammary tumors that continue to grow and fail to regress after hormonal deprivation possess a wide range of estrogen- and cAMP-binding activities that do not change after ovariectomy or DBcAMP treatment. These findings, then, suggest that the integrity of both cAMP-binding proteins and estrogen receptors is probably essential for the control of hormone-dependent mammary tumor growth. Estrogen receptors may promote growth, while cAMP-binding proteins may play a role in the regression phase of these tumors similar to the pivotal role of cAMP-binding proteins demonstrated in the growth arrest of other tumor models (7–9, 12). Further investigations of the phosphorylation of nuclear proteins in conjunction with cAMP binding and estrogen binding in the nuclei would help to elucidate the mechanism of hormone-dependent mammary tumor growth and regression.

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