THE ROLE OF cAMP ON NEOPLASTIC CELL GROWTH AND REGRESSION.

III. ALTERED cAMP-BINDING IN DBcAMP-UNRESPONSIVE WALKER 256 MAMMARY CARCINOMA

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SUMMARY: An exogenous supply of $N^6,O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) in vivo produces regression of one type of Walker 256 mammary carcinoma cell population (DBcAMP-responsive); a second type of cell population continues to grow despite DBcAMP treatment (DBcAMP-unresponsive). A correlation was found between altered cAMP-binding of the tumor cytosol and DBcAMP-unresponsiveness. It was found that there was: a) a higher apparent dissociation constant (Kd) for cAMP-binding in unresponsive tumor cytosol in vitro, and b) unsaturability of cAMP-binding by unresponsive tumor cytosol in response to elevated cAMP levels in vivo. Cycloheximide abolished the saturation of cAMP binding in vivo as well as tumor regression produced by DBcAMP.

INTRODUCTION: Previous work from this laboratory showed that cAMP derivatives can produce in vivo inhibition of tumor growth and that this inhibition varies with tumor type (1). Selection of two cell populations from hormone-independent Walker 256 rat mammary carcinoma (W256), one regressing (DBcAMP-responsive) and the other growing (DBcAMP-unresponsive) under DBcAMP treatment, offered a biochemical tool for studying the cAMP system in relation to in vivo control of tumor growth (2).

This paper presents evidence that DBcAMP-unresponsiveness of W256 may be correlated with a reduced ability of specific tumor proteins to bind cAMP.

Previous studies showed that 6 days of DBcAMP treatment (10 mg/0.1 ml of 0.85% NaCl/day/rat injected subcutaneously) resulted in a 2-fold elevation of cAMP concentration in both DBcAMP-responsive and -unresponsive W256 tumors but regression occurred only in the responsive tumor. Thus, a simple increase of cAMP level in the tissue could not explain the inhibitory effect on tumor growth (1). This observation is similar to that found in many

other systems (3-7) where a correlation between intracellular cAMP levels and the ultimate metabolic effects was also difficult to obtain. Two possible explanations for this lack of correlation are a decrease in the activation of the enzymes or proteins which mediate cAMP action within the cell, or a compartmentalization of the intracellular nucleotide. It seemed necessary, therefore, to measure directly the degree to which the first step of the activation sequence, i.e., cAMP-binding to receptor protein (8), would reflect the apparent intracellular cAMP concentrations. Experiments were designed to show if a rise in tissue cAMP concentration was paralleled by an increase in the amount of cAMP bound to receptor protein of the tumor cell after a single injection of DBcAMP.

RESULTS AND DISCUSSION: Specific cAMP binding by cytosol of the responsive tumor decreased sharply to 50% of basal binding activity after 30 minutes of DBcAMP treatment when the cAMP level of the tumor had reached a peak. On the contrary, cAMP binding activity of the unresponsive tumor cytosol remained low and did not change despite elevation of the tumor cAMP level (Fig. 1). Thus, when the responsive tumor is stimulated by DBcAMP which apparently increases the intracellular cAMP level, there is a rise in the amount of receptor proteins endogenously saturated by cAMP, as indicated by a decrease in their ability to bind exogenously added ^{3}H-cAMP. Confronted with elevated cAMP levels, however, the unresponsive tumor seems unable to saturate its cAMP-binding receptors.

Actinomycin D and cycloheximide have been found to interfere with a normal increase in cAMP concentration in some systems (12,13) but not in others (14-16). When animals bearing responsive tumors were treated first with cycloheximide and then with DBcAMP, the level of tumor cAMP failed to increase, the depression of residual binding failed to occur (Fig. 1), and the tumor continued to grow. Thus, cycloheximide interfered with the DBcAMP effect. This interference was not observed when a low dose of actinomycin D was substituted for cycloheximide. Elevation of cAMP levels and regression

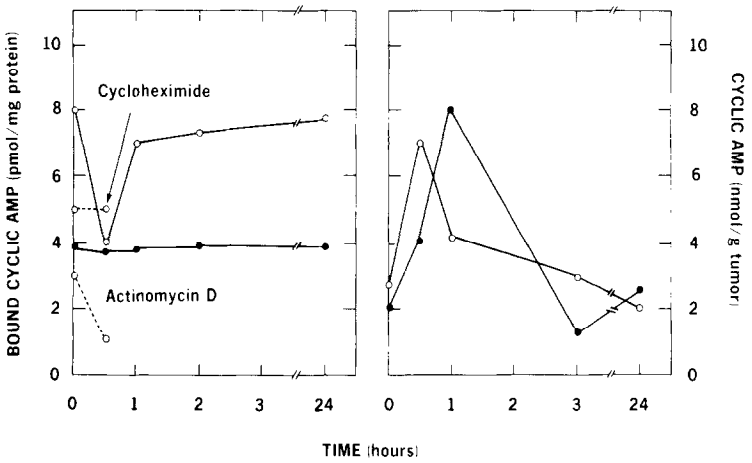

Figure 1. Cyclic AMP binding activity and cAMP content of DBcAMP-responsive (O) and -unresponsive (●) Walker 256 tumors after single injection of DBcAMP. DBcAMP 10 mg/0.1 ml of 0.85% NaCl/rat (bearing 2-3 g tumors) was injected s.c. Cycloheximide or actinomycin D, 40 µg/day/rat s.c. for 5 days and on the 6th day DBcAMP was injected (10 mg/rat s.c.). At the indicated times of post DBcAMP injection, rats were killed, 4 tumors per group were pooled, then homogenized with 5 volumes of tris-HCl, 10 mM, pH 7.5. Cyclic AMP binding to tumor cytosol (105,000 x g, 1 hr) was measured by the competition assay of Gilman (9). The reaction mixture (0.2 ml final volume) contained: 50 mM sodium acetate buffer (pH 4.5), 1 mM theophylline, 10^{-6}M, [^{3}H]cAMP (27 ci/mmol), and ±1 mM non-radioactive cAMP. The reaction was started by addition of 20 µl (70-120 µg protein) of tumor cytosol. The binding reaction was performed at its optimal pH of 4.5. The binding was expressed as the specific binding: calculated by the subtraction of a blank value (the amount of [^{3}H]cAMP bound in the presence of excess non-radioactive cAMP) from the value obtained with radioactive nucleotide alone (10). Cyclic AMP levels in tumors were measured by Gilman's competitive protein binding method (9). The binding protein was purchased from Calbiochem, San Diego, Calif. A tumor fragment (0.2-0.5 g) was dipped into liquid nitrogen, weighed, and homogenized in 5 volumes of 5% trichloracetic acid and the ether-extracted trichloracetic acid supernatant was used in the determinations. Protein concentrations were determined by the method of Lowry et al (11).

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did occur and residual binding of cAMP was decreased significantly (Fig. 1)

We next inquired whether impaired saturation of cAMP-receptor protein of the unresponsive tumor in response to elevated cellular cAMP *in vivo* could be reflected in specific cAMP-binding activity of tumor cytosol *in vitro*. Cytosols from untreated responsive and unresponsive tumors were, therefore, tested for specific cAMP binding at various concentrations of [^{3}H]cAMP. At all cAMP concentrations tested, there was considerably less

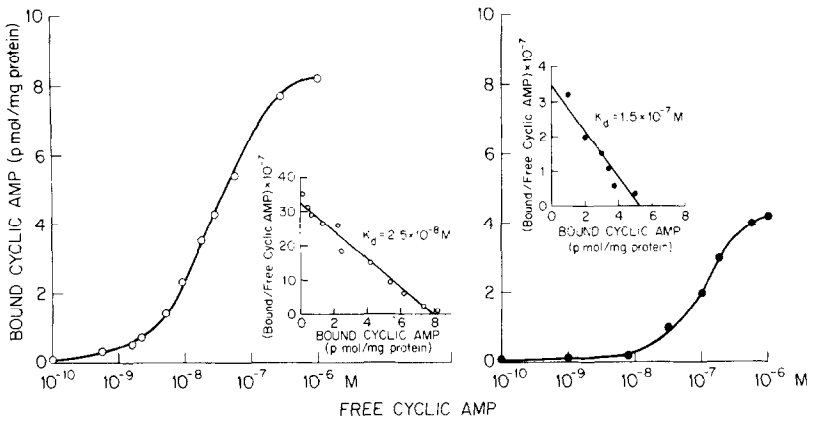

Figure 2. Specific cAMP binding by cytosol of untreated DBcAMP-responsive (O) and -unresponsive (●) Walker 256 tumors at various cAMP concentrations. Reaction mixture is the same as in legend of Fig. 1 except for various concentrations of [^{3}H]cAMP with or without 1 mM non-radioactive cAMP. The binding was expressed as specific binding (see the definition of specific binding in legend of Fig. 1). The concentration of free [^{3}H]cAMP was obtained by subtraction of the total bound [^{3}H]cAMP from total [^{3}H]cAMP present in the incubation mixture. A duplicate set of experiments showed the reproducible cAMP-saturation curves.

specific cAMP binding by unresponsive tumor cytosol (Fig. 2). The specific binding data were plotted by the Scatchard technique (17) and yielded a single straight line, indicating that only a single class of specific binding sites were detected by the assay. The equilibrium dissociation constants (Kd) for the reaction: cAMP + binding protein $\rightleftharpoons$ cAMP-binding protein complex, were calculated to be 2.5×10^{-8}M and 1.5×10^{-7}M for responsive and unresponsive tumors, respectively (Fig. 2), indicating a significantly lower affinity of cAMP-binding protein toward cAMP in the unresponsive tumor. Thus, in addition to the quantitative difference, there might also be a qualitative difference between "responsive" and "unresponsive" binding proteins.

Recent reports have emphasized the correlation between cAMP-binding proteins and altered cAMP metabolism. A deficiency in cAMP-binding protein, the regulatory subunit of cAMP-activated protein kinase, has been shown in DBcAMP-resistant lymphoma cells (18). Cultured hepatoma cells, which

are insensitive to glucagon or catecholamine, have been shown to possess different cAMP-binding proteins from those of liver cells (19,20). Whether regulation of tumor growth by cAMP *in vivo* will ultimately be determined by cyclic nucleotide stimulation of phosphorylation (21,22) or by gene interaction of cAMP-receptor protein per se, as in the bacterial system (23), remains to be clarified.

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