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Dibutyryl Cyclic-AMP Mimics the Effect of Ovariectomy on a Dehydrogenase in Hormone-Dependent Mammary Tumors*

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Abstract—*Growth of 7,12-dimethylbenz(α)anthracene(DMBA)-induced mammary carcinoma is arrested by either ovariectomy or treatment with* $N^6,O^{2'}$ *dibutyryl cyclic adenosine 3′, 5′-monophosphate (DBcAMP). When this occurs, a change in the isozyme pattern of glucose-6-phosphate dehydrogenase (G6PD) is observed. In growing tumors, 4 major molecular forms of glucose-6-phosphate dehydrogenase (G6PD), designated G6PD-1, -2, -3 and -4, have been identified by their distinct electrophoretic mobilities, subcellular localizations, and substrate and cofactor specificities. In regressing tumors, due to either ovariectomy or DBcAMP treatment, G6PD-3 (microsome-associated) activity disappears. This activity reappears when tumor growth is resumed by either the injection of estradiol-valerate or cessation of DBcAMP treatment. A change in G6PD-3 activity does not occur in those DMBA tumors that continue to grow, rather than regress, after ovariectomy or DBcAMP treatment. G6PD-1 and -2 (post microsomal supernatant-associated) activities decrease only during ovariectomy-induced tumor regression and G6PD-4 (mitochondria-associated) activity remains inaltered during DMBA tumor regression.*

The disappearance of G6PD-3 activity appears to be a common biochemical parameter found in the regression of a hormone-dependent mammary carcinoma following either ovariectomy or DBcAMP treatment of the host.

INTRODUCTION

OUR PREVIOUS study [1] showed that disappearance of microsomal glucose-6-phosphate dehydrogenase activity correlates with $N^6,O^{2'}$-dibutyryl cyclic adenosine 3′,5′-monophosphate (DBcAMP)-induced growth arrest [2] of MTW9, a transplantable, hormone-dependent rat mammary tumor. Hershey *et al.* [13] identified 2 molecular forms of this enzyme in both normal and neoplastic rodent mammary tissues, and Richards and Hilf [4, 5] found that one of these forms decreased in activity during the regression of carcinogen-induced rat mammary tumors following hormone withdrawal (ovariectomy). Growth of 7,12-dimethylbenz(α)anthracene (DMBA)-induced tumors is arrested by either ovariectomy of the host]6, 7] or treatment with DBcAMP [2]. In this paper we examine further the correlation between growth arrest and G6PD activities, in particular disappearance of microsomal G6PD, of the primary DMBA-induced, hormone-dependent rat mammary carcinomas during their regression due to either ovariectomy or DBcAMP treatment.

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MATERIALS AND METHODS

Tumor models

Primary DMBA-induced [6, 7] mammary carcinoma in Sprague–Dawley random-bred females was used as an experimental model of a hormone-dependent tumor. Tumor-bearing animals, 200–250 g and 3–4 months old, were kept in wire cages in a 24°C air-conditioned room and fed Purina rat chow with water *ad libitum*. Tumor volumes, used to assess the growth response to ovariectomy or DBcAMP treatment, were calculated from daily tumor measurements by Vernier caliper [8].

Preparation of whole tumor extract and subcellular fractionation

All procedures were performed at 0° to 4°C. Tumors from rats, killed by decapitation, were immediately excised, weighed, minced, and homogenized in a ground glass homogenizer with 2 vol of 10 mM Tris–HCl buffer, pH 7.6, containing 1 mM EDTA, 5 mM $MgCl_2$ and 0.5% Triton X-100 [9]. Homogenates were centrifuged at 105,000 ***g*** for 60 min, and the clear supernatant was designated as whole tumor extract.

Cell fractionation was carried out by a slightly modified procedure of Hogeboom [10]. Tumors were homogenized in a Teflon-glass homogenizer with 2 vol of 0.44 M sucrose, containing 1 mM EDTA and 10 mM Tris-HCl buffer, pH 7.6. Nuclei were removed after 2 centrifugations at 700 ***g*** for 10 min. The second supernatant was centrifuged at 9000 ***g*** for 10 min, then at 105,000 ***g*** for 60 min, and the final supernatant (post microsomal fraction) was used for soluble G6PD measurement. The sediments were washed three times with sucrose-EDTA-Tris–HCl buffer and designated as crude mitochondrial and microsomal fractions, respectively. To release the enzyme, the particulate fractions were frozen and thawed, then homogenized in a ground glass homogenizer with 2 vol of 10 mM Tris–HCl buffer, pH 7.6, containing 1 mM EDTA, 5 mM $MgCl_1$ and 0.5% Triton X-100 [9]. The clear supernatants after centrifugation were used for the measurement of mitochondrial- and microsomal-associated G6PD, respectively.

Acrylamide gel electrophoresis

Disc gel electrophoresis was performed by the method of Davis [11]. Fifty microliters of tumor extract (500 μg protein) were subjected to electrophoresis on 7% polyacrylamide gels (7 × 25 mm) for 2 hr at 2 ma/gel at 4°C in a Tris–glycine buffer system, pH 8.3, using a Canalco 1200 apparatus. Gels were developed for enzyme activity in the dark at 37°C for 30 min in an incubation medium described by Beitner and Naor [9]. The medium contained 0.3 M Tris–HCl buffer, pH 7.6; 7 mM $MgCl_2$, 0.4 mM $NADP^+$ or 0.4 mM NAD^+; 0.1 mM phenazine methosulfate; 0.4 mM nitroblue tetrazolium; and 1.6 mM glucose-6-phosphate or 1.6 mM galactose-6-phosphate. After incubation, gels were washed thoroughly with distilled water and immersed in 7% acetic acid to terminate the reaction.

Enzyme assay

Activity of G6PD was determined by following the change in absorbance at 340 nm due to NADPH production in a Gilford Model 240 spectrophotometer. The conditions of the assay assured zero-order kinetics for at least 5 min. The method used was the double-substrate technique of Glock and McLean [12] in which the blank contained 6-phosphogluconate; G6PD activity was calculated after subtraction of the blank value. One unit of enzyme activity is defined as 1 μmole of pyridine nucleotide reduced per min at 37°C in the assay system.

Chemicals and reagents

Acrylamide and bisacrylamide were obtained from Eastman Co., New York, N.Y. $NADP^+$, NAD^+, glucose-6-phosphate, 6-phosphogluconic acid, nitroblue tetrazolium, galactose-6-phosphate and phenazine methosulfate were purchased from Sigma Chemical Co., St. Louis, Mo.; DBcAMP was from JEM Research Products, Kensington, Md., and Delestrogen (estradiol-17-*n*-valerate in sesame oil) was obtained from Squibb and Sons, New York, N.Y.

Abbreviations used are: DBcAMP, N^6, $0^{2'}$-dibutyryl cyclic adenosine 3′, 5′-monophosphate; G6PD, glucose-6-phosphate dehydrogenase;DMBA,7,12dimethylbenz(α)anthracene.

Qualitatively identical electrophoretic bands were reproduced in the protein range of 25 ~ 750 μg.

Enzyme activity in gel b was developed for 15 min longer to be certain that the disappearance of band 3 after DBcAMP treatment was not an artifact due to the interference of the enzyme activity development in gels.

Under suitable conditions soluble G6PD (G6PD-1 and 2) can utilize NAD^+ in place of $NADP^+$ (Levy, H.R., J. Biol. Chem. 238, 775, 1963) and galactose-6-P (the enzyme showed Km values of 3.3×10^{-3} M, and 3.3×10^{-5} M for galactose-6-P and glucose-6-P, respectively (Bodwin, J.S. and Cho-Chung, Y.S., Proc. Am. Assoc. Cancer Res. 16, 89, 1975).

Particulate bound G6PD (G6PD-3 and -4) showed Km values of 1.6×10^{-5} M and 1.1×10^{-5} M, for glucose-6-P and galactose-6-P, respectively (Bodwin, J.S. and Cho-Chung, Y.S., Proc. Am. Assoc. Cancer Res. 16, 89, 1975).

G6PD-3 is similar to the autosomally-linked hexose-6-P dehydrogenase (that is, glucose dehydrogenase) with respect to its subcellular

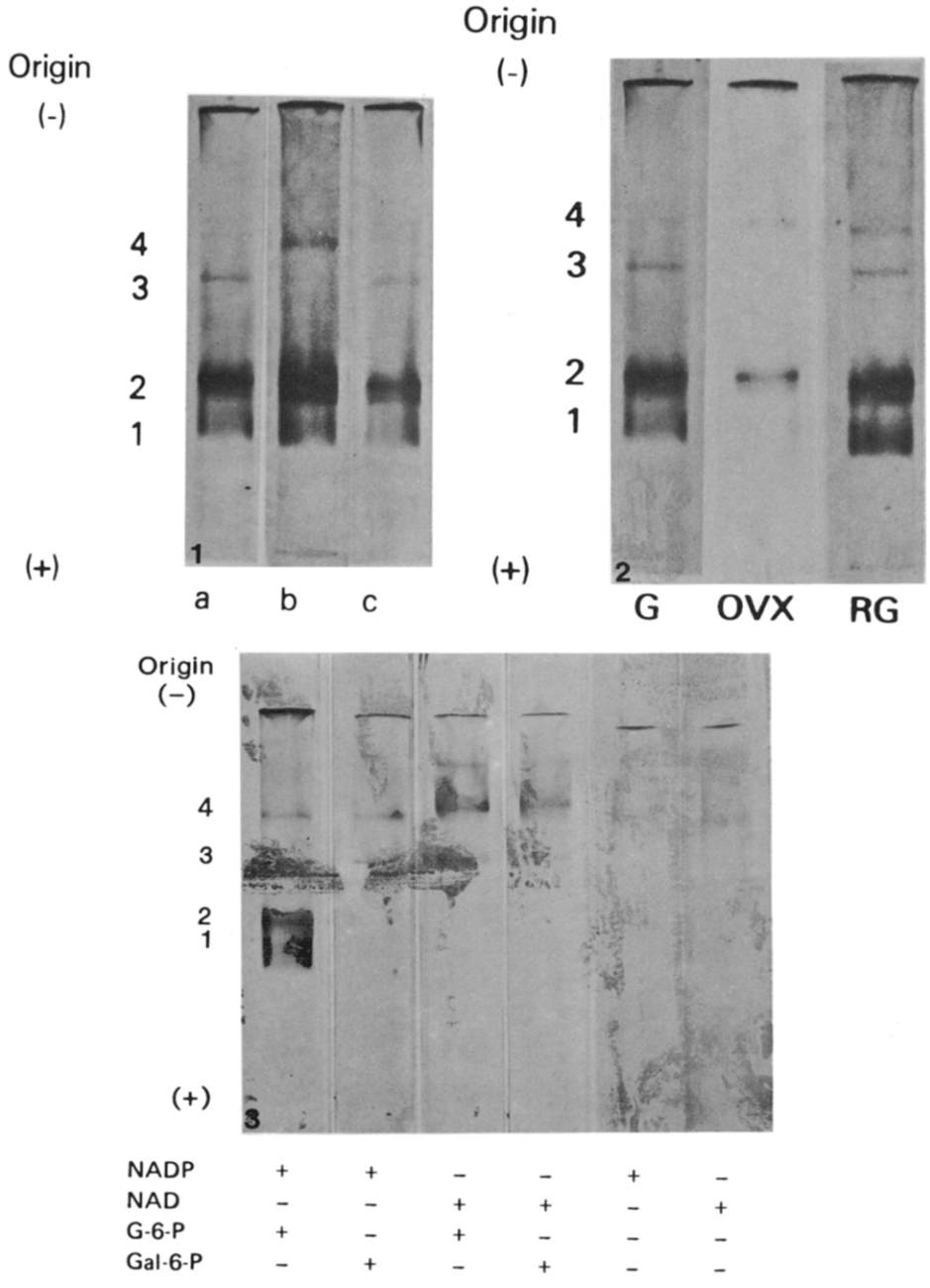

Fig. 1. *Effect of DBcAMP treatment on multiple molecular forms of G6PD in DMBA tumors. a, control, tumors growing; b, DBcAMP treatment* (10 mg/0.1 ml 0.85% NaCl/day/200 g *rat, s.c.*) [2], *for* 5 days (0% vol *change of initial tumor sizes*); *c, DBcAMP treatment for* 5 *days* (0% vol *change of initial tumor sizes*), *then the treatment was stopped for* 5 days (50% *increase of initial tumor sizes*). *At the end of each treatment, whole tumor extracts were prepared for electrophoresis and the gels were incubated to develop the activity as described under "Materials and Methods" except that gel "b" was incubated* 15 min *longer. The incubation medium contained glucose-6-phosphate as the substrate and NADP*$^+$ *as the cofactor. Numbers* 1–4 *refer to G6PD-*1,2,3 *and* 4 *with* 1 *being the fastest migrating anodal band. Each gel represents* 1 *of* 15 *identical gels.*

Fig. 2. *Effect of ovariectomy on multiple molecular forms of G6PD in DMBA tumors. G, tumors growing; OVX,* 5 days *after ovariectomy of host rat* (50% *decrease of initial tumor sizes*); *RG, regrowing,* 5 days *after ovariectomy* (50% *decrease of initial tumor sizes*), *a single injection of delestrogen* (33 μg/0.1 ml *sesame oil*/200 g *rat, s.c.*) [2] *was given, then tumors were removed* 5 days *later when tumor volumes were increased by* 30% *over those at* 5 days *after ovariectomy. Preparation of tumor extracts, subsequent electrophoresis, and activity development of gels are the same as described in the legend to Fig.* 1. *See the legend to Fig.* 1 *for numbers,* 1–4. *Each gel represents* 1 *of* 15 *identical gals.*

Fig. 3. *Substrate and cofactor specificities of multiple molecular forms of G6PD in DMBA tumors. Whole tumor ectracts were subjected to electrophoresis and the gels were incubated to develop the activity as described under "Materials and Methods." +, or − indicates presence or absence of substrate or cofactor in the incubation medium. See the legend to Fig.* 1 *for numbers* 1–4. *Results were identical in* 7 *experiments.*

localization and reactivity with galactose-6-P and NAD^+. However, evidence suggesting that G6PD-3 is not H6PD concerns its lack of reactivity with either glucose (Table 1, footnote ‡) or 2-deoxy-glucose-6-P, its fluctuation of activity with hormonal changes, and its significantly different pH optimum for glucose-6-P and galactose-6-P.

RESULTS

Effect of DBcAMP treatment and hormonal deprivation on multiple molecular forms of G6PD in DMBA mammary tumors

Whole extracts from growing DMBA tumors exhibited 4 major activity bands of G6PD in polyacrylamide-gel electrophoresis (Fig. 1a). In growth-arrested tumors following DBcAMP treatment, the activity band of G6PD-3 disappeared while the other 3 activity bands remained (Fig. 1b). Cessation of DBcAMP treatment restored the G6PD-3 activity band (Fig. 1c) as well as tumor growth [2].

The effect of hormonal deprivation (ovariectomy) on G6PD activity bands of DMBA tumor extracts is shown in Fig. 2. Ovariectomy resulted in the disappearance of the G6PD-3 band and an appreciable decrease of G6PD-1 and -2 activity bands (Fig. 2, OVX) in regressing tumors. Injection of estradiol-valerate into the ovariectomized host restored these activity bands (Fig. 2, RG) as well as tumor growth [2]. The changes in the activity bands were not observed when DMBA tumors ($\sim 12\%$) failed to regress but continued to grow after ovariectomy or DBcAMP treatment.

Spectrophotometric analysis of G6PD activities in subcellular fractions of DMBA tumors during DBcAMP treatment and ovariectomy is shown in Table 1. Activity in the microsomal fraction decreased to 40% of the control activity (Table 1, no treatment) at 1 day after DBcAMP treatment and by the 5th day no activity was detectable. G6PD activities in soluble and mitochondrial fractions remained unaltered. As previously reported [2], growth arrest of tumors was appreciable by the 3rd day of treatment.

G6PD activity in the microsomal fraction also decreased following hormonal deprivation: 3 days after ovariectomy the activity decreased to 45% of the control value and by the 5th day, no activity was measurable (Table 1). The enzyme activity in the soluble

Table 1. Effect of DBcAMP treatment and ovariectomy on G6PD activities of subcellular fractions in DMBA tumors

Treatment	Glucose-6-P dehydrogenase activity			
	TE	S_3	P_3	P_2
	units/g tumor			
none	5.3±0.8*	3.6±0.6†	0.22±0.03‡	0.21±0.03
DBcAMP, 1 day	5.2±0.8	3.7±0.6	0.09±0.01	0.21±0.03
DBcAMP, 5 day	4.9±0.7	3.6±0.5	n.m.	0.22±0.02
Stop DBcAMP§	5.3±0.8	3.6±0.7	0.23±0.03	0.20±0.03
OVX, 3 day	3.5±0.6	2.2±0.3	0.10±0.02	0.21±0.02
OVX, 5 day	1.3±0.2	0.7±0.1	n.m.	0.22±0.03
OVX + Est.¶	5.9±0.9	4.0±0.7	0.27±0.04	0.20±0.03

Tumors from treated and control rats were fractionated by the method of Hogeboom [10] as described under "Materials and Methods." Each fraction was assayed for enzyme activity as described under "Materials and Methods." TE, whole tumor extract (see "Materials and Methods"); S_3, post microsomal supernatant; P_3, microsomal extract; P_2, mitochondrial extract; n.m., not measurable. The protein concentration of microsomal and mitochondrial extracts from growing and regressing tumors was similar. Seventy-five to eighty per cent recovery of the total activity after fractionation may indicate the incomplete extraction and/or loss of enzyme activity during the preparation.

*Mean±S.E. (6 tumors in each group).

†The value did not change when assayed in the presence of 0.5% Triton X-100.

‡The assay for glucose dehydrogenase [13] showed activity similar to the blank value of G6PD assay.

§Treatment as in the legend to Fig. 1, c.

¶Treatment as in the legend to Fig. 2, RG.

fraction also decreased markedly with only 20% of the initial activity measurable at 5 days after ovariectomy, whereas the enzyme activity in the mitochondrial fraction remained unchanged (Table 1). Tumors decreased in size by 15 and 40% at 3 and 5 days after ovariectomy, respectively. Cessation of DbcAMP treatment or injection of estradiol-valerate to the ovariectomized host restored G6PD activities in both the microsomal and soluble fractions (Table 1). Similar results were obtained when G6PD activities were expressed per mg of subcellular fractions (data not shown). Thus microsome-associated G6PD activity disappeared in regressing tumors following either DBcAMP treatment or ovariectomy, just as the G6PD-3 activity band of whole extracts (see Figs. 1 and 2). The results of the electrophoretic analyses of subcellular fractions from growing DMBA tumors were similar to those found in growing MTW9 tumors (1): G6PD-1 and -2 in the post-microsomal supernatant (treatment with 0.5% Triton X-100 did not alter G6PD-1 and -2 activity bands), G6PD-3 in the microsomal fraction, and G6PD-4 in the mitochondrial fraction (data not shown).

Substrate and cofactor specificities of multiple forms of G6PD in DMBA tumors

As shown in Fig. 3 G6PD-1 and -2 activity bands appeared only when both glucose-6-phosphate and $NADP^+$ were present in the incubation medium. G6PD-3 and -4 activity bands, however, were seen in the presence of either glucose-6-phosphate or galactose-6-phosphate and either $NADP^+$ or NAD^+, although the G6PD-4 band was darker in the presence of $NADP^+$ and either substrate than with NAD^+ and substrate (Fig. 3). The tumor extracts also contained NAD^+-dependent, but not $NADP^+$ dependent, other dehydrogenases that migrate slower than G6PD-4 (Fig. 3).

DISCUSSION

This study shows that the change in the activity of one molecular form of G6PD (G6PD-3) is a parameter closely associated with growth control of a hormone-dependent mammary tumor. The disappearance of G6PD-3 activity during DBcAMP-induced growth arrest of tumors may be due to enzyme repression by cAMP. Glucagon and cAMP have been shown to prevent the induction of rat liver G6PD which normally occurs when fasted rats are fed a high-carbohydrate, non-fat diet [14]. It was suggested that the increased level of cAMP may repress the synthesis of lipogenic enzymes [14]. Since the cAMP level was also increased in regressing DMBA tumors following ovariectomy [15, 16], the disappearance of G6PD-3 after ovariectomy may also be due to enzyme repression. Our experimental data suggest that G6PD-3 may be localized in the microsomal compartment of cells in DMBA tumors, as in MTW9 and hepatoma 5123 [1]. The ultimate proof that G6PD-3 is microsomal G6PD, however, requires purification and physico-chemical characterization of the isolated enzyme. Such an approach has been pursued for the identification of microsomal G6PD in rat liver [17].

We have shown previously that disappearance of microsomal G6PD activity is a unique event in DBcAMP-induced tumor regression since the enzyme in the host liver remained unchanged during DBcAMP treatment [1]. We now show that the disappearance of G6PD-3 (microsomal G6PD) is a common biochemical expression of tumor regression produced by either hormonal deprivation or DBcAMP treatment. An increase of acid RNase synthesis [2, 18] and phosphorylation of specific nuclear proteins (RAP) [19] are also common biochemical parameters that were found in regressing mammary tumors following either ovariectomy or DBcAMP treatment. Thus it appears that regression of DMBA tumors following either DBcAMP treatment or hormonal deprivation may be due to a similar, if not identical, mechanism. It is important to point out that the disappearance of G6PD-3 is an early event of tumor regression since it occurs when the structures of regressing and growing tumor cells can not be distinguished by light or electron microscopic examination (J. S. Bodwin *et al.*, unpublished observations). Moreover, the disappearance of this specific enzyme is associated only with hormone-dependent mammary tumors. That G6PD-3 disappears earlier after DBcAMP treatment than after ovariectomy could suggest a difference in the rates of synthesis and degradation of this enzyme in the respective tumor regression processes. Further studies on the synthesis and degradation of G6PD-3 in DMBA tumors during their growth and regression would help to elucidate the role of this enzyme in the control of hormone-dependent mammary tumor growth.

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