THE ROLE OF CYCLIC AMP IN NEOPLASTIC CELL GROWTH AND REGRESSION

II. GROWTH ARREST AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOZYME SHIFT BY DIBUTYRYL CYCLIC AMP

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SUMMARY: $N^6,O^{2'}$-Dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) injected into rats bearing MTW9 mammary carcinoma resulted in an early disappearance of tumor microsomal glucose-6-phosphate dehydrogenase activity while mitochondrial and supernatant isozyme activities were not affected. Prolonged DBcAMP treatment of rats bearing 5123 hepatoma significantly decreased all glucose-6-phosphate dehydrogenase isozyme activities but did not alter host liver isozyme activities or liver regeneration. Since DBcAMP treatment arrested growth of these tumors, the loss of microsomal glucose-6-phosphate dehydrogenase may be an early event in the inhibition of tumor growth *in vivo*.

INTRODUCTION: Previous work (1,2) from this laboratory demonstrated that DBcAMP produced growth arrest in mammary tumors. The experiments reported here show that disappearance of the microsomal glucose-6-P dehydrogenase isozyme is an early event following DBcAMP treatment and correlates with growth arrest of these tumors. Hershey et al. (3) found 2 glucose-6-P dehydrogenase isozymes in both normal and neoplastic rodent breast tissues and Richards and Hilf (4) described these isozymes in carcinogen-induced rat mammary tumors. They also observed that during tumor regression due to hormone withdrawal one of the isozymes decreased in activity. In an attempt to elucidate the mechanism of growth inhibition produced by DBcAMP in tumors, glucose-6-P dehydrogenase isozyme activities were studied.

MATERIALS AND METHODS: MTW9 mammary carcinoma of Wistar rats, hormone-dependent and transplantable (5) and Morris 5123 hepatoma of Buffalo/N rats, hormone-insensitive and transplantable, were used. Animals, 170-200 g, each

bearing a 2-3 g tumor growing subcutaneously, were randomly divided into 2 groups. Animals of one group were given subcutaneous injections of 10 mg DBcAMP in 0.1 ml 0.85% NaCl per day. Animals of the second (control) group received subcutaneous injections of 0.1 ml 0.85% NaCl per day.

RESULTS AND DISCUSSION: DBcAMP treatment resulted in the disappearance of activity of one glucose-6-P dehydrogenase isozyme from tumors while the liver isozyme activities in the same rat remained unaltered (Fig. 1). The intracellular distribution pattern of the isozymes in MTW9 showed isozymes 1 and 2 in the supernatant, isozyme 3 in the microsomal fraction, and isozyme 4 in the mitochondrial fraction (Fig. 2). Although particle-associated isozyme activities are low, their existence can be supported by the following observations: a) Preliminary studies (10) with sodium dodecyl sulfate treatment of total extract indicate that the fast moving anodal bands (supernatant isozymes) are not a simple dissociation product of slow moving bands (particulate isozymes). b) Kimura and Yamashita showed (11) that microsomal and supernatant glucose-6-P dehydrogenase isozymes of rat liver are not identical in terms of physicochemical and immunological characteristics. c) Beitner and Naor reported (8) that the particle-associated forms of glucose-6-P dehydrogenase isozyme activities in rat adipose tissue were only detected in the presence of excess Mg^{++} while supernatant isozyme was not affected. It was concluded from data shown in Fig. 2 that the isozyme which disappeared following DBcAMP treatment was the microsomal enzyme. Glucose-6-P dehydrogenase activities of subcellular fractions from tumors treated with DBcAMP (Table I) were further evaluated by a spectrophotometric assay (12). Microsomal isozyme activity decreased significantly to 40% of the control activity (Table I, 0 time) 24 hours after DBcAMP treatment; no activity was detectable by the third day. However, isozyme activities of the supernatant and mitochondrial fractions remained unaltered. As previously reported (1) growth arrest of tumors by DBcAMP was appreciable by the third day.

DBcAMP produced only the response of tumor microsomal isozyme but not

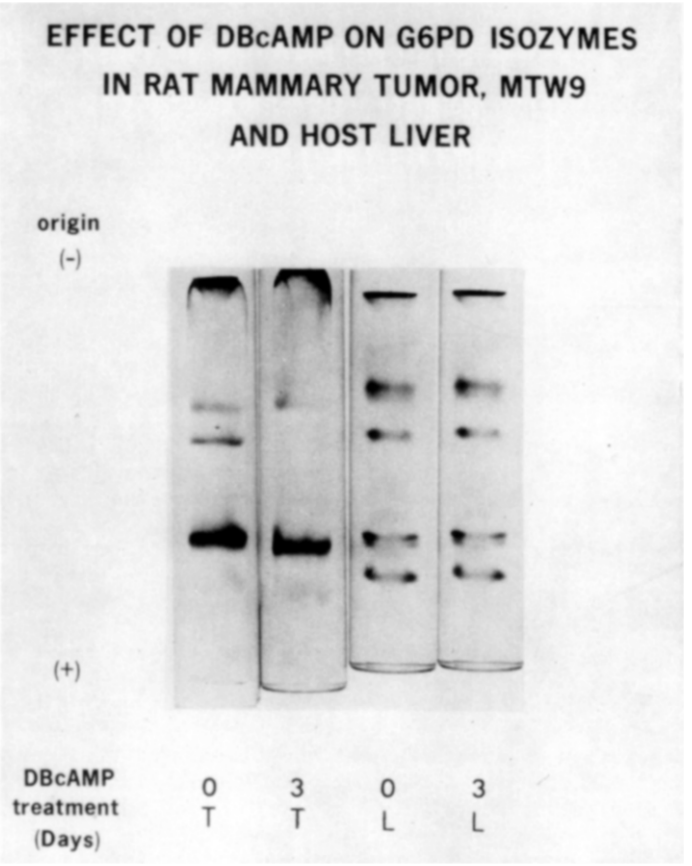

Figure 1. Effect of DBcAMP on glucose-6-P dehydrogenase isozyme activities in rat mammary tumor MTW9 and host liver. Animals bearing MTW9 tumors were treated with DBcAMP (see "Materials and Methods") for 3 days then killed. Tumors and livers from treated and control rats were homogenized immediately after removal in 2 volumes of cold deionized water containing 1 mM EDTA, 5 mM $MgCl_2$, and 0.5% Triton X-100. Homogenates were centrifuged for 60 min at 105,000 x g and supernatants (200-300 µg protein) were subjected to electrophoresis on 7% acrylamide gel with a current of 2 ma/gel for 45 min at 4 C by the method of Ornstein (6) and Davis (7). Gels were developed for enzyme activity at 37 C for 30 min in an incubation medium described by Beitner and Naor (8) (0.3 M tris-HCl buffer, pH 7.6, 7 mM $MgCl_2$, 0.4 mM $NADP^+$, 0.1 mM phenazine methosulfate, 0.4 mM nitro-blue tetrazolium and 1.5 mM glucose-6-P). After incubation, gels were washed thoroughly with distilled water and immersed in 7% acetic acid to terminate the reaction. Longer staining of gels for up to 16 hr did not change the basic band patterns but 2 fast moving anodal bands diffused and became darker. Seven tumors and livers from each group showed identical basic patterns of isozyme activities. T, tumor; L, liver.

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that of liver isozyme. To determine if the same phenomenon would occur in normal growing tissue, regenerating liver was tested together with 5123 hepatoma. DBcAMP treatment for 7 days resulted in the disappearance of both

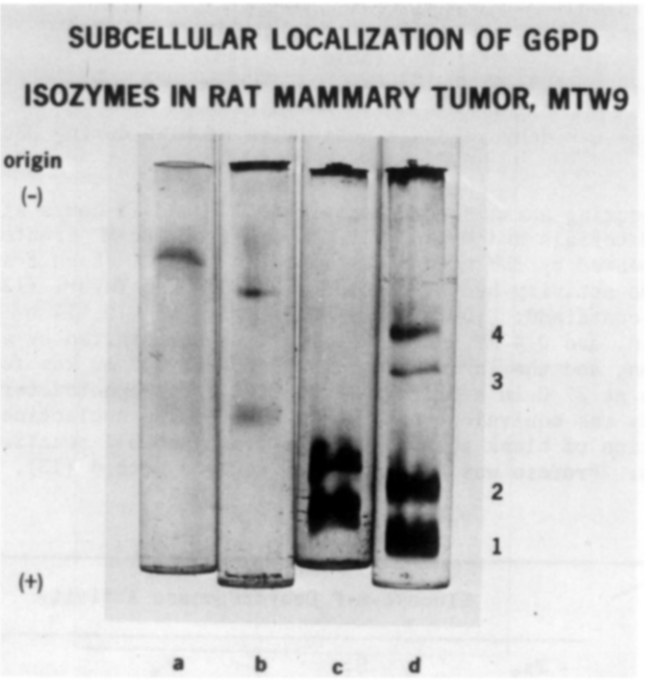

Figure 2. Subcellular localization of glucose-6-P dehydrogenase isozymes in rat mammary tumor MTW9. Cell fractionation was carried out by a slightly modified procedure of Hogeboom (9). Tumors were homogenized in 2 volumes of cold 0.44 M sucrose containing 1 mM EDTA. Nuclei were removed by centrifuging twice at 700 x g for 10 min. The supernatant was subjected to successive centrifugations at 9000 x g for 10 min and 105,000 x g for 60 min. The sediments were washed twice with sucrose and designated as mitochondrial and microsomal fractions, respectively. To release the enzyme the particulate fractions were frozen, thawed, and homogenized in 2 parts of cold deionized water containing 1 mM EDTA, 5 mM $MgCl_2$ and 0.5% Triton X-100. The clear supernatants after centrifugation, as well as the supernatant from the microsomal fraction, were subjected to electrophoresis. For preparation of whole tissue extract, a piece of tumor was frozen, thawed and homogenized in cold distilled water containing 1 mM EDTA, 5 mM $MgCl_2$ and 0.5% Triton X-100. The homogenate was centrifuged at 105,000 x g for 60 min and the supernatant subjected to electrophoresis. After 60 min of electrophoresis (at 4 C) the gels were stained for enzyme activity for 90 min in the medium described in Fig. 1. a, mitochondrial fraction; b, microsomal fraction; c, post microsomal supernatant; d, whole tissue extract. Isozymes numbered 1-4, 1 being the fastest migrating anodal band. At the end of electrophoresis, the bromphenol blue marker band on gels a and b had moved to about one-fourth the distance from the anodal end while gels c and d showed the marker to be at the anodal end.

microsomal and mitochondrial glucose-6-P isozyme activities as well as a significant decrease in supernatant isozyme activity of 5123 hepatoma, but regenerating liver isozymes remained unchanged (Fig. 3). Moreover, growth of 5123 hepatoma was inhibited while liver regenerated at the same rate as in control animals (Fig. 3). We have not established whether DBcAMP induced

Table I

Subcellular glucose-6-P dehydrogenase activities of MTW9 during DBcAMP treatment.

MTW9 tumor-bearing animals were decapitated 24 and 72 hours after DBcAMP treatment (see "Materials and Methods"). Tumors from DBcAMP treated and control rats were fractionated by the method described in Fig. 2. Each fraction was assayed for enzyme activity by the method of Matsuda and Yugari (12). The reaction mixture contained: 100 mM tris-HCl buffer, pH 8.0, 14 mM $MgCl_2$, 0.8 mM glucose-6-P, and 0.4 mM NADP. The reaction was started by adding the enzyme preparation, and the increase in absorbance at 340 mμ was followed for the first few min at 37 C in a Gilford Model 2000 spectrophotometer. One unit of enzyme activity was equivalent to 1 μmole of pyridine nucleotide reduced per hr after subtraction of blank values. Blanks contained all reaction mixtures except the enzyme. Protein was determined by Lowry's method (13).

Hours post DBcAMP treatment	Glucose-6-P Dehydrogenase Activity			
	Ts_3	S_3	P_3	P_2
	units/mg protein			
0	10.7 ± 1.0[a]	7.2 ± 0.8	1.0 ± 0.09	0.9 ± 0.08
24	10.0 ± 0.9	7.0 ± 0.7	0.4 ± 0.03[b]	1.1 ± 0.09
72	9.5 ± 0.9	7.0 ± 0.8	n.m.	1.0 ± 0.10

[a]Mean ± S.E. (5 tumors in each group).

[b]This value is more significant if one considers the fact that the microsomal fraction contains an appreciable amount of supernatant isozyme activity (Fig. 2b) which does not change during regression. P value compared to the control was 0.01.

Recovery of total activity after fractionation was usually 85%. TS_3, total extract; S_3, post microsomal supernatant; P_3, microsomal fraction; P_2, mitochondrial fraction; n.m., not measurable.

disappearance of microsomal isozyme activity by decreasing its synthesis or by inactivating the enzyme. However, reports on prevention of glucose-6-P dehydrogenase induction by cAMP, DBcAMP, and glucagon (14) suggest that inhibition of enzyme synthesis is most probable. The relationship between the microsomal isozyme disappearance and tumor growth arrest is not clear. Although the activity of liver soluble glucose-6-P dehydrogenase was shown to be modified

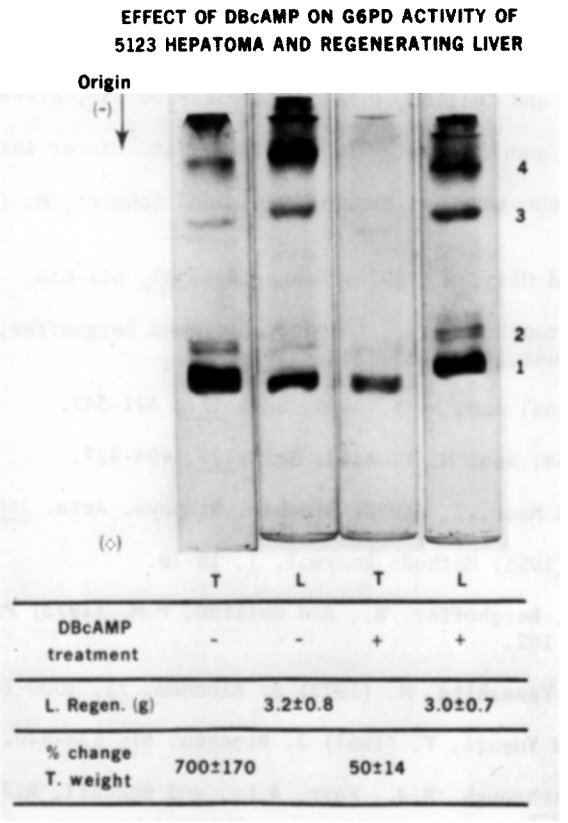

Figure 3. Effect of DBcAMP on glucose-6-P dehydrogenase isozyme activities and host liver regeneration. Five animals bearing 5123 hepatoma were treated with DBcAMP for 3 days, then hepatectomized and further treated with DBcAMP for 4 more days. Five control animals bearing 5123 hepatoma were hepatectomized only. At 4 days post hepatectomy both groups of animals were decapitated, extracts were prepared from pools of 5 tumors and 5 livers, respectively. The extracts were subjected to electrophoresis and developed for isozyme activity as shown in Fig. 1. T, tumor; L, regenerating liver.

by hormonal and dietary manipulations (15,16), the liver microsomal isozyme was found to remain fairly constant under various conditions but did show fluctuations in activity during fetal development (11). Changes in glucose-6-P dehydrogenase isozyme patterns have also been observed during rat mammary gland differentiation (17). Thus, the disappearance of microsomal isozyme in DBcAMP treated tumors may mimic the conditions occurring during cell differentiation.

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