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Effect of Estrogen Treatment on DMBA-Induced Mammary Tumor Growth and Biochemistry in Intact and Diabetic Rats[1] (38535)

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We reported that about 60% of DMBA-induced mammary tumors in Sprague–Dawley rats regress after the animal has been made diabetic by injection of streptozotocin (1). These data agree in general with the earlier reports by Heuson and his coworkers (2–4), who used alloxan to induce diabetes; they reported 90% of the DMBA-induced tumors regressed after induction of diabetes. However, the diabetic animals demonstrated significant weight loss, which may have contributed to the considerably higher number of regressing tumors, compared to our report, in which no weight loss of diabetic animals occurred. Nevertheless, it would appear that a certain population of the carcinogen-induced neoplasms may be classified as insulin-dependent, just as these tumors have earlier been classified as estrogen-dependent and prolactin-dependent.

Many studies have shown that DMBA-induced tumors will regress after treatment with pharmacologic doses of estrogen (5, 6), either as a direct effect or indirectly through interference with prolactin action at the tumor cell (7). As an extension of our earlier studies, it seemed logical to determine if estrogen treatment would have any further effect on growth patterns of DMBA-induced tumors in diabetic rats. The results presented here indicate that all of the tumors were observed to regress in estrogen-treated diabetic rats, suggesting that insulin deprival plus estrogen therapy may have additive effects.

Materials and Methods. Fifty-day-old Sprague–Dawley rats were intubated with 7,12-dimethylbenz(a)anthracene dissolved in sesame oil. Each rat received a total of 25 mg of DMBA, in doses of 5 mg/ml once weekly for 5 wk; tumors arose 8–15 wk after initial administration of DMBA. Tumors were measured by calipers to obtain two perpendicular diameters; tumor size, expressed as tumor surface (diameter 1 × diameter 2 = $cm^2 \times \pi$). Animals were utilized for experiments when the first tumor to appear reached approximately 2.0 × 2.0 cm. Estradiol valerate, when administered, was injected subcutaneously at a dose of 1 mg/animal/wk; treatment was initiated one day after streptozotocin was injected. Tumor regression was defined as a decrease in tumor surface of at least 20% and tumor growth defined as an increase of at least 20% during the period of observation.

Diabetes was induced by one intravenous injection of streptozotocin to animals fasted overnight. Streptozotocin (Upjohn Co., Kalamazoo, MI) was dissolved in saline, rapidly adjusted to pH 4.5 with citric acid, and administered at a dose of 50 mg/kg body weight in a volume of about 0.5 ml. After treatment, animals were allowed access to food and water. Blood glucose levels were monitored twice weekly, using a microversion of the Nelson–Somogyi method (8). Urinary glucose was determined by Clinistix (Ames). Animals were classified as diabetic if blood glucose levels were 250 mg/100 ml or higher and urinary glucose exceeded 0.5%.

Tissues, obtained at sacrifice of animals and stored at −20°, were thawed and homogenized in cold 0.05 *M* Tris buffer, pH 7.4, to produce a final 10% homogenate (w/v). Aliquots of the homogenates were taken for determination of nucleic acids, which were extracted by a modification of the Schneider method (9). RNA was measured by the

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orcinol reaction as described by Ceriotti (10) and DNA was determined by the diphenylamine reaction according to the method of Dische (11).

Enzyme assays were performed on the supernatant of the homogenate after centrifugation at 29,000 *g* for 20 min at 4°. Each enzyme assay was performed under identical conditions by measuring the absorbancy change at 340 nm due to production of NADPH or oxidation of NADH. Under these conditions of zero order kinetics, enzyme values are comparable and are expressed as μmoles of NADPH produced/min/mg DNA or as μmoles NADH oxidized/min/mg DNA. The enzymes measured, by procedures previously cited (12), were: pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40); phosphofructokinase (ATP:D - fructose - 6 - phosphate phosphotransferase, EC 2.7.1.11); hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1); glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate dehydrogenase: NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase, decarboxylating, EC 1.1.1.44). Aliquots of the supernatants were also taken for protein determination according to the method of Lowry *et al.* (13).

TABLE I. EFFECT OF ESTROGEN ON GROWTH OF DMBA-INDUCED TUMORS IN DIABETIC RATS.

Animal	Dose of Estradiol valerate	% Growing	% Static	% Regressing
Intact control (362)[a]	—	54	29	17
Diabetic (33)	—	24	18	58[b]
Intact (8)	1.0 mg/wk	25	0	75[b]
Diabetic (8)	1.0 mg/wk	0	0	100[b]

[a] Numbers in parentheses indicate number of tumors observed in various groups. Figures for intact control animals include reports by Teller *et al.* (5) and Griswold *et al.* (6) in addition to our observations.

[b] Significant difference ($P < 0.05$) in distribution as compared to the intact control animals according to chi square analysis.

Data are presented as the mean ± SEM; significance according to the Student's *t* test was designated as $P < 0.05$. Analysis of differences of tumor growth patterns was performed by chi square test; significance was accepted at $P < 0.05$.

Results. Table I summarizes the effects of estrogen treatment on tumor growth in intact and diabetic rats. Diabetes, as reported previously, resulted in a significant increase in the number of regressing tumors compared to intact control tumor-bearing animals (5, 6). Administration of estradiol valerate caused regression of 75% of the tumors studied whereas treatment of diabetic rats with estradiol valerate caused regression of all the tumors studied. Thus, estrogen treatment enhanced the effects of diabetes in producing tumor regression.

Table II presents a summary of tumor

TABLE II. THE EFFECT OF ESTROGEN ON THE WEIGHT OF DMBA-INDUCED TUMORS AND UTERINE WEIGHT IN INTACT AND DIABETIC RATS.

Animal	Dose of estradiol valerate (mg/wk)	Body weight (gm)	Tumor weight (gm)	Uterine weight (mg)	Blood glucose (mg/100 ml)
Control	0	278 ± 11	5.08 ± 1.13[a]	603 ± 58	124 ± 13
Diabetic	0	256 ± 18	1.76 ± 0.56[b, c]	396 ± 33[c]	384 ± 12[c]
Control	1.0	268 ± 16	2.20 ± 0.95	1400 ± 290[c]	145 ± 1
Diabetic	1.0	229 ± 5[c]	0.39 ± 0.09[c, d]	780 ± 30[c, d]	409 ± 2[c, e]

[a] Data represented as the mean ± SEM.

[b] This weight represents the average weight of DMBA-induced tumors which are insulin-dependent; the average weight of the insulin-independent tumors was 5.64 ± 2.10 g.

[c] Significant difference ($P < 0.05$) from the Control.

[d] Significant difference ($P < 0.05$) from the Diabetic.

[e] Significant difference ($P < 0.05$) from the Control + Estrogen (1 mg/wk).

TABLE III. THE EFFECT OF ESTROGEN ON PROTEIN AND NUCLEIC ACID LEVELS IN DMBA-INDUCED TUMORS IN INTACT AND DIABETIC RATS.

Animal	Dose of estradiol valerate (mg/wk)	Protein (μg/mg)	RNA (μg/mg)	DNA (μg/mg)	RNA/DNA
Control (15)	0	85.0 ± 7.0[a]	6.60 ± 0.61	7.33 ± 0.37	0.88 ± 0.06
Diabetic (11) (dependent)	0	79.0 ± 5.0	4.59 ± 0.37[b]	9.62 ± 0.87[b]	0.52 ± 0.06[b]
Control (7)	1.0	82.0 ± 4.0	9.47 ± 0.79[b]	7.70 ± 1.08	1.14 ± 0.09[b]
Diabetic (6)	1.0	66.0 ± 3.0[b, c, d]	6.79 ± 0.90[c, d]	10.16 ± 0.86[b]	0.67 ± 0.07[b, d]

[a] Data represented as the mean ± SEM; the numbers in parentheses indicate the number of tumors per group.

[b] Significant difference ($P < 0.05$) from the control.

[c] Significant difference ($P < 0.05$) from the diabetic.

[d] Significant difference ($P < 0.05$) from the control + estrogen (1 mg/wk).

weights obtained in these animals, sacrificed 13 to 18 days after streptozotocin treatment to induce diabetes. The average body weight of the control animals, diabetic animals and intact estrogen-treated animals were similar; diabetic rats receiving estrogen therapy showed a slight weight reduction. As was previously shown (1), the insulin-dependent tumors (tumors regressing in the diabetic animal) were reduced in weight compared to tumors of control animals whereas, insulin-independent tumors (those which showed less than a 20% reduction in size) were comparable in weight to the tumors of intact animals. Estrogen administration (1 mg/wk) appeared to cause a reduction in tumor weight in both control and diabetic animals, however, this decrease was only significant in the diabetic animals. Uterine weights were reduced in the diabetic animals, however the uterotrophic response to estrogen was comparable to that observed in the intact animals. Blood glucose levels of the diabetic rats were elevated about threefold compared to the intact animals; estrogen treatment had no effect on blood glucose levels.

The levels of RNA and DNA in tumors from these animals are presented in Table III. Regressing tumors in diabetic rats had a reduction in RNA levels and an increase in DNA levels, resulting in a decrease in the RNA/DNA ratio. Similar responses have been reported earlier (12). Administration of estradiol valerate increased RNA levels and RNA/DNA ratios in tumors. It is interesting that estrogen treatment of diabetic rats, which resulted in regression of all tumors studied, resulted in an increase in DNA levels and a reduced RNA/DNA ratio in neoplasms when compared to intact control (growing tumors) animals. However, the decrease in RNA caused by insulin lack and the increase in RNA produced by administration of estrogen were apparently offset by one another in the tumors from diabetic animals treated with estrogen; the resulting level of RNA in these neoplasms was similar to that found in tumors from intact control animals. These data suggest that the mechanism whereby insulinemia and estrogen therapy cause tumor regression is different. Protein levels in cytoplasmic fractions of the tumors were similar except for a reduced level in tumors from diabetic rats treated with estrogen.

Since one of the major physiological roles of insulin is directed towards regulation of carbohydrate metabolism, we also examined the activities of certain enzymes selected on the basis of their roles in glycolysis (PK, PFK and HK) and oxidative glucose metabolism (G6PD and 6PGD). These results are given in Table IV. The activities of PK, PFK, G6PD and 6PGD were reduced in regressing tumors from diabetic animals, whereas no significant effects on enzyme ac-

TABLE IV. THE EFFECT OF ESTROGEN ON CERTAIN ENZYME ACTIVITIES[a] IN DMBA-INDUCED TUMORS IN INTACT AND DIABETIC RATS.

Animal	Dose of estradiol valerate (mg/wk)	PK	HK	PFK	6PGD	G6PD
Control (15)	0	11.83 ± 0.85[b]	0.120 ± 0.011	0.266 ± 0.026	0.327 ± 0.021	0.461 ± 0.036
Diabetic (11) (dependent)	0	7.90 ± 1.09[c]	0.080 ± 0.020	0.191 ± 0.019[c]	0.194 ± 0.036[c]	0.164 ± 0.036[c]
Control (7)	1.0	9.31 ± 1.63	0.102 ± 0.024	0.266 ± 0.048	0.401 ± 0.014	0.564 ± 0.092
Diabetic (7)	1.0	3.30 ± 0.45[c, d, e]	0.059 ± 0.003[c]	0.104 ± 0.019[c, d, e]	0.081 ± 0.021[c, d, e]	0.320 ± 0.075

[a] Activity expressed as μmoles NADH oxidized/min/mg DNA or μmoles NADPH produced/min/mg DNA.
[b] Data represented as the mean ± SEM; the numbers in parentheses indicate the number of tumors per group.
[c] Significant difference ($P < 0.05$) from the control.
[d] Significant difference ($P < 0.05$) from the diabetic.
[e] Significant difference ($P < 0.05$) from the control + estrogen (1 mg/wk).

tivities were found after administration of estradiol valerate. However, tumors from diabetic animals treated with estrogen demonstrated activities of PK, PFK and 6PGD that were significantly reduced below those found in tumors of diabetic rats, indicating that estrogen had augmented the response due to lack of insulin. It should be noted that HK activity was significantly lower in tumors from diabetic estrogen-treated animals, a response not seen in tumors from diabetic rats. Estrogen treatment, which has in the past led to increased G6PD activity in DMBA-induced tumors, did not significantly alter G6PD activity in the neoplasms studied here; however, estrogen treatment did reverse the reduction in G6PD activity brought about by insulinemia.

Discussion. The data presented here are an extension of our earlier findings that a considerable number of DMBA-induced mammary tumors (>50%) regress after endogenous insulin is removed by making the animals diabetic (1–4). As compared to the more toxic manifestations of alloxan as the diabetogenic agent (14), streptozotocin treatment did not lead to significant decreases in body weight in tumor-bearing animals and the effects on tumor growth would not be directly attributable to inanition. It thus appears that insulin may be required for maintaining growth of these carcinogen-induced mammary tumors, a finding not unlike the earlier reports on the requirement of estrogens and prolactin for neoplastic growth (15). It is of interest that the effects of pharmacologic doses of estrogen, which have been reported to produce tumor regression, were also seen in the diabetic rat. The fact that all of the tumors observed in this study were found to regress in estrogen-treated diabetic rats suggests an additive effect of these hormone manipulations and perhaps, the existence of cell populations possessing different hormonal requirements for growth. Indeed, Heuson and Legros (3) reported that insulin treatment could not prevent regression of tumors after ovariectomy nor could estrogen treatment prevent tumor regression after induction of diabetes.

Some of the biochemical data presented here lend support to different mechanisms whereby tumor regression was induced. Removal of endogenous insulin by induction of diabetes resulted in tumor regression in which the regressing neoplasms demonstrated decreases in RNA levels, increases in DNA levels, and decreased activities of glycolytic enzymes and enzymes of the hexose-monophosphate pathway. In contrast, regression of tumors by estrogen therapy was accompanied by an increase in RNA levels with no change in DNA levels and no significant alterations in the enzyme activities measured (PK and HK activities showed a modest decline and G6PD and 6PGD activities showed a modest increase but none of the changes were significant at the 95% confidence limit). However, estrogen administration to tumor-bearing diabetic rats produced regression of all tumors and these tumors showed a decrease in RNA/DNA ratios, cytoplasmic protein content and striking reduction in the activities of the

glycolytic enzymes PK and PFK, as well as 6PGD, the enzymes being reduced to levels below that found in regressing tumors of untreated diabetic rats. It appears that the effects of estrogen in the diabetic rat enhance some of the responses induced by the diabetic state, perhaps due to a greater sensitivity of tumors to estrogen therapy in the absence of the growth promoting effects of insulin.

In extensive studies of mammary tumor regression by Gullino *et al.* (16, 17, 18), castration-induced regression of DMBA-induced tumors was shown to be reflected by a modest decrease in RNA/DNA ratios and a slight increase in DNA content per g of tissue, results which were also seen here in the diabetic rats. Glucose utilization by MTW9 mammary tumors, regressing after ovariectomy, was essentially unchanged (17) but in an earlier report from our laboratory, production of $^{14}CO_2$ from glucose substrate *in vitro* was significantly reduced in DMBA-induced tumors from diabetic rats (1). The fact that activities of glycolytic enzymes were reduced in regressing tumors indicates that these may reflect tumor growth, a conclusion postulated earlier by Weber (19) after examining hepatomas and by Knox *et al.* (20–22) after examination of a series of transplantable mammary tumors of different growth rates. Studies are currently in progress to examine more carefully the role of insulin on mammary tumor growth and metabolism.

Summary. Sixty percent of DMBA-induced mammary tumors in Sprague–Dawley rats regress after the animals were made diabetic by treatment with streptozotocin. Administration of estradiol valerate, 1 mg/wk/animal, caused regression of 75% of tumors in intact rats but regression of all tumors in diabetic rats. Estrogen treatment appeared to enhance the effects of diabetes, which by itself resulted in decreased activities of glycolytic enzymes. A role of insulin in growth of DMBA-induced mammary tumors may be warranted.

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