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Hormonal Effects on Thymidine Kinase and Thymidylate Kinase Activity of Estrogen-dependent Tumors in the Rat[1]

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SUMMARY

In estrogen-induced, estrogen-dependent, mammary and adrenocortical carcinomas, the specific activities of the thymidine kinase and thymidylate kinase decreased significantly within 2 to 4 days after removal of the source of estrogen of the host. After 7 days, these changes were even more marked in the adrenal tumors. Similar changes were not observed in the activity of these enzymes in autonomous tumors in response to alterations in the supply of estrogen to the host. The treatment with antiestrogenic compounds of estrogenized animals bearing dependent adrenal tumors also caused a decrease in the activity of the two enzymes. The replacement of estrogen by treatment with adrenocorticotropic hormone in animals with adrenal tumors caused a stimulation of kinase activity at 2 days but did not prevent the decrease observed at 7 days after the removal of estrone. Growth of the mammary and adrenocortical carcinomas, as indicated by activity of the kinases, was maintained after estrogen removal by treatment of the host with prolactin.

INTRODUCTION

Carcinomas of several endocrine organs have been induced in rats by prolonged exposure to estrogen, administered as a s.c. implanted pellet of estrone. Transplantable carcinomas of the mammary gland, anterior pituitary, and adrenal cortex have been established (17). A significant proportion of these tumors appears to be estrogen dependent, in that growth will occur in ovariectomized female or intact male hosts if a source of estrogen is provided. Upon removal of the estrogen, tumor growth is arrested, and regression eventually occurs. While this implies that estrogen is required to maintain tumor growth, a direct action of estrogen on the tumor is not necessarily indicated, since variation in estrogen levels may influence the synthesis and/or secretion of pituitary hormones such as ACTH[2] (11), prolactin (3, 14), and luteinizing and follicle stimulating hormones (5). This suggests that the tumors may not depend directly on estrogen but, rather, may require a pituitary hormone, produced in abnormal amounts in response to the elevated estrogen concentrations, to maintain their growth. The purpose of th experiments described here was to investigate this possibility. The effects of estrogen withdrawal on the growth rate of dependent and autonomous mammary and adrenocortical carcinomas have been studied, and attempts to maintain the growth of dependent tumors in the absence of estrogen by administering other hormones are also described.

In these experiments, the activities of 2 enzymes associated with DNA precursor synthesis, the thymidine and thymidylate kinases, have been used as indicators of tumor growth rate. Previously, it has been shown that the activity of the thymidine kinase varied with growth rate of the adrenocortical tumors and that the activity of the enzyme decreased rapidly on removal of the estrogen from the host (7). This decrease occurred significantly before changes in physical dimensions could be reliably detected. In these studies, the thymidylate kinase has been included to provide an additional indication of the rate of growth of the tumor and to supplement the information obtained by measuring the thymidine kinase, since the significance of the latter enzyme in cellular metabolism is not known.

MATERIALS AND METHODS

Growth of Tumors. Four transplantable tumors were used in these studies—a dependent and an autonomous mammary tumor and a dependent and an autonomous adrenocortical tumor. The tumors were obtained originally from the collection of Dr. R. L. Noble. The carcinomas had been induced in hooded rats by prolonged treatment with estrogens and were carried as s.c. transplants in young intact males of the inbred strain of Hooded rats. In some experiments, female hosts were used for transplantation of the mammary tumors. At the time of transplant, a 20- to 25-mg pellet of estrone and cholesterol (9:1, by weight) was implanted s.c. in the flank of the host, and the tumor was transplanted s.c. into the back of the neck. Tumors were classed as autonomous if they grew in host animals lacking a source of estrogen, or, in pellet-treated hosts, if they continued to grow after depelleting. Dependent tumors were those that grew only in estrogenized hosts and that regressed on removal of the source of estrogen.

Treatment of Animals. To study the effects of estrogen withdrawal on the enzyme activities of the tumors, we removed the estrone pellets using ether anesthesia. In experiments designed to determine the ability of other hormones to replace estrogen in maintaining tumor growth, estrone pellets were removed in the morning, and treatment with hormones was begun 5 to 6 hr later. Prolactin and ACTH

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[2] The abbreviation used is: ACTH, adrenocorticotropic hormone.

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were administered by s.c. injection, while pelleted and depelleted controls received injections of 0.9% NaCl solution. The effects of the estrogen antagonist (U-11,100 A) and of testosterone were determined in pelleted animals. The antagonist was administered by s.c. injection, and we administered testosterone by implanting a pellet in the flank opposite to that in which the estrone pellet was located. Each experimental group consisted of 3 to 4 animals. Enzyme assays were done on individual tumors or, in some cases, on the pooled tumors from the whole group of animals.

Enzyme Assays. Tumors were removed from decapitated animals, weighed, and homogenized singly or by group in a Servall homogenizer at 16,000 rpm in 10 volumes of ice-cold 50 mM potassium phosphate buffer, pH 7. The supernatant fraction from centrifugation of the homogenate at 100,000 × *g* for 1 hr was used as the enzyme source. Thymidine kinase was determined as previously described (7). Thymidylate kinase was assayed in a similar manner, but the concentrations of the components of the reaction mixture differed from those used for the thymidine kinase. A 0.2-ml aliquot of enzyme solution that contained 0.3 to 1.0 mg protein was added to 0.3 ml of a solution containing 2 μmoles of ATP, 4 μmoles of magnesium acetate, 25 μmoles of potassium phosphate buffer (pH 7), and 12 nmoles of dTMP, including 0.1 μCi of dTMP-2-^{14}C, to form the complete reaction mixture. Aliquots of each extract, or an appropriate dilution, were incubated for 7 and 15 min at 37°. Incubation was terminated by immersion of the tube in boiling water for 3 min, followed by rapid cooling in ice. The procedure was then identical to that used in the thymidine kinase assay. Acid-soluble material was extracted, and the nucleoside and nucleotide components were separated by paper chromatography and located by autoradiography. Areas of paper corresponding to thymine plus thymidine, dTMP, dTDP, and dTTP were eluted into vials with 1.0 ml of 1.0 M NH_4HCO_3, and radioactivity was determined with the use of Bray's solution (1) in a Packard liquid scintillation spectrometer with 70% efficiency. Results are expressed as nmoles of dTMP converted to dTDP + dTTP/hr/mg protein. The activity of each extract was determined from reactions in which conversion of dTMP was linear with time and protein concentration.

Protein Concentration. The protein content of each extract was determined by the method of Lowry *et al.* (13).

Reagents and Hormones. Thymidine-2-^{14}C, 50 to 60 mCi/mmole, was purchased from Amersham/Searle, Toronto, Canada; dTMP-2-^{14}C, 44-50.8 mCi/mmole, was purchased from New England Nuclear, Montreal, Canada; ACTH was purchased from Schwarz/Mann, Orangeburg, N. Y.; and estrone and testosterone were from Sigma Chemical Co., St. Louis, Mo. Ovine prolactin was a gift from the Endocrinology Study Section, NIH, Bethesda, Md. The estrogen antagonist, U-11,100 A, was a gift from the Upjohn Company, Kalamazoo, Mich., courtesy of Dr. P. W. O'Connell.

RESULTS

Effects of Estrogen Withdrawal

The activities of the thymidine and thymidylate kinases were determined in preparations of dependent and autonomous mammary and adrenocortical carcinomas. Marked decreases in activity were observed in the dependent tumors by 2 to 4 days after the host animals were depelleted. In 1 experiment, the specific activity of thymidine kinase of pooled dependent adrenal tumors from hosts depelleted 2 days earlier was 1.9 nmoles/hr/mg protein, compared with 4.8 nmoles in tumors from estrone-pelleted animals. The values for dTMP kinase activity were 7.2 nmoles/hr/mg protein for tumors from depelleted hosts and 14.5 nmoles for growing control tumors. The tumors used in this particular experiment were from a transplant generation that grew more rapidly than usual, which accounts for the somewhat higher specific activities of the enzymes in the growing tumors. Table 1 shows the results of a series of experiments with adrenal tumors, and 70 to 80% decreases in activity of the kinases were observed by 4 to 7 days after removal of estrogen from the host. This procedure had no effect on the enzyme activities in the autonomous tumors. Table 2 shows the results of a similar series of experiments with the dependent and autonomous mammary tumors. Again, removal of the source of estrogen of the host caused decreases of 85 to 90% in the specific activities of the kinases in dependent tumors in 4 days but produced no alteration in enzyme activities in the autonomous tumors.

The involvement of estrogen in maintaining the growth of the dependent adrenal tumors is also demonstrated (Table 3). Treatment of host animals with the estrogen antagonist U-11,100 A or with testosterone, an antiestrogen of unknown mechanism, decreased the activity of the 2 enzymes. A significant effect (40% decrease in activity) was evident 1 day after implantation of a testosterone pellet, while the administration of U-11,100 A daily for 7 days caused a 50% drop in activity of the enzymes.

Effects of Exogenous Hormones after Estrogen Withdrawal

Prolactin. Growth of both dependent adrenal and mammary carcinomas, as indicated by the activities of the 2 kinases, was maintained by treating the hosts with prolactin after the source of estrogen was removed (Table 4). The specific activities of the thymidine and thymidylate kinases of growing mammary tumors were, respectively, 9.17 and 41.9 nmoles of substrate converted per hr per mg protein. The activities of the enzymes in tumors in hosts depelleted 4 days previously were 0.26 and 2.68 nmoles/hr/mg protein, while treatment of depelleted hosts with prolactin for 4 days maintained the activities at 8.6 and 35.9 nmoles/hr/mg protein. Similar results were obtained in experiments with dependent adrenal tumors. Enzyme activities decreased by 50 to 60% in 2 days after removal of estrogen from the hosts but were maintained at almost control levels by treatment of the depelleted hosts with prolactin.

As young intact male rats (5 to 6 weeks old) were used as hosts in the experiments with the adrenocortical carcinomas, it seemed possible that the apparent regression that followed removal of estrogen from the host was actually a response to increasing testosterone levels, since rapid regeneration of the testes might occur in these animals. For an investigation of this possibility, tumors were grown in estrogenized male rats that had been castrated several days before implantation of the

Table 1

Effect of withdrawal of estrogen from the host on the activity of thymidine and dTMP kinases of adrenal tumors

Enzyme assays were done on tumors from rats containing estrone pellets and on tumors from hosts from which the estrogen had been removed for the period indicated. Differences due to removal of estrogen were statistically significant; $p < 0.01$, determined by Student's t test.

Type of tumor	Hormonal state of host	Specific activity of (nmoles/hr/mg protein)	
		Thymidine kinase	Thymidylate kinase
Dependent	Estrone present	3.62 ± 0.44[a]	7.11 ± 0.44[a]
	Estrone removed		
	2 days	1.71	ND[b]
	4 days	1.62	ND
	7 days	1.02 ± 0.10[a]	1.84 ± 0.44[a]
Autonomous	Estrone present	3.73	9.36
	Estrone removed, 4 days	3.29	9.33

[a] Mean ± S.E. Data were obtained from 3 to 7 experiments, with 3 to 4 animals per group in each experiment. The tumors were homogenized individually. Other data (not keyed with[a]) were obtained from experiments in which tumors from 2 to 4 animals per group were pooled.
[b] ND, not determined.

Table 2

Effect of withdrawal of estrogen from the host on the activity of thymidine and dTMP kinases of mammary tumors

Enzyme assays were done on tumors from rats containing estrone pellets and on tumors from hosts from which the estrogen had been removed for the period indicated.

Type of tumor	Hormonal state of host	Specific activity (nmoles/hr/mg protein) of	
		Thymidine kinase	dTMP kinase
Dependent	Estrone present	12.01 ± 1.61[a]	31.91 ± 5.70[a]
	Estrone removed, 4 days	0.71 ± 0.06[a]	3.69 ± 0.88[a]
Autonomous	Estrone present	5.76[b]	5.70[b]
	Estrone removed, 3 days	6.52[b]	6.70[b]

[a] Mean ± S.E. Data were obtained from 3 to 7 experiments, with 3 to 4 animals per group in animals/group. Tumors were homogenized individually for enzyme assay. The differences between values for groups with and without estrone are statistically significant; $p < 0.01$.
[b] Values were obtained from experiments in which tumors from hosts given the same treatment were pooled for enzyme assay.

estrone and tumor. The tumors developed normally in these hosts, and the results (Table 5) indicate that the kinase activities decreased after removal of the estrogen but that they were maintained at levels characteristic of growing tumors by the injection of prolactin. These data signify that testosterone levels were not involved in the decrease in activity of the enzymes in tumors after the removal of estrogen from intact male hosts, nor was testosterone involved in the action of prolactin.

ACTH:ACTH + Prolactin. A decrease in thymidine kinase activity in dependent adrenal tumors during treatment of depelleted hosts for 7 days with ACTH has been reported (7). The present results (Table 6) of studies in which 2 enzyme activities were used as parameters of growth are in agreement with the earlier report, but the marked stimulation of kinase activity after 2 days of treatment with ACTH was an unexpected finding. It is a quantitatively significant effect, however, and may represent a short-term response of adrenal tissue, normal or malignant, to its trophic hormone. Stimulation of DNA synthesis, thymidine kinase, and DNA polymerase activity in guinea pig adrenal in response to ACTH has been reported (15).

The data in Table 6 demonstrate further that growth of the dependent adrenocortical tumor is maintained by prolactin in the absence of estrogen. Tumors in prolactin-treated rats had the same level of enzyme activities as did tumors in estrogenized hosts and did not show the marked decreases found in tumors in animals depelleted for 2 or 7 days (40 to 90%). In depelleted animals given combined ACTH and prolactin, the effect of ACTH predominated at both time intervals studied. The enzyme activities were increased over those in growing control tumors (in estrogenized hosts) at 2

Table 3

Effect of estrogen antagonists in vivo on the activity of thymidine and dTMP kinases in dependent adrenocortical carcinomas

Enzyme assays were done on tumors from estrone pellet-treated hosts given the treatment indicated. Animals that were given injections of 0.9% NaCl solution served as controls for U-11,100 A-treated rats. Controls for testosterone-treated hosts were given no treatment. The 0.9% NaCl solution or U-11,100 A was administered s.c.

Treatment of host	Length of treatment (days)	Relative specific activity (% of growing control) of	
		Thymidine kinase	dTMP kinase
None, or 0.9% NaCl solution injection		100	100
U-11,100A			
0.75 mg/kg daily	7	98	100
2.0 mg/kg daily	7	49	50
Testosterone pelleted	1	59	ND[a]
	4	58	ND
	7	28	19
	14	37	30
	19	37	18
	23	28	40

[a] ND, not determined.

Table 4

Effects of replacement of estrogen by prolactin on the activity of thymidine and dTMP kinases in dependent mammary and adrenocortical carcinomas

Activity of the kinases was determined on tumors from animals treated as shown in the table. Growing control tumors were obtained from 0.9% NaCl solution-treated, estrone pellet-treated animals. The effects of estrone removal were determined with tumors from depelleted, 0.9% NaCl solution-treated animals, and the effects of prolactin were measured with tumors from depelleted, prolactin-treated hosts. Estrone was removed from the appropriate groups of animals at 9 to 10 a.m., and all treatments were begun at 4 p.m. on the same day. The results were obtained from 2 experiments, each involving 3 to 5 animals/group.

Type of tumor	Treatment of host	Relative specific activity (% of growing control) of	
		Thymidine kinase	dTMP kinase
Mammary[a]	Estrone present + 0.9% NaCl solution	100	100
	Estrone removed 4 days + 0.9% NaCl solution	3	6
	Estrone removed, 4 days + prolactin[b]	88	83
Adrenal	Estrone present, + 0.9% NaCl solution	100	100
	Estrone removed, 2 days + 0.9% NaCl solution	38	49
	Estrone removed, 2 days + prolactin[b]	73	82

[a] Female hosts were used for this experiment.
[b] Animals received 3 mg/day s.c.

days, but they decreased to the level of regressing tumors (depelleted hosts) after 7 days. These results duplicate the effects of ACTH alone and suggest that ACTH actively reverses the positive effect of prolactin on tumor growth.

DISCUSSION

These experimental results demonstrate the validity of using the activities of the thymidine and thymidylate kinases as indicators of the effect of hormone manipulation on tumor growth. In the dependent tumors, a significant decrease in activity of the enzymes occurred at very short intervals after removal of the estrogen supply from the host. Since similar effects were not observed in autonomous tumors in response to the same hormonal changes in the host, the enzyme assays provide accurately measurable parameters of growth that reflect the biological properties of the tumors.

That estrogen functions in maintaining the growth of the dependent tumors is shown by the effects of its removal on the activities of the 2 kinases and by the decrease in their activities in dependent adrenal tumors following treatment of

Table 5

Effects of estrogen withdrawal and replacement with prolactin on activity of kinases of dependent adrenal tumors in castrated male hosts

Activity of the kinases was determined in tumors from castrated male animals given the treatment indicated. All treatments were given for 7 days.

Treatment of host	Relative specific activity (% of growing control) of	
	Thymidine kinase	dTMP kinase
Estrone present + 0.9% NaCl solution	100	100
Estrone removed, 7 days + 0.9% NaCl solution	27	32
Estrone removed, 7 days + prolactin[a]	117	78

[a] Each animal received 3 mg prolactin daily s.c.

Table 6

Effects of replacement of estrogen by ACTH and/or prolactin on activity of thymidine and dTMP kinases of dependent adrenocortical carcinomas

Kinase assays were done on tumors from host rats treated as shown in the table for 2 or 7 days. The results were obtained from 2 or 3 experiments.

Treatment of host[a]	Relative specific activity (% of growing control) of	
	Thymidine kinase	dTMP kinase
Estrone present + 0.9% NaCl solution	100	100
Estrone removed, 2 days + 0.9% NaCl solution	59	58
Estrone removed, 2 days + ACTH[b]	239	278
Estrone removed, 2 days + prolactin[c]	109	102
Estrone removed, 2 days + prolactin + ACTH[d]	207	147
Estrone removed, 7 days + 0.9% NaCl solution	40	11
Estrone removed, 7 days + ACTH[b]	23	20
Estrone removed, 7 days + prolactin[c]	140	110
Estrone removed, 7 days + prolactin + ACTH[d]	31	39

[a] Refers to presence or absence of estrone pellet and to treatment given animals daily during the course of the experiment.
[b] Each animal received 10 i.u./day s.c.
[c] Each animal received 3 mg prolactin per day s.c.
[d] Each animal received 10 i.u. ACTH and 3 mg prolactin per day s.c.

the host with antiestrogenic compounds. Although the mechanism of testosterone is unknown, it is considered to be antiestrogenic because it has been used successfully in treating some human breast cancers (10) and can inhibit the initiation of mammary carcinomas in rats (4). However, a direct competition of testosterone for estrogen-binding sites in the tumors need not be implicated in the opposing effects of the hormones on their growth. Testosterone may produce its effect by altering pituitary function, and for this reason it has been called antiestrogenic, rather than an estrogen antagonist. The estrogen antagonist U-11,100 A competed with estrogen for binding sites in target tissues (9) and decreased the growth rate of 7,12-dimethylbenzanthracene-induced mammary tumors in rats (21). However, our results do not specify that estrogen acts directly on the dependent tumors in maintaining their growth. Estrogen is bound to pituitary tissue (6, 12, 20) and can influence the synthesis and secretion of pituitary hormones (3, 5, 11, 14) which, in turn, could act directly on the tumors. The 7,12-dimethylbenzanthracene-induced, estrogen-dependent mammary tumors (8) constitute a similar biological system. Although these tumors contain specific estrogen-receptor proteins (16), it has been evident for some time that the pituitary plays a role in mediating the effects of estrogen. Growth of the tumors could not be supported by estrogen in hypophysectomized hosts (18, 19), and regression of the tumors in response to treatment of the host with antibodies to prolactin has recently been demonstrated (2).

The results presented in this paper indicate that prolactin is active in maintaining the growth of the estrogen-induced tumors. Maintenance of the activity of the kinases in tumors by injection of prolactin into hosts lacking a supply of estrogen suggests that the pituitary hormone acts directly on the tumor and, in fact, seems to affect the activity of at least part of the DNA-synthetic process. The results imply that prolactin, increased in concentration due to the chronic administration of estrogen, is involved in the maintenance of growth of these estrogen-induced and -dependent carcinomas. Experiments are in progress to investigate the possible role of prolactin in promoting the growth of other sublines of the estrogen-induced carcinomas. Although the work described here does not provide a definitive answer, it is possible that prolactin is also involved in the induction of the tumors.

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