Glandular Kallikrein in Estrogen-induced Pituitary Tumors: Time Course of Induction and Correlation with Prolactin

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ABSTRACT

Glandular kallikrein (a trypsin-like serine protease) is an estrogen-induced and dopamine-repressed protein in the rat anterior pituitary which appears to be associated with lactotrophs. This study examined glandular kallikrein levels in diethylstilbestrol (DES)-induced pituitary tumors in F344 rats and compared it to plasma and pituitary prolactin, and pituitary wet weight. Ovariectomized F344 rats were implanted with Silastic tubes containing 0 or 5 mg DES for 1, 3, 5, 7, or 9 weeks. Glandular kallikrein was measured by microenzymatic assay using D-valylleucylarginyl-p-nitroanilide following trypsin treatment of extracts to activate latent forms of glandular kallikrein. Prolactin was measured by radioimmunoassay. DES induced steady time-dependent increases in pituitary wet weight with 7- and 16-fold increases observed by 5 and 9 weeks, respectively. Growth rates averaged 11.4 mg/week during the first 5 weeks of DES exposure, and then increased to 23.2 mg/week between weeks 5 and 9. Glandular kallikrein total activity (nmol/min/pituitary) increased 130- and 240-fold after 3 and 5 weeks of DES exposure respectively, and then abruptly plateaued. The specific activity (nmol/min/mg protein) of glandular kallikrein peaked at 3–5 weeks (36-fold increase compared to controls) and then declined as pituitary protein but not glandular kallikrein continued to increase. Total pituitary prolactin constantly rose during DES exposure with 12- and 26-fold increases after 5 and 9 weeks, respectively. Plasma prolactin levels also continuously rose during exposure to DES with 130- and 290-fold increases after 5 and 9 weeks, respectively. No major strain differences were found with regard to sensitivity to the acute effects of estrogen or dopaminergic stimulation on glandular kallikrein induction. DES-induced pituitary tumors in F344 rats are well known to arise via lactotroph proliferation, and the striking elevation in glandular kallikrein and prolactin during the early phases of tumor growth provide further support for a localization of glandular kallikrein in lactotrophs. However, the abrupt stabilization in glandular kallikrein levels by week 5 was unexpected and may signal a biochemical transformation of the tissue during tumor progression.

INTRODUCTION

Glandular kallikrein is a trypsin-like serine protease characterized by an ability to generate kinins (potent vasodilatory peptides) from kininogens (plasma glycoprotein precursors). Although classically postulated to function in local blood flow regulation via kinin generation, increasing evidence has suggested a role in the processing of precursors to diverse biologically active molecules (for review see Refs. 1 and 2). In addition, other enzymes which are closely related to glandular kallikrein appear to be involved in the processing of nerve growth factor and epidermal growth factor from their respective precursors (3).

Glandular kallikrein has recently been discovered in the intermediate and anterior lobes of the rat pituitary (4–8). In the anterior pituitary, glandular kallikrein is a major estrogen-induced protein with levels about 20 times higher in females than in males (5, 6). Glandular kallikrein mRNA levels are also subject to estrogen regulation in the anterior lobe, indicating that the increased enzyme levels are due to increased gene expression (9, 10). The dynamics of the estrogen induction of glandular kallikrein enzyme activity is consistent with such a mechanism (11).

The estrogen induction of glandular kallikrein is inhibited by dopaminergic neuroendocrine systems (12). The dual regulation of anterior pituitary glandular kallikrein by estrogen and dopamine uniquely parallels the regulation of prolactin. This has suggested that glandular kallikrein may originate from lactotrophs (prolactin-containing cells). Indeed, we reported that glandular kallikrein levels are markedly elevated in pituitary tumors induced in F344 rats by DES2 implants (13). These pituitary tumors arise from lactotroph proliferation and are widely used models for the study of pituitary hyperplasia and neoplasia. The purpose of the present study was to examine the time course of glandular kallikrein induction during development of estrogen-induced pituitary tumors and to correlate it with the induction of prolactin. In addition, the F344 rat is uniquely sensitive to pituitary tumor induction by estrogens. Thus, we also compared the F344 rat with tumor-resistant rat strains to determine whether strain differences in pituitary tumor susceptibility may be related to altered hormonal and neuroendocrine regulation of glandular kallikrein.

MATERIALS AND METHODS

Animal and Treatment Protocols. Weanling female F344 rats (CDF strain) were purchased from Charles River Laboratories (Wilmington, MA). All rats were ovariectomized at 23 days of age and were implanted with Silastic tubes containing 0 or 5 mg DES at the time of ovariectomy or 2, 4, 6, or 8 weeks later. All rats were killed 9 weeks after ovariectomy to give age-matched groups that had been exposed to DES for 0, 1, 3, 5, 7, or 9 weeks (6–7 rats per group). Implants were prepared as described by Wiklund et al. (14), and were massaged periodically (moved under the skin) to prevent encapsulation by connective tissue. Such encapsulation may limit DES diffusion.

In a second experimental set, different rat strains were compared with regard to their sensitivity to the estrogen induction and dopaminergic modulation of anterior pituitary glandular kallikrein. Female F344 (CDF strain), Sprague-Dawley (CD strain), and Holtzman (CDH strain) rats (Charles River Breeding Laboratories), 60–70 days old were ovariectomized 2 weeks prior to drug treatment. For the estrogen-sensitivity experiment, estradiol benzoate was administered to each of the three rat strains at doses of 1.0 or 10 µg/rat (0.1-ml volumes) every 48 h for 10 days (5 injections). In addition, rats also received bromocriptine (Sigma Chemical Co., St. Louis, MO) at a dose of 0.1, or 6 mg/kg twice daily for the 10-day dosing period (5–6 rats/group). Estradiol benzoate was dissolved in benzyl alcohol, diluted in sesame oil, and injected s.c. in 0.1-ml volumes/100 g body weight. Bromocriptine was dissolved in a vehicle composed of 10% ethanol, 0.8 HIMHC1, and 1% benzyl alcohol, and injected i.p. in volumes of 0.1 ml/100 g body weight.

Tissue Preparation. Rats were anesthetized with sodium pentobarbi-
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Time Course of DES Effects on Pituitary Wet Weight, Protein Content, and Macroscopic Appearance. The time course for the DES induction of pituitary wet weight and total pituitary protein with significant increases (63 and 60%, respectively) noted by 1 week of DES exposure. Pituitary wet weight exhibited 7- and 16-fold increases by 5 and 9 weeks, respectively, as compared to the control. Total pituitary protein exhibited 7- and 17-fold increases at 5 and 9 weeks, respectively. Pituitary growth rates markedly accelerated during the latter period of tumor growth. Thus, growth rates averaged 11.4 mg/week during the first 5 weeks of DES exposure, and then increased to 23.2 mg/week between weeks 5 and 9 of DES exposure.

After week 5, pituitary protein content rose at a slightly faster pace than pituitary wet weight. This probably results from the transition of the pituitary to a hemorrhagic state (see below), in which small amounts of blood protein were trapped in the tissue and escaped removal by the perfusion technique.

Other than an increase in size, pituitaries had a normal macroscopic appearance through the first 3 weeks of DES exposure. The tissue retained its characteristic shape and its color was ivory white, indicative of complete removal of blood from the tissue by the perfusion technique. Also, there was little evidence of compression of the medial basal hypothalamus by the pituitary mass. However, by week 5 the macroscopic appearance of the pituitary was extremely abnormal. The tissue was hemorrhagic and upon dissection cords of freshly clotted blood were present. The shape of the normal pituitary was lost and was replaced by a large gelatinous mass of variable shape. The medial basal hypothalamus was noticeably indented, indicating compression by the tumor mass. However, the pituitary remained within its capsule and no infiltration of adjacent tissues by the tumor was evident. This description is concordant with the observations of others regarding estrogen-induced pituitary tumors in the rat (14, 17–19).

Time Course of DES Effects on Glandular Kallikrein and Prolactin. Fig. 2 compares the time course of DES induction of glandular kallikrein total activity (nmol/min/pituitary) to total pituitary prolactin (μg PRL-RP-3/pituitary) and plasma prolactin (μg PRL-RP-3/ml). All three measures were significantly higher than controls as early as 1 week following exposure to DES. Glandular kallikrein total activity increased 130- and 240-fold relative to control after 3 and 5 weeks of DES exposure, respectively, and then abruptly plateaued. In contrast, total pituitary prolactin and plasma prolactin constantly rose during exposure to DES. Total pituitary prolactin exhibited 12- and 26-fold increases after 5 and 9 weeks, respectively. Plasma prolactin levels exhibited 130- and 290-fold elevations after 5 and 9 weeks, respectively. It was noted that the rate of increase in pituitary prolactin markedly accelerated during the latter stages of tumor growth in synchrony with increases in tumor mass. Thus, following an initial induction phase during the first week of DES exposure (+162.9 μg), prolactin increases averaged 41.6 μg/week between weeks 1 and 4, and then accelerated to 97.1 μg/week between weeks 5 and 9.

The specific activity of glandular kallikrein (nmol/min/mg protein) peaked at 3–5 weeks, with a 36-fold elevation observed (as compared to control), and then declined 59% between weeks 5 and 9 (Fig. 3). The decline in glandular kallikrein specific activity is due to the continued increase of total pituitary protein (Fig. 1), despite the leveling off of total glandular kallikrein content following 5 weeks of DES exposure (Fig. 2). In contrast to glandular kallikrein, the concentration of pituitary prolactin peaked following 1 week of exposure to DES, with a 4-fold increase, and then sharply declined between weeks 1 and 5.

Fig. 1. Time-course comparison of pituitary wet weight and total pituitary protein of 12-week-old ovariectomized F344 rats exposed to 5 mg DES for 0, 1, 3, 5, 7, or 9 weeks. Points, mean of determinations on 6–7 pituitaries; bars, SE. * P < 0.01 versus control; ** P < 0.05 versus control.

Fig. 2. Comparison of time course of DES induction of glandular kallikrein total activity (nmol/min/pituitary) to total pituitary prolactin (μg PRL-RP-3/pituitary) and plasma prolactin (μg PRL-RP-3/ml). All three measures were significantly higher than controls as early as 1 week following exposure to DES. Glandular kallikrein total activity increased 130- and 240-fold relative to control after 3 and 5 weeks of DES exposure, respectively, and then abruptly plateaued. In contrast, total pituitary prolactin and plasma prolactin constantly rose during exposure to DES. Total pituitary prolactin exhibited 12- and 26-fold increases after 5 and 9 weeks, respectively. Plasma prolactin levels exhibited 130- and 290-fold elevations after 5 and 9 weeks, respectively. It was noted that the rate of increase in pituitary prolactin markedly accelerated during the latter stages of tumor growth in synchrony with increases in tumor mass. Thus, following an initial induction phase during the first week of DES exposure (+162.9 μg), prolactin increases averaged 41.6 μg/week between weeks 1 and 4, and then accelerated to 97.1 μg/week between weeks 5 and 9.

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Fig. 2. Time-course comparison of total glandular kallikrein activity, total pituitary prolactin, and plasma prolactin in pituitaries of ovariectomized F344 rats exposed to 5 mg DES for 0, 1, 3, 5, 7, or 9 weeks. Pituitary homogenates were trypsin activated and assayed for kallikrein activity (S2266 hydrolysis). Total pituitary prolactin and plasma prolactin were measured by radioimmunoassay. Points, mean of determinations on 6-7 pituitaries; bars, SE. * P < 0.01 versus control.

Fig. 3. Time-course comparison of glandular kallikrein specific activity and prolactin concentration in pituitaries of ovariectomized F344 rats exposed to 5 mg DES for 0, 1, 3, 5, 7, or 9 weeks. Pituitary homogenates were trypsin activated and assayed for kallikrein activity (S2266 hydrolysis). Pituitary prolactin concentration was measured by radioimmunoassay. Points, mean of determinations on 6-7 pituitaries; bars, SE. * P < 0.01 versus control; ** P < 0.01 versus all other groups.

(-56%) and stabilized between weeks 5 and 9 at levels 50% above controls (Fig. 3).

Immunoprecipitation analyses of glandular kallikrein in 5- and 9-week tumors were performed to determine if tumor progression alters the ratio of immunological activity to enzymatic activity. Fig. 4 illustrates results of immunoprecipitation analysis of identical amounts of glandular kallikrein activity of a 5-week pituitary tumor, 9-week pituitary tumor, and rat urine (as a standard) following incubation with various dilutions of antiglandular kallikrein antiserum. Immunoprecipitation curves for glandular kallikrein from the three sources were indistinguishable. This indicates that the measured activity was due to immunoreactive glandular kallikrein, and that glandular kallikrein in 5- and 9-week tumors did not differ in their ratio of immunological activity to enzymatic activity. Nonimmune serum had no effect on glandular kallikrein activity. Also, extracts of 5- and 9-week tumors were mixed and incubated together to determine if 9-week tumors contain kallikrein inhibitors which reduce 5-week tumor glandular kallikrein activity. Glandular kallikrein activity of 5-week tumor extracts was not inhibited by 9-week tumor extracts (data not shown). This indicates that the decline in glandular kallikrein specific activity during the latter stages of tumor growth is not due to the presence of kallikrein inhibitors.

Comparison of Different Rat Strains for Sensitivity to Estrogen Induction of Glandular Kallikrein and Its Dopaminergic Modulation. Fig. 5 (left) compares the dose-response curves for the estrogen induction of anterior pituitary glandular kallikrein in F344, Sprague-Dawley, and Holtzman rats over a 10-day dosing period. Estradiol benzoate produced dose-dependent increases in glandular kallikrein activity for all strains. Although the F344 strain exhibited a slightly greater sensitivity to the 1-µg dose of estradiol benzoate, no strain differences were found with regard to sensitivity to the 10-µg dose of estrogen. Dopamine receptor stimulation with bromocriptine markedly attenuated glandular kallikrein activity in both F344 and Sprague-Dawley rats treated with estrogen (Fig. 5, right), with both strains demonstrating similar sensitivities to bromocriptine. The slightly greater sensitivity of the F344 rat to the lower dose of estrogen (adjusted for body weight differences in this experiment) was again evident.

DISCUSSION

We have previously established that glandular kallikrein in the female rat anterior pituitary is under powerful stimulatory

Fig. 4. Immunoprecipitation of glandular kallikrein of a 5-week pituitary tumor, 9-week pituitary tumor, and rat urine with antiserum against rat urinary kallikrein. Tissue extracts or diluted urine containing identical glandular kallikrein activities (200–210 pmol/min/50 µl) were incubated with various dilutions of antiserum or nonimmune serum. Immune complexes were precipitated with a protein A suspension and the supernatant was assayed for kallikrein activity (S2266 hydrolysis).

Fig. 5. (left) compares the dose-response curves for the estrogen induction of anterior pituitary glandular kallikrein in F344, Sprague-Dawley, and Holtzman rats over a 10-day dosing period. Estradiol benzoate produced dose-dependent increases in glandular kallikrein activity for all strains. Although the F344 strain exhibited a slightly greater sensitivity to the 1-µg dose of estradiol benzoate, no strain differences were found with regard to sensitivity to the 10-µg dose of estrogen. Dopamine receptor stimulation with bromocriptine markedly attenuated glandular kallikrein activity in both F344 and Sprague-Dawley rats treated with estrogen (Fig. 5, right), with both strains demonstrating similar sensitivities to bromocriptine. The slightly greater sensitivity of the F344 rat to the lower dose of estrogen (adjusted for body weight differences in this experiment) was again evident.

DISCUSSION

We have previously established that glandular kallikrein in the female rat anterior pituitary is under powerful stimulatory
regulation by ovarian estrogens and that this induction is subject to inhibitory modulation by dopaminergic neuroendocrine systems (5, 6, 12). This regulation uniquely parallels that of prolactin (20), and has suggested that glandular kallikrein may be coordinately expressed and regulated with prolactin in lactotrophs. DES-induced pituitary tumors in F344 rats are well known to arise from lactotroph proliferation on the basis of elevated prolactin content and biosynthesis, morphological features of the tumor cells, and the preponderance of cells immunocytochemically staining for prolactin (14, 17, 19, 21, 22).

Thus, our laboratory has used this tumor model as a simple physiological test for the hypothesis that anterior pituitary glandular kallikrein arises from lactotrophs. In a preliminary communication we reported markedly elevated levels of glandular kallikrein in DES-induced pituitary tumors (13), evidence which clearly supported a localization in lactotrophs. In particular, 7-fold increases in pituitary weight and protein content were associated with 250-fold increases in glandular kallikrein content, 12-fold increases in pituitary prolactin content, and 130-fold increases in plasma prolactin levels. Glandular kallikrein increases of such magnitude would only be expected if it was being expressed in lactotrophs. At this juncture, it should be noted that glandular kallikrein has recently been reported in prolactin-producing GH1 cells (23), and that recent immunocytochemical studies have also localized glandular kallikrein in lactotrophs but not in other cells of the rat anterior pituitary. Thus, multiple independent approaches uniformly point to the lactotroph as the cell of origin of glandular kallikrein in the rat anterior pituitary.

The present study also yielded an unexpected and intriguing observation: glandular kallikrein and prolactin levels markedly diverged during the latter stages of growth of the DES-induced pituitary tumors. During the first 5 weeks of tumor growth (up to 7-fold increases in pituitary weight), increases in glandular kallikrein were generally paralleled by increases in prolactin. However, glandular kallikrein levels abruptly plateaued at week 5 and were stable thereafter, whereas tumor growth rates and increases in pituitary prolactin accelerated. Thus, some type of biochemical transformation appeared to occur in the tissue at the stage of tumor progression marked by an accelerated growth rate. This observation may signal a neoplastic transformation of the tissue, and should provide a useful biochemical marker for further studies aimed at elucidating the mechanisms and consequences of this transformation.

The stabilization of glandular kallikrein levels at week 5 is unlikely to reflect a termination of lactotroph proliferation, since tumor mass and prolactin levels continued to increase. Moreover, others have reported that the latter stages of tumor growth continue to be associated with increases in DNA content (14), an elevated rate of DNA synthesis (17, 24), as well as increases in total cell number and the percentage of lactotrophs (21). One explanation for the stabilization of glandular kallikrein levels after 5 weeks of DES exposure is the selective proliferation of lactotrophs with low or no glandular kallikrein content during the latter periods of tumor growth. It is possible that lactotrophs expressing glandular kallikrein do not themselves proliferate but arise from lactotrophs not expressing glandular kallikrein through an estrogen-dependent differentiation pathway. This differentiation may be lost following neoplastic transformation. This hypothesis would explain the stability of glandular kallikrein levels during the latter stages of tumor growth, and would also explain the more rapid growth rate of the tumor following transformation (lactotrophs no longer differentiating into a nonproliferating population).

Comparison of glandular kallikrein levels from the 8-week DES-induced tumors previously reported (13), and the tumors reported in the present study, suggest that stabilization of glandular kallikrein content may not be strictly dependent on time of exposure to estrogen. Rather, it may be more related to tumor size. In both studies, nearly identical elevations of glandular kallikrein total activity (220- to 240-fold increases) and specific activity (32- to 36-fold increases) were found at similar tumor sizes (65.8–67.2 ng) despite different times of exposure to DES (5 versus 8 weeks). The reason for the different growth rates in the two studies is unclear.

Two other events first became notable after 5 weeks of DES exposure. First, the pituitaries became grossly hemorrhagic. The hemorrhagic nature of estrogen-induced pituitary tumors has been frequently noted (14, 17–19, 25) and appears to result from tumor growth which disrupts the parenchymal-vascular interface, causing leakage of blood into extravascular spaces. Second, by week 5 the medial basal hypothalamus was severely compressed by the growing tumor mass. Either of these events could interfere with neuroendocrine and hormonal control mechanisms required for the normal growth and differentiation of the pituitary.

The marked elevation in the glandular kallikrein content of DES-induced pituitary tumors raised the possibility that glandular kallikrein may be causally linked in some fashion to the increased tumor susceptibility of F344 rats. Comparison of the tumor-sensitive F344 strain with the tumor-resistant Sprague-Dawley and Holtzman strains revealed no major differences with regard to the ability of estrogen to induce glandular kallikrein, or of bromocriptine to modulate such inductions during...
acute exposures. Thus, the propensity of F344 rats to develop tumors appears unlikely to involve primary alterations in the mechanisms underlying the estrogen induction of glandular kallikrein or its neuroendocrine modulation. Rather, glandular kallikrein levels appear to be secondarily affected by a biochemical transformation of the tissue arising during tumor development.

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