Direct arterial vascularization of estrogen-induced prolactin-secreting anterior pituitary tumors

(microsphere/arteriogenesis/vascular cast/hypophyseal portal blood supply)

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ABSTRACT The rat anterior pituitary gland (AP) receives all of its blood supply via the hypophyseal portal circulation. We now report, in rats with estradiol (E_2)-induced prolactin-secreting tumors, that newly formed arteries directly supply the AP and that this arteriogenesis is closely correlated with the sensitivity of two strains of rats to the tumorigenic action of E_2. Fischer 344 rats, a strain extremely sensitive to E_2, and Sprague-Dawley rats, a less sensitive strain, were ovariectomized and implanted with E_2-filled or empty Silastic capsules. Ten to 63 days later, microspheres (15 μm) were injected into the heart. Normally microspheres do not reach the AP because they are trapped in the primary portal capillary plexus. Some animals were also perfused with vascular cast material. In Fischer rats, after 63 days of E_2, the pituitary weight, serum prolactin, and number of microspheres in the AP were 5-, 42-, and 18-fold greater than control values, respectively. The same parameters in E_2-treated Sprague-Dawley rats were 2-, 27-, and 7-fold greater than control values. Vascular casts from E_2-treated Fischer rats revealed numerous arteries entering the AP. No arteries to the AP were observed in Sprague-Dawley controls. These results show that E_2-induced tumorigenesis of the AP is associated with the development of a direct arterial blood supply. We hypothesize that the regions supplied by these new arteries would receive systemic blood containing subphysiological concentrations of dopamine. The loss of dopaminergic inhibition in concert with E_2 stimulation may lead to tumor formation.

Prolactin-secreting pituitary tumors can be induced in rats by prolonged treatment with estrogen (1–3). The size of the anterior pituitary gland (AP) of estrogen-treated animals is markedly increased, which is associated with increased mitotic activity (4). The primary cells affected are prolactin (PRL)-producing cells, lactotrophs. Estrogen stimulates both PRL synthesis and proliferation of lactotrophs (4–7). Different strains of rats have different susceptibilities to the tumorigenic action of estrogen, with the Fischer 344 strain being particularly sensitive (8, 9).

The mechanisms by which estrogen mediates tumor induction most likely include direct effects on lactotrophs, as well as indirect effects via alterations in the hypothalamic regulation of lactotrophs. PRL secretion (10) and synthesis (11) have been shown to be tonically inhibited by dopamine produced in the tuberoinfundibular neurons. Dopamine also appears to be involved in the control of the cell division of lactotrophs. Destruction of the tuberoinfundibular neurons results in an increased density of lactotrophs (12) and the blockade of dopamine receptors with antagonists increases DNA synthesis and the mitotic index of the AP (13). These findings have led us and others (14) to hypothesize that tumor development could involve escape of the AP from hypothalamic dopamine regulation.

Dopamine is released from hypothalamic neurons into the hypophyseal portal vascular plexus. The volume of the portal plexus is small, maintaining a physiologically active concentration of dopamine in the AP (15). The AP of the rat receives its blood supply exclusively via the portal circulation (16), thereby maintaining a tonic inhibition of PRL secretion. If a region of the AP were to receive a direct arterial blood supply, lactotrophs supplied by that artery would escape hypothalamic regulation because the level of dopamine in systemic blood is not sufficient to suppress PRL secretion (15). The tumorigenic action of estrogen could involve vascularization of the AP by arteries (arteriogenesis) and the resultant escape of that region from dopaminergic regulation. Changes in the blood supply—i.e., vascularization of solid tumors—have been well established as a sequela in tumorigenesis (17). However, these changes usually represent a proliferation of the existing blood supply (angiogenesis).

We (18) and others (19) have shown that microspheres (15 μm) injected into the left ventricle of the heart do not reach the AP because they are too large to pass through the primary portal capillaries in the median eminence and posterior pituitary gland. If a region of the AP were to receive a direct arterial blood supply, microspheres should now reach that region (Fig. 1). In the present study, we have demonstrated, using microspheres alone and in combination with a vascular cast technique, that the sensitivity of Fischer 344 and Sprague-Dawley rats to the tumorigenic action of estradiol is correlated with its ability to induce a direct arterial blood supply to the AP.

MATERIALS AND METHODS

Animals. Sprague-Dawley (S–D) and Fischer 344 (F344) rats were obtained from Simonsen Laboratories (Gilroy, CA) and Harlan Laboratories (Madison, WI), respectively. Animals were housed in a controlled 14-hr light/10-hr dark photoperiod with food and water available ad lib. Rats (180–200 g) were bilaterally ovariectomized and implanted with 1-cm Silastic capsules (Dow; o.d., 0.125 inch; i.d., 0.062 inch) containing 17β-estradiol (E_2; Sigma) or left empty.

Microsphere Injection. After 10, 26, or 63 days rats were anesthetized with pentobarbital (42 mg/kg of body weight), the thoracic cavity was opened, and 0.5 ml of saline containing 2 million microspheres (15.3 $\pm$ 0.7 μm; 3M Company, St. Paul, MN) was injected into the left ventricle of the heart. After a few minutes, the right atrium was severed and the blood was collected. The blood was centrifuged (10 min, 1500 $\times$ g) and the serum was frozen for subsequent radioimmunoassay for PRL. After decapitation of the rat, the entire pituitary gland was removed and weighed. In some cases, the total pituitary gland was fixed for 72 hr in 0.1 M cacodylate buffer/2% paraformaldehyde/0.1% glutaraldehyde, pH 7.4, and then cleared in methyl salicylate (Fisher). These

Abbreviations: AP, anterior pituitary gland; PRL, prolactin; E_2, 17β-estradiol; S–D, Sprague-Dawley; F344, Fischer 344.
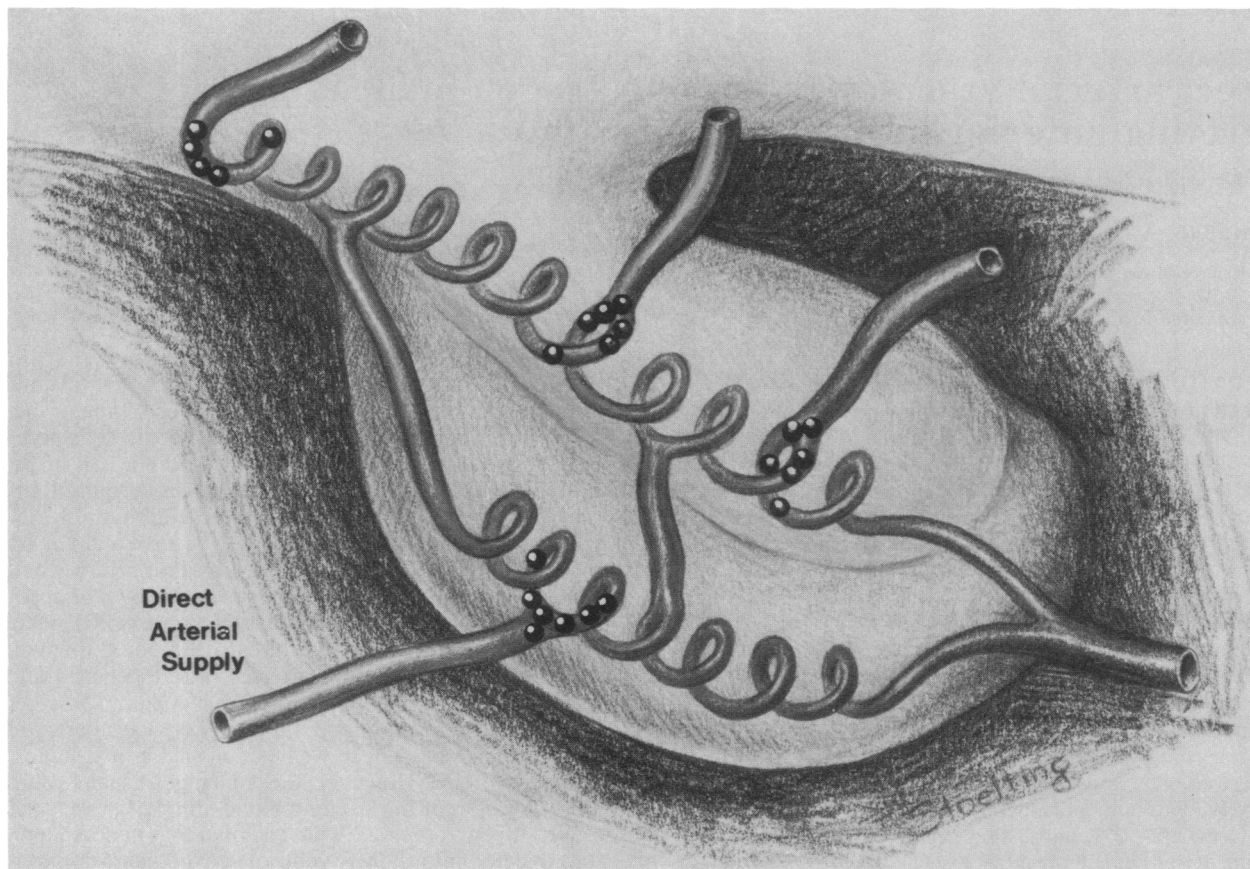
FIG. 1. Diagrammatic representation of the hypophyseal-portal blood supply of the rat. An aberrant artery is shown directly supplying the anterior pituitary. Microspheres are shown trapped in both the primary portal capillaries, as would normally occur, and the region of the anterior pituitary supplied by the aberrant vessel.

pituitary glands were transilluminated and the microspheres were counted with the aid of a stereomicroscope. In most cases, the posterior and anterior glands were separated and digested with 2 ml of ethanol/5 M NaOH (1:1) at 37°C. After centrifugation (10 min, 1500 × g), the pellet containing the microspheres was suspended in distilled H_2O and spread onto a glass slide, and the microspheres were counted with the aid of a light microscope.

Vascular Casts. To clarify the anatomy of the arteries by which microspheres enter the AP, we performed vascular cast experiments. Rats implanted with E_2-filled or empty Silastic capsules for 26 or 55 days were anesthetized with pentobarbital (42 mg/kg of body weight). The thoracic cavity was opened and 0.2 ml of heparin (1000 units/ml) was injected into the left ventricle of the heart. Animals were sequentially injected with microspheres and perfused with 20 ml of a silicone rubber injection compound (Microfil MV-112, Canton Bio-medical Products, Boulder, CO) directly into the ascending aorta at a pressure of 120–160 mm Hg (1 mm Hg = 133 Pa). The perfused rats were stored at 4°C for 24 hr to allow the silicone to solidify. The skull was dissected free of soft tissue, and the cranium was opened and immersed in 10% formalin for 24 hr. With the aid of a stereomicroscope, the AP was exposed to reveal the silicone in the vascular system. Photomicrographs were taken through a Nikon HFX dissecting microscope.

Radioimmunoassay. Serum prolactin concentrations were measured in 1-, 10-, or 100-μl aliquots by a double-antibody radioimmunoassay with materials and protocols provided by the Rat Pituitary Hormone Distribution Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Results are expressed in terms of NIADDK rat PRL RP-1.

Statistics. Statistical comparisons of data were carried out by one-way analysis of variance. Post hoc Newman–Keuls tests were then made, and P values < 0.05 were considered statistically significant (20).

RESULTS

In S–D rats, implantation of E_2-containing Silastic capsules for 10 days resulted in a significant increase ($P < 0.05$) in total pituitary weight and serum PRL concentration compared with rats implanted with empty capsules (Fig. 2A). The values from control rats given empty implants for all time periods were not significantly different ($P > 0.05$) and were combined. After 63 days of E_2 treatment, the pituitary weight was two times that of control animals and the serum PRL was >1500 ng/ml. The number of microspheres observed in the AP gland of control S–D rats was not significantly different ($P > 0.05$) from that of E_2-treated rats except after 26 days of E_2 treatment.

In E_2-treated F344 rats, the total pituitary gland weight was significantly increased by 10 days ($P < 0.05$) and it continued to increase at subsequent time intervals, reaching a size five times that of control animals after 63 days (Fig. 2B). The serum PRL from these animals was significantly increased ($P < 0.05$) by 10 days and at all subsequent time intervals. Higher numbers of microspheres were observed in the AP of control F344 rats (22 ± 12; $n = 13$) than were seen in the control S–D rats (12.8 ± 0.7, $n = 15$). Most APs from the control F344 rats contained very few microspheres, 11 APs had fewer than 16 microspheres while 2 glands contained 104 or 130 microspheres. After 10 days of E_2 treatment, there were more microspheres in the AP (56.6 ± 12; $n = 5$) but not until 26 days of E_2 treatment was there a dra-

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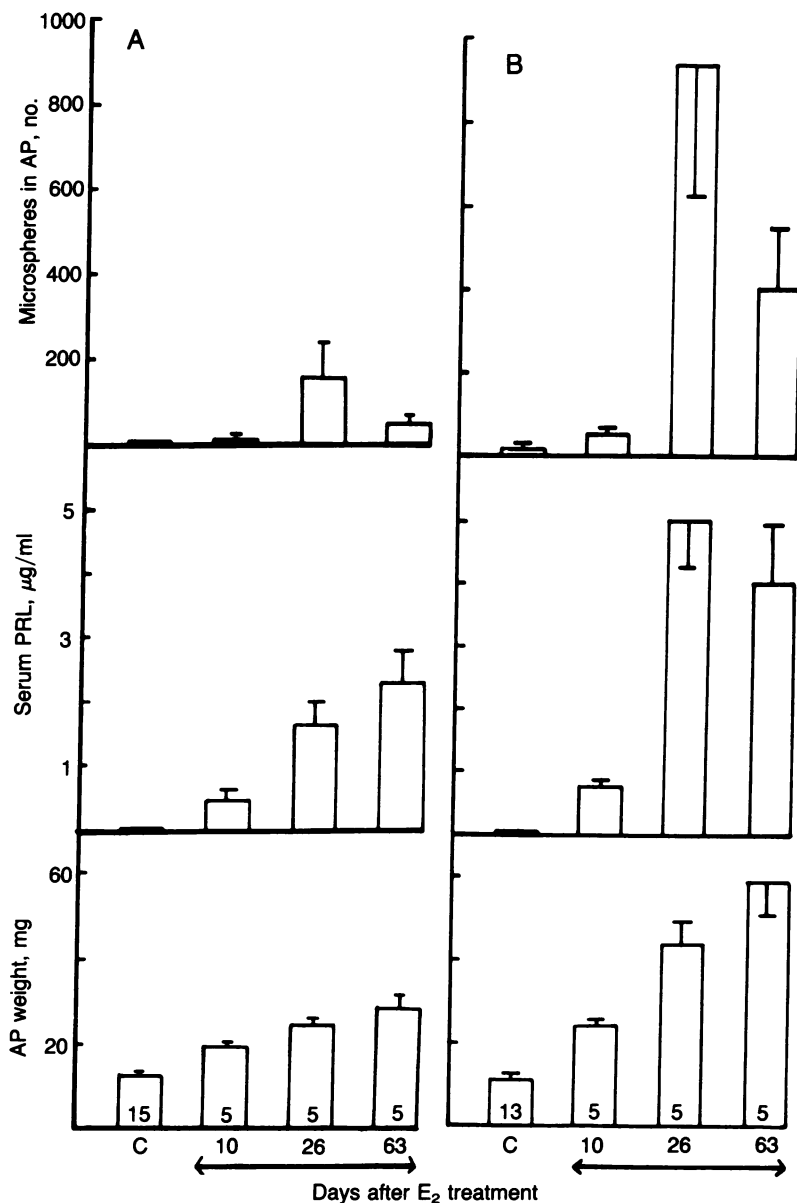*Proc. Natl. Acad. Sci. USA 81 (1984)* 4551

FIG. 2. AP weight, serum prolactin levels, and number of microspheres in the AP of ovariectomized S–D (A) and F344 (B) rats implanted with blank or E_2-filled Silastic capsules for various times. Results represent mean ± SEM; numbers in bars represent numbers of animals.

matic and significant ($P < 0.05$) increase in the number of microspheres (935 ± 307; $n = 5$). After 63 days of E_2 treatment, the number of microspheres in the AP gland was not significantly different from APs after 26 days of E_2 treatment.

The number of microspheres observed in the posterior pituitary gland of F344 rats implanted with blank (474 ± 72; $n = 4$) or E_2-containing capsules (400 ± 70; $n = 3$) for 63 days was not significantly different ($P > 0.05$).

To clarify that the microspheres were reaching the AP via newly formed arteries, we performed vascular cast experiments. Animals were sequentially injected with microspheres and perfused with microphil cast material. In control S–D rats, few if any microspheres were observed in the AP and no vessels were observed entering the gland (Fig. 3). Large numbers of microspheres can be seen in the median eminence near the terminal branches of the superior hypophyseal arteries. After 63 days of E_2, the APs of F344 rats were greatly enlarged and contained extensive areas of necrosis. Large numbers of microspheres were observed in the noninfarcted regions of the AP. Vessels filled with cast material could be observed entering the AP and contained microspheres in the vicinity of their terminal branches (Fig. 4).

Arteries were observed entering the AP from the dura. The bilateral dural vessels in this photomicrograph had their origin from the basilar artery. In other instances (not shown), dural vessels were shown to be branches of the internal carotid and posterior communicating arteries. In APs that were sectioned microspheres could be observed throughout the gland.

In one out of four F344 rats analyzed with the vascular cast technique, there were a few vessels filled with cast material that contained small numbers of microspheres. This is consistent with the data presented above that some of the APs of untreated F344 rats contain small numbers of microspheres.

DISCUSSION

These studies show the formation of new arteries (arteriogenesis) that directly enter the APs of rats with PRL-secreting tumors induced by E_2. The exclusive blood supply of the normal rat AP is via the hypophyseal portal venous system (16). Thus injection of microspheres into the left ventricle of the heart in control rats results in the presence of microspheres only in the median eminence and posterior pituitary
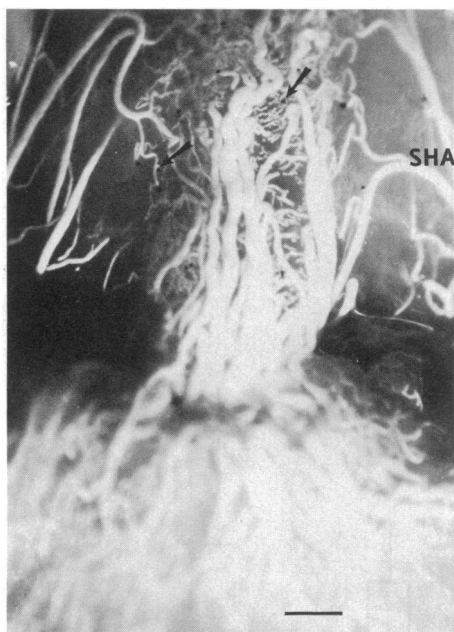
FIG. 3. Photomicrograph of the central view of a control S–D rat brain. Animals were injected sequentially with microspheres and microphil cast material. Microspheres (arrows) can be seen trapped in the median eminence in the vicinity of the small terminal branches of the superior hypophyseal artery (SHA). No microspheres can be seen in the anterior pituitary. (Bar = 100 μm; × 75.)

FIG. 4. Photomicrograph of the ventral surface of a female F344 rat treated with E_2 for 55 days. The dura (D) has been reflected. Arteries filled with cast material can be seen coursing from the dura to the AP. Microspheres (arrows) can be observed in the AP in the region of the fine branches of the arteries. In subsequent dissections the arteries supplying the dura were shown to be branches of the basilar artery. The dark appearance of extensive regions of the AP is the result of infarctions. (Bar = 100 μm; × 60).

gland because the microspheres are too large to transverse the capillaries and reach the AP. In rats with E_2-induced PRL-secreting tumors, arteries were shown to directly supply the AP by the observation that microspheres can now reach the AP and by direct visualization of arteries by vascular casts. The degree of arteriogenesis of the AP is in close agreement with the sensitivity that two strains of rats exhibit to the tumorigenic actions of E_2. In F344 rats, a strain very sensitive to the actions of E_2, pituitary weight increased 6-fold following 63 days of E_2 treatment accompanied by an 18-fold increase in the number of microspheres observed. In S–D rats, a less sensitive strain, the pituitary weight and microsphere number increased 2-fold and 7-fold, respectively. The numbers of microspheres counted in the posterior pituitary gland of E_2-treated or control rats were not significantly different, indicating no increase in the arterial blood supply to that area.

In all instances the new arteries to the AP coursed within the dura and penetrated the AP from its ventral and lateral aspects. These arteries were observed to originate from the basilar, internal carotid and posterior communicating arteries. It is also likely that the AP is supplied by arteries from additional origins—e.g., the inferior hypophyseal artery—since these vessels would be difficult to observe if they entered the AP from a dorsal aspect. These observations are consistent with an earlier report of dural vessels entering the AP of long-term estrogen-treated rats (21). In this study, India ink was used to visualize blood vessels, however, no comment was made as to the origin or directionality of blood flow in the dural vessels. In the present study, by sequential injection of microspheres and vascular cast material, we have shown that the cast-filled vessels entering the AP are arteries.

These data do not establish a cause-and-effect relationship between arteriogenesis of the AP and tumor formation. The changes in vascularization described would result in an escape from tonic inhibitory dopaminergic regulation. Removal of dopaminergic inhibition results in increased PRL secre-
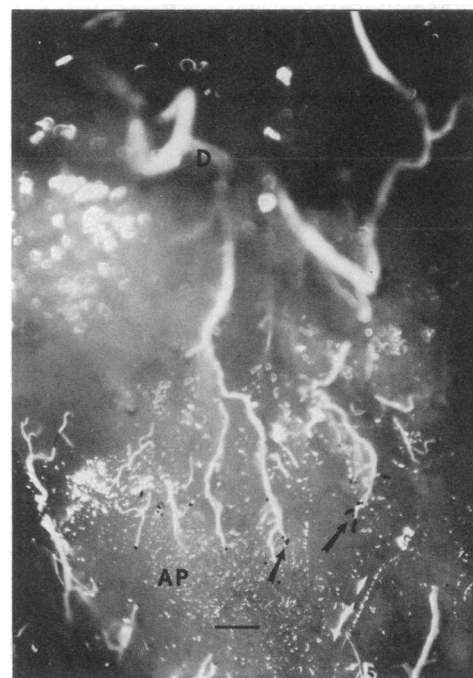
tion and mitotic activity of lactotrophs (13, 22). However, removal of dopamine inhibition by itself does not appear to result in tumor formation. In S–D rats with lesions of the dopamine-producing neurons, there is hyperplasia of lactotrophs but the size of the AP does not increase and no tumors are formed after 21 days (12). However, it is possible that removal of dopaminergic inhibition coupled with estrogenic stimulation would result in tumor induction.

Estrogen has multiple sites of action in the tumorigenic process. It should be noted that the method used for inducing tumors is nonphysiological because of the continuous unopposed high-estrogenic stimulus. Estrogen stimulates mitotic activity in the AP (13) either by direct action on the lactotrophs or via production of an estromedin (23). Damage to the tuberoinfundibular dopamine-producing neurons has been shown by a decrease in dopamine fluorescence in aged female rats with spontaneous PRL-secreting tumors as well as in young females given E_2 (24). In rats with chronic hyperprolactinemia induced by E_2 dopamine concentrations in the median eminence are significantly reduced while norepinephrine and serotonin concentrations are unchanged (25). This reduction in hypothalamic dopamine may be due to the hyperprolactinemia that develops, although other studies (26) implicate a direct action of E_2 on the brain. Therefore, arteriogenesis in concert with these other mechanisms may result in tumorigenesis.

Interestingly, the numbers of microspheres observed after 26 days of E_2 treatment appears to be greater than after 63 days of E_2 treatment although the pituitary is only two-thirds the size. In human AP tumors, Schechter (27) has shown a disruption of the tissue organization at the parenchymal–pericapillary interface as a result of tumor growth; this causes breakdown of the capillary walls and leads to the formation of extravasations and decreased velocity of the blood

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flow. In rats with pituitary tumors, the blood flow to the pituitary (blood flow/pituitary weight ratio) was reduced compared with controls (28). The hemorrhagic appearance of the long-term E_2-treated AP is well known (8, 21, 28). This could explain our results after 63 days of E_2 treatment. The AP has increased in size resulting in the formation of extravasations. The blood flow is decreased and fewer microspheres enter the gland.

In control F344 rats, almost all (85%) of the APs had fewer than 16 microspheres but two APs had more than 100 microspheres. One possible explanation for the latter is that the F344 strain is so sensitive to E_2 that endogenous E_2 has initiated tumorigenesis prior to ovariectomy in a small percentage of the animals. Another explanation is that a higher percentage of this strain have vascular anomalies.

The mechanism(s) involved in arteriogenesis is/are unknown; however, the process appears analogous to angiogenesis [formation of capillary sprouts with eventual development of a microcirculatory network (29)]. We have no evidence of a proliferation of the capillary plexus of the AP because our techniques show only the induction of a new and previously nonexistent arterial blood supply. However, it is logical to propose that the growth of arteries into the AP could be due to stimulation of a factor by E_2 that in turn stimulates the migration and proliferation of vascular endothelial cells. The growth of numerous solid tumors has been shown to be associated with production of angiogenic factors that act in this fashion (17, 30).

We speculate that a direct arterial supply may be partially responsible for human PRL-secreting adenomas. Possibly rather than induction of an arterial supply by E_2 these vessels may occur in a percentage of humans as a vascular anomaly. In postmortem studies, approximately 22.5% of all pituitaries have been shown to contain tumors (31). Most human PRL-secreting adenomas are small, focal, and slow growing (32). These observations are consistent with the existence of a vessel supplying one small region of the AP and thus allowing it to escape from dopaminergic inhibition. Patients with PRL-secreting adenomas do not show an increase in PRL following challenge with a dopamine antagonist, supporting the idea that the tumor has escaped from dopaminergic inhibition (33). Although the current study verifies the induction of a direct arterial blood supply to the AP in rats, it remains to be shown that vascular changes are involved in the etiology of human adenomas.

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