Modulation of Plasminogen Activator in Rodent Mammary Tumors by Hormones and Other Effectors

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ABSTRACT

The production of plasminogen activator (PA) and its regulation by hormones and other effectors were studied in organ cultures of primary rat and mouse mammary tumors. PA was quantitated using the radioiodinated fibrin plate method. The level of PA in tumor tissue was 10-100-fold higher than that in normal rat or mouse mammary glands; the rates of PA secretion were 10-1000-fold higher in the tumor cultures. PA production was stimulated by prolactin and pituitary extracts in N-nitrosomethylurea- and 7,12-dimethylbenz(a)anthracene-induced rat tumors but not in mammary tumor virus-induced mouse tumors; hydrocortisone inhibited PA production in all three tumor categories. Sex hormones and agents such as cholera toxin and retinoic acid effectively modulated enzyme production by some tumors.

Three major points of interest emerge from our findings: (a) the pattern of tumor PA response to hormones differs qualitatively and quantitatively from that previously determined for the normal mammary; (b) the profile of responses of tumor PA and tumor growth to hormones shows numerous correlations suggesting that these two parameters may be coordinately regulated; (c) pituitary extracts contain an apparently novel factor that stimulates rat mammary tumor PA synthesis.

INTRODUCTION

The mammary gland becomes a target of hormonal control at the earliest stages of its development in the embryo (8, 9, 17) and, so far as we know, all stages of postnatal mammary development are regulated and coordinated by complex, multiple hormonal interactions (see Ref. 40 for a comprehensive review). Recent work in this area has pointed to the likely existence of some hormonal "stop" and "go" signals. The results have also underlined the obligatory sequential nature of the developmental program in that only specific sequences of hormonal stimuli produce appropriate responses (40). The progress achieved thus far is due in good part to the possibility of studying the responses of intact glands in organ culture, a condition that permits the expression of complex physiological and developmental functions.

In the absence of a comparably useful experimental system, there has been no equivalent progress in defining and analyzing the patterns of mammary tumor responses to hormones. This lag is partly attributable to the difficulty of maintaining and/or propagating mammary tumor cells, especially those of human origin. A common approach has been to study the mitogenic effect of hormones on selected tumor-derived cell populations, especially permanent cell lines. These observations, while often of interest, are necessarily made in the absence of the epithelio-mesenchymal interactions that are known to be important for the behavior of tumors in general, and particularly so for normal and neoplastic mammary tissue. It is also difficult to extrapolate results obtained with cell lines, cloned from a single cell, to the behavior of intact tissue in vivo. In addition, given the wide range of phenotypic differences among primary tumors, it would be useful to develop an experimental system that permits the results of in vitro experiments on individual tumors to be referred to the original tumor-bearing host.

Relatively little effort has been directed toward the study of tumor characteristics not obviously or directly associated with growth. One such potentially useful function appears to be the production of PA by tumors since it is strongly associated with the neoplastic state. Most primary malignant tumors of human and animal origin contain and secrete elevated levels of PA (7, 12, 13, 22, 28, 30, 31, 34). PA production and its regulation by hormones have been tightly correlated with a variety of examples of normal tissue remodeling including ovulation (38), trophoblast implantation (39), and mammary involution (29) and the migration of normal cells such as hemopoietic stem cells (43) and inflammatory cells (44). More recently, direct evidence has been obtained that PA activity is essential for metastasis by a human tumor (HEP 3) in the chick embryo.3

Apart from these observations, there is an intriguing body of evidence which suggests that tumor growth and PA production may be under coordinate regulation, at least in some tumors. This was first suggested by Mak et al. (20) who demonstrated that, in the Shionogi murine mammary carcinoma, which is androgen dependent for growth, PA production in vitro was stimulated by androgens but not by estrogens. Their observations were confirmed by reports (5, 6) that in MCF-7 cells, which require both estrogen and progesterone for growth in nude mice, PA production in vitro was stimulated by estrogen and further potentiated by progesterone. Conversely, tumor growth and PA were modulated in parallel in a mouse melanoma, both being inhibited in cells exposed to bromodeoxyuridine (10).

The preceding considerations prompted us to use PA produc-

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tion as a sensitive probe with which to explore the hormonal
responses of mammary tumors, having the following aims in
mind: (a) to compare the hormonally regulated patterns of PA
synthesis that occur in neoplastic and normal mammary tissue,
being particularly attentive to those that might be characteristic
of either state; (b) to obtain a larger data base for testing the
scope of the coordinate modulation of tumor growth and PA
synthesis; and (c) to initiate a search for new hormones that
might regulate the properties of tumors. To acquire a normal
base line for the comparative studies, we first examined the
hormonal regulation of PA secretion in the normal lactating and
involuting rodent mammary gland (29). This work established a
putative role for PA in postpartum and post-lactation gland
involution, and it also defined the hormonally modulated patterns
of enzyme synthesis in several physiological states. PA produc-
tion precisely reflected the hormonal environment both in vivo
and in vitro, with enzyme content and secretion decreasing or
increasing in response to hormone combinations that permitted
lactation or involution, respectively.

This paper describes our initial studies on the hormonal mod-
ulation of PA secretion in mammary tumors. For comparison with
normal tissue we have thus far examined exclusively primary
rodent tumors, freshly explanted from the original host. We have
studied 2 models of hormone-responsive tumors induced by
NMU and DMBA in rats and one ostensibly hormone-independent
type induced by MTV in mice. Using an organ culture system
somewhat modified from that previously applied to normal tissue,
we have assayed the effects of the major mammotrophic hor-
mones, several hormone-like agents, and some tissue extracts.

MATERIALS AND METHODS

Chemicals and Reagents. Hormones were obtained as follows: bo-
vine insulin, 17β-estradiol, methionine-enkephalin, and hydrocortisone
from Sigma Chemical Co. (St. Louis, Mo.); 8-arginine vasopressin and chola toxin from
Schwarz/Mann (Orangeburg, N. Y.); 8-arginine vasotocin, thyrotropin-
releasing hormone, and luteinizing hormone-releasing hormone from
Bachem (Torrance, Calif.); oxytocin from Parke-Davis (Detroit, Mich.);
follicle-stimulating hormone from Collaborative Research (Walsham, Mass.);
prolactin, ovine growth hormone, ovine follicle-stimulating hor-

mone, ovine thyroid-stimulating hormone; human adrenocorticotropi
corticotropin hormone-(1-39), and human β-melanocyte-stimulating hormone-(1-22),
all gifts from the National Pituitary Agency, NIAMDD, NIH; ovine pituitary
powder from Phoenix Chemicals (Christchurch, New Zealand); highly
purified ovine luteinizing hormone and the residue from an acid-acetone
extraction of swine pituitary powder, gifts from Dr. D. Burleigh (ICN, Inc.,
Terre Haute, Ind.). Pituitary Extract A was prepared from this residue,
and Extract B was prepared from unfraccionated ovine pituitary powder.

Extracts were prepared as follows. Twenty mg of powder were added
to 1 ml of alkaline-0.9% NaCl solution (NaOH added to pH 10 to 11),
the suspension was homogenized and centrifuged at 15,000 rpm/5 min,
and the supernatant was sterilized by filtration (an aliquot assayed for protein)
and kept for no longer than 1 week at 4°C (pH 8 to 9) before use. Pituitary
polypeptide hormones were dissolved according to instructions from the
NIAMDD. Insulin was first dissolved at 5 mg/ml in 5 mx HCl and then
diluted with culture medium to 1 mg/ml stock solution. Steroid hormones
were dissolved in absolute ethanol at 10^-3 M and diluted with medium to
desired concentration. The maximum ethanol concentration in the
medium (0.1%) was not deleterious to cultures, as determined in preliminary
experiments. Except for insulin (kept at 4°C) and steroid hormone stock
solutions (kept at -20°C), all other hormones were prepared within the
same week of use. All-trans-retinoic acid was obtained from Eastman
(Rochester, N.Y.) and was always handled in dim light; the preparation
of solutions and other considerations were as for steroid hormones
except that fresh solutions were always used.

Reagents for the 125I-fibron plate assay were obtained as described
previously (38). Reagents for protein and DNA assays were obtained as
follows. Bio-Rad dye reagent concentrate from Bio-Rad (Richmond, Calif.);
crystallized BSA from Armour Pharmaceuticals (Phoenix, Ariz.);
diphenyldimine and acetaldehyde from Eastman; calf thymus DNA from
Worthington (Freehold, N.J.); Ultrafluor from National Diagnostics (Som-
merville, N.J.) and [methyl-3H]thymidine from Amersham (Arlington
Heights, Ill.).

Other reagents were obtained as follows: NMU was from K and K,
ICN Pharmaceuticals (Plainview, N.Y.); ZPCK, BSA for use in culture
medium (Catalogue No. A-7511), and Triton X-100 were from Sigma; the
source of casein was nonfat dry milk from Carnation Co. (Los Angeles,
Calif.); culture media were from Grand Island Biological Co. (Grand Island,
N.Y.), sera were from Reheis (Division of Armour Pharmaceuticals,
Phoenix, Ariz.); and antibiotics were from Pfizer (New York, N.Y.).

Additional reagents were of the highest grade available from standard
sources.

Buffers. PBS++ and extraction buffer (0.5% Triton X-100-0.1 M Tris-
HCl, pH 8.1) were used.

Tumors. MTV tumor-bearing female BALB/c x DBA/ F1 (hereafter
called CDBF) mice (37) were a gift from Dr. D. S. Martin (St. Anthony’s
Medical Center, Woodhaven, N.Y.). DMBA induced-tumor bearing female
Sprague-Dawley rats were obtained from Hazleton Laboratories (Vienna,
Va.). Virgin female Bu/N rats were obtained from Charles River (Wil-
lington, Mass.), and mammary tumors were induced according to the
method of Guilino et al. (15) except that either one or, less frequently, 2
injections (1 month apart) were given. Rats 50 to 56 days old were
anesthetized with sodium pentobarbital and given 0.5 ml/100-g-body
weight injections of a fresh solution of NMU (10 mg/ml) into a tail vein
using a stainless steel 27-gauge needle. Injected animals were fed Purina
rat chow ad libitum. Mammary tumors first appeared approximately 3
months after injection. By 10 months, 80% of the animals had developed
one or more tumors. Survival during the latency period was usually better
than 80%. Most tumors grew rapidly and progressively while some,
when they reached a size of 2 to 4 g, showed no further increase in
mass. These static tumors were never seen to regress. Since rats treated
with DMBA or NMU often developed multiple tumors, occasionally up to
3 tumors from the same animal were taken for study. In such instances,
it was found that the tumors exhibited a wide range of responses, similar
in its heterogeneity to that of tumors from different hosts.

Normal mammary tissue was obtained from virgin female Bu/N rats
(Charles River) and female CDBF mice (from Dr. D. S. Martin’s labora-

Organ Culture. Tumors or normal tissue (third mammary gland pair)
were dissected aseptically from rats anesthetized with sodium pento-
barbital and exsanguinated or from mice killed by cervical dislocation.
The tumors were transferred to PBS++ supplemented with antibiotics
(0.5 × 10^6 units penicillin per liter and 200 mg streptomycin per liter),
trapped free of connective tissue and any necrotic centers, rinsed free
of blood, and minced with sharp scissors to yield fragments 0.5 to 1.0
mm in diameter averaging 20 µg of protein each. A sample of the tumor
was stored at -20°C in extraction buffer for future determination of PA content.

For organ culture, 25 explants (mouse tumors and normal tissue) or
50 explants (rat tumors) were transferred to sterile siliconized lens paper
rafts (41) floating over, respectively, 2 ml (35-mm dish) or 4 ml (60-mm
dish) of basal culture medium (Waymouth 752/1) supplemented with
NaHCO3, antibiotics as above, BSA (0.5 mg/ml), and insulin (1 µg/ml) or
basal medium further supplemented with the hormones which had effects
on PA production that we wished to study (alternatively, hormones were
added following 2 to 3 days of preincubation in basal medium). The large
number of explants assured good agreement between replicate cultures
(±10%) and were always used unless otherwise indicated. Each data
point shown is the mean of results from duplicate cultures. Tumor
cultures were incubated at 37°C in a humid atmosphere of 95% air-5% CO₂. Cultures of normal tissue were maintained in 95% O₂-5% CO₂. Conditioned medium was harvested daily (samples were kept at −20°C until assayed for PA) and replaced by an equal volume of basal medium to which hormones were added from fresh stocks. When cultures were terminated, the explants were rinsed with PBS**, transferred to extraction buffer, and stored at −20°C. Later, the explants were thawed and homogenized, and aliquots of the homogenate were used to determine DNA content. After centrifugation (1500 rpm/5 min), aliquots of the supernatants were assayed for protein.

Since for obvious reasons we opted to culture tumors in a well-defined, serum-free medium, preliminary experiments were carried out to determine the minimal hormonal replacements required to ensure tissue viability over a 4- to 5-day culture period. Insulin, at 1 µg/ml, was found to satisfy this requirement as described in detail elsewhere (25). The excellent cell viability provided by insulin medium is illustrated by the absence of any significant leakage of lactate dehydrogenase into the extracellular medium throughout 5 days of culture (Chart 1); further, in insulin medium, PA is synthesized and secreted at very fast linear rates, the total amount of exported PA reaching 44 times the intracellular level after 5 days (Chart 1). That being so, we have used PA production in basal medium as the base line against which the effect of hormones on PA production was measured.

Since the in vivo environment of tumor cells is hypoxic (45), it seemed appropriate to examine the effect of the O₂ tension on culture viability. As shown for NMU-induced tumor organ cultures in Table 1, both the rate of PA production and that of [3H]thymidine incorporation were much faster in tumors grown in 95% air than in tumors grown in 95% air-5% CO₂. The use of a gas mixture containing only 5% O₂ (5% O₂-90% N₂-5% CO₂), however, was not beneficial (not shown). Similar results were obtained for MTV- and DMBA-induced tumors. Therefore, tumors were always cultured in 95% air-5% CO₂.

**Primary Culture.** Tumors were digested with collagenase as described (30); 1 to 2 x 10⁵ cells were seeded per well of multiwell (1.4-cm-diameter) Linbro plates in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and incubated at 37°C in humid air supplemented with 5% CO₂. After 36 hr, the cultures were washed, and fresh Dulbecco’s medium (described above) was added; after a further 48 hr, the cultures were washed with Dulbecco’s medium once, and prolactin or pituitary extract were added in Dulbecco’s medium containing BSA (0.5 mg/ml). Conditioned medium was collected after 72 hr and stored at −20°C until assayed for PA.

**Protein Assay.** Protein was assayed by the Bio-Rad dye method according to the manufacturer’s instructions.

**DNA Assay and [³H]Thymidine Incorporation.** Samples of homogenate were precipitated with 0.1 volume of concentrated perchloric acid for 30 min at 0°C. The precipitate was collected on 0.45-µM filters under suction and hydrolyzed in 0.5 M perchloric acid at 70°C for 1 hr. DNA was assayed according to the colorimetric method of Burton (4) using hydrolyzed calf thymus DNA as standard. To determine [³H]thymidine incorporation, aliquots of the hydrolysate were mixed with Ultrafluor and counted in a scintillation counter.

**PA Assay.** The 125I-fibrin plate assay was used as described previously (30, 38). For the measurement of PA content, the tissue was homogenized in extraction buffer and centrifuged at 1500 rpm/5 min, and 0.025-ml aliquots of supernatant at 0.5, 0.25, and 0.125 mg/ml were assayed with or without plasminogen. In the absence of plasminogen, no fibrinolytic activity was detected except in the case of NMU-induced tumors. This plasminogen-independent activity could be blocked by inhibitors of chymotrypsin; its probable source was ruptured mast cell granules since these contain a chymotryptic activity (18) and plasminogen-independent fibrinolysis occurred only in mast cell-rich tumors. This problem was overcome by the routine addition of ZPCK to the extraction buffer (0.03 ml aliquots of supernatant at 0.5, 0.25, and 0.125 mg/ml were assayed with or without plasminogen). This is an oversaturating concentration which completely inhibits the chymotryptic activity but does not interfere with PA or plasmin-catalyzed fibrinolysis or with the protein assay. For the measurement of PA secretion, 0.025-ml aliquots of conditioned medium were assayed in duplicate. All of the fibrinolytic activity in tumor-conditioned medium was invariably plasminogen dependent. A urokinase standard curve was included in every assay. Unless otherwise stated, PA measurements were expressed in Ploog (32) millunits of urokinase secreted per hr per mg of explant protein.

**Lactic Dehydrogenase Assay.** Fresh aliquots of undiluted conditioned media or appropriately diluted cell extracts were assayed as described (33).

**Electrophoresis and Zymography.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as published (14) except that the stock solution contained 50% acrylamide and 5% bis-acrylamide and casein-agar underlays were used. These were prepared as follows: 0.8 ml of 8% casein, 1.0 ml of human plasminogen (0.2 mg/ml), and 2.2 ml of 0.02% sodium azide were combined and heated to 45°C; 2.2 ml of 2.6% agar were boiled and quickly added to the casein-plasminogen, mixed, and poured over a leveled 4 x 4-inch glass plate. Caseinolysis was allowed to proceed at 37°C and was checked regularly against a dark field. When effects were maximal, the polyacrylamide gel was discarded, and the casein-agar plate was stained and destained as described (14). The casein-agar layer was then allowed to dry at room temperature, yielding a permanent record of the experiment.

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**Table 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor wt (g)</th>
<th>PA, 95% air/95% O₂</th>
<th>[³H]Thymidine, 95% air/95% O₂</th>
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<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>1.4</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>1.9</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>2.2</td>
<td>3.6</td>
<td>NT*</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>1.8</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>8.1</td>
<td>8.9</td>
<td>30</td>
</tr>
</tbody>
</table>

* NT, Not tested.
RESULTS

Tissue Content of PA and Rate of Secretion in Organ Culture

Total enzyme content was measured in detergent extracts of freshly excised mammary tissue. Chart 2 shows the values obtained for normal glands and primary mammary tumors induced by MTV, DMBA, or NMU. Relative to PA levels in normal tissue, the enzyme content of tumors was 1 to 3 orders of magnitude higher.

The rate of enzyme secretion was estimated from the amount of enzyme released into the medium during the first day of organ culture in basal (insulin-containing) medium. In this system, insulin appears to provide a general supportive function in maintaining tissue viability and has no effect on tumor enzyme secretion per se. No other hormones were present as a component of the basal culture medium, but it is likely that some were carried over with the explants. Hence, we in fact measured a rate of secretion which reflected the influence of the hormonal milieu of the host prior to explantation. These rates of PA production by normal glands and the 3 different kinds of mammary tumors are presented in Chart 3. All tumors secreted the enzyme at much higher rates than did normal mammary tissue, with the mean difference being close to (DMBA-induced tumors) or greater than (MTV and NMU) 2 orders of magnitude. Among chemically induced tumors, individual values extended from <1 to >3 orders of magnitude above normal levels. This heterogeneity was apparent even when the tumors, which covered a broad size range (0.5 to 8 g), were subgrouped according to weight. This is not surprising. Tumors arising in response to treatment with chemical carcinogens vary greatly in their properties, including malignancy, and would be expected also to vary in PA output. On the other hand, virus-induced mammary tumors formed a more homogeneous group, with all tumors secreting at least 100-fold more enzyme than normal glands.

Mammary tumors maintained their high rates of secretion for at least 5 days in culture, while intracellular enzyme content remained constant (Chart 1), suggesting that tumor enzyme secretion required de novo enzyme synthesis. This expectation was corroborated directly by the finding that cycloheximide (10 μg/ml) rapidly and completely inhibited PA production (data not shown). It can also be seen that throughout this period intracellular lactic dehydrogenase was maintained at high levels, and very little of this enzyme (about 6% of the total) appeared in the extracellular medium. This result attests to the viability of the tissue, and it also demonstrates that the appearance of PA in the medium is not attributable to leakage from damaged or moribund tissue.

Hormonal Modulation of PA Secretion

Effect of Prolactin. The first test of hormonal modulation of tumor PA concerned the action of prolactin on NMU tumors as follows. Briefly, tumors were explanted directly into basal medium or prolactin medium (basal medium supplemented with 1.1 to 4.3 × 10^{-7} mM prolactin) and maintained for 3 to 4 days. Prolactin stimulated PA secretion in 22 of a total of 27 tumors tested and was without effect on 5 tumors; maximal responses ranged from 1.5- to 3.6-fold increases and were more commonly observed late in culture (days 3 or 4), suggesting that the explants might have been responding maximally to circulating prolactin in the host at the time of explanting.

The effect of prolactin was then tested on cultures preincubated in basal medium; this reduced the basal rate of enzyme secretion as the effect of carried-over hormones decayed and therefore allowed the study of added hormones in the absence
of lingering \textit{in vivo} effects. In 5 of 7 tumors tested in this way, prolactin induced an absolute increase in PA secretion, whereas no response was observed in 2 tumors; 3 examples of responsive and one of a nonresponsive tumor are illustrated in Chart 4, A to D. Using the latter protocol of delayed hormonal stimulation, the effect of prolactin was also tested on DMBA- and MTV-induced tumors. The results show that DMBA-induced tumors (Table 2) resemble the NMU group in that a majority (4 of 5) responded with 1.6- to 7-fold increase in enzyme secretion. On the other hand, no response was detected in any of the 6 MTV-induced tumors tested (2 such experiments are shown in Chart 4, E and F). It is of interest that the growth of these tumors \textit{in vivo} is known to be independent of prolactin (24, 26, 46).

As seen in Chart 4 and Table 2, there was an apparent inverse correlation between the basal rate of tumor PA secretion and response to prolactin. No such correlation was evident when the effect of prolactin was tested in combination with either retinoic acid (Table 4; Chart 6) or cholera toxin (Table 5).

Effect of Hydrocortisone. The effect of hydrocortisone was of special interest, since this hormone strongly suppresses both PA production and involution in normal mammary tissue.

PA secretion in MTV-induced tumors was inhibited by $10^{-7}$ M hydrocortisone in 26 of 30 and not changed in 4 of 30 of the tumors tested. Examples of hormone-responsive and -refractory behavior are illustrated in Chart 5, A to C.

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Among NMU-induced tumors, the same concentration of hydrocortisone produced a variable degree of inhibition, from better than 60% (2 of 11 tumors) to no inhibition (2 of 11), with an overall mean of 32% ($n = 11$). However, when hydrocortisone was added to cultures together with prolactin, the mean inhibition for the same group of tumors increased to 54%, and all tumors showed better than 30% inhibition. Thus, prolactin sensitized the NMU tumors to inhibition by glucocorticoids, an effect illustrated for one tumor in Chart 5D) for which the basis has not been analyzed and deserves further investigation. As might have been expected, the effect of hydrocortisone on MTV-induced tumors, which are prolactin unresponsive, was not affected by prolactin.

The action of $10^{-7}$ M hydrocortisone was also tested on 3 DMBA tumors in prolactin medium. Enzyme secretion was decreased in 2 tumors (58 and 75% inhibition) and unchanged in the third.

At the concentration used in the above experiments, hydrocortisone, as already noted, invariably suppresses enzyme secretion by the normal lactating rodent mammary gland to the extent of at least 90% (29). There is a sharp contrast, therefore, between the predictability and extent of the hydrocortisone effect on normal tissue and the variable and more limited response of neoplastic tissue. Even so, the qualitative similarity in response to hydrocortisone is somewhat noteworthy, however, since it is exceptional among the various effectors tested (see "Discussion").

Effect of Estradiol and Progesterone. When tested on NMU-induced tumor cultures maintained in absence of prolactin, 17$\beta$-estradiol ($10^{-8}$ or $10^{-9}$ M) or progesterone ($10^{-8}$ or $10^{-7}$ M) did not affect PA production throughout the standard 4-day period of culture (2 tumors). However, in an NMU-induced tumor in which prolactin alone induced a 2-fold increase in PA secretion, pretreatment with 17$\beta$-estradiol ($10^{-8}$ M) sensitized the tumor to prolactin which then produced a 4-fold stimulation of PA secretion. Conversely, with this same tumor, progesterone ($10^{-8}$ M) completely blocked the PA response to prolactin. This experiment indicates that, at least in some tumors and at the level of the PA response, the actions of estradiol and progesterone are, respectively, synergistic and antagonistic with that of prolactin.

We have not attempted to determine whether the steroid effects described here are correlated with the presence of the usual cytoplasmic receptor molecules in the tumor cells.

Table 3 summarizes the results obtained with a total of 4
tumors that were tested specifically to obtain further evidence bearing on possible interactions in the effects of estradiol and progesterone with that of prolactin. Here various hormone combinations were added to rat tumor cultures preincubated in basal medium; as can be seen, in all tumors, addition of estradiol together with prolactin stimulated enzyme secretion beyond the level seen in prolactin alone, although the magnitude of the effects was variable; progesterone with prolactin was again inhibitory (2 DMBA-induced tumors) or without effect (NMU-induced Tumor 1, 2, and 3 of Table 2).

Effect of Retinoic Acid. Retinoic acid, particularly in combination with prolactin, stimulated PA secretion in all NMU-induced tumors tested (Table 4). The interaction between retinoic acid and prolactin may in some cases be slightly more than additive, but not conspicuously so, and these agents appear to increase enzyme secretion through at least partly independent pathways, since one tumor that responded well to retinoic acid was inert to prolactin (Table 4, Tumor 4).

Chart 6 illustrates the combined effect of retinoic acid and prolactin in greater detail. It can be seen that, relative to control cultures in basal medium, cultures treated with retinoic acid and prolactin secreted PA at a faster rate throughout the culture period (Chart 6A); when exposure to these effectors was delayed until the third day of culture, their increased acceleration of enzyme secretion within 48 hr to a level equaling that of cultures continuously exposed to the 2 agents (B).

Retinoic acid (10⁻⁷ M) did not affect enzyme secretion by MTV-induced tumors (n = 3, not shown).

Effect of Cholera Toxin. Exposure of NMU-induced tumors to cholera toxin produced a wide range of response in PA secretion; these included, respectively, in the 3 tumors tested, either no effect or a mild (38%), or a good (158%) increase (Table 5). Combined treatment with cholera toxin and prolactin, however, gave 3-fold increases in every case (Table 5); the effects of cholera toxin and prolactin were additive in the one tumor that responded well to cholera toxin alone and moderately synergistic in the other 2 tumors.

Cholera toxin (10⁻⁷ M) alone did not modulate enzyme secretion in any of 3 MTV-induced tumors tested (not shown).

Combined exposure to prolactin, cholera toxin, and retinoic acid was tested on 2 NMU-induced tumors; this combination was found to be potently synergistic, yielding 6- and 8-fold increases over enzyme secretion in basal medium (compare with increases obtained by single treatment with either prolactin,

### Table 3

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<tr>
<th>Tumor</th>
<th>PA (milliunits urokinase/hr/mg protein)</th>
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<tr>
<td>DMBA-induced</td>
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<tr>
<td>Tumor 1</td>
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<tr>
<td>Tumor 2</td>
<td>140</td>
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<tr>
<td>Tumor 3</td>
<td>78</td>
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* P, 2.2 x 10⁻⁷ M prolactin; E, 10⁻⁸ M 17β-estradiol; PG, 10⁻⁷ M (NMU-induced tumor) progesterone; NT, not tested.

### Table 4

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<th>Tumor 4</th>
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<td>Tumor 3</td>
<td>94</td>
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* NT, not tested.

### Table 5

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<th>Tumor 5</th>
<th>PA (milliunits urokinase/hr/mg protein)</th>
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<td>Basal</td>
<td></td>
</tr>
<tr>
<td>Tumor 1</td>
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</table>

The concentration in basal medium of retinoic acid is 10⁻⁷ M, and that of prolactin is 2.2 x 10⁻⁷ M. Tumors were cultured in the indicated media from Day 0 (Tumors 1 and 2) or following 2 days of culture in basal medium (Tumors 3 and 4). The PA secretion shown is for the second day of treatment; duplicate cultures of 50 explants were used for each determination.

The concentration in basal medium of cholera toxin is 10⁻⁸ M and that of prolactin is 2.2 x 10⁻⁷ M. Tumors were cultured in the indicated media immediately following explantation. The PA secretion shown is for the first day of culture and treatment; duplicate cultures of 50 explants were used for each determination.
Effect of Pituitary Extracts. The growth properties of experimental rodent mammary tumors cannot easily be rationalized in terms of the effects of known hormones (16, 19, 36), and we have therefore explored the possibility of applying PA responses in organ culture as a probe for detecting potentially new hormones acting on mammary tumors. With this in mind, we first tested the ability of crude pituitary extracts to modulate PA production in experimental mammary tumors. To exclude any contribution by the prolactin present in the extracts, these were always tested in medium containing a concentration of prolactin (2.2 x 10^{-7} M, 5 μg/ml) that was saturating for the stimulation of PA production. This concentration is 3 orders of magnitude above the dissociation constant for prolactin binding to its receptor in NMU-induced tumor membranes (42); when higher concentrations (4.3 and 8.7 x 10^{-7} M) were tested, PA secretion was inhibited 40 to 50% relative to the level obtained with 2.2 x 10^{-7} M prolactin.

Treatment of rat mammary tumors with 70 μg of unrefractionated pituitary extract per ml (Extract B; see "Materials and Methods") increased enzyme secretion more than 2-fold above the level seen in organ cultures treated with prolactin alone: this pattern was obtained with 10 of 13 NMU-induced tumors and 4 of 5 DMBA-induced tumors (Chart 7 shows the effect on one DMBA- and 2 NMU-induced tumors); the remaining tumors were unresponsive. None of the 3 murine MTV-induced tumors tested was affected by pituitary extracts, as illustrated for one tumor in Chart 7D. This finding is consistent with the pituitary independence of MTV-induced tumors for growth in vivo (27). Pituitary extract was also tested on primary monolayer cell cultures of 3 NMU tumors: here, the responses gave 1.5-, 1.9-, and 2.3-fold increases in enzyme secretion. The dose dependence of this effect in one such experiment is presented in Chart 8.

Direct documentation that prolactin was not responsible for the effect of pituitary extract was obtained in 2 ways: (a) even with tumors unresponsive to prolactin, pituitary extract strongly stimulated enzyme secretion (Chart 9); (b) prolactin-depleted extracts (Extract A; see "Materials and Methods") had the same effect as total extracts at one-half the concentration (Chart 10).

To determine if any of the known anterior pituitary hormones could be responsible for the stimulation seen with extracts, purified hormones were added to prolactin medium at concentrations sufficient to elicit a response in specific target organs. The effect of the hormones on PA secretion by NMU-induced tumor organ cultures was then compared with that produced by the extract. Table 6 shows that neither follicle-stimulating hormone, nor thyroid-stimulating hormone, nor adrenocorticotropic hormone were effective substitutes. The experiment of Chart 10 shows that the same was true for growth hormone and hypothalamic releasing factors (thyrotropin-releasing hormone and luteinizing hormone-releasing hormone), methionine-enkephalin, and β-melanocyte-stimulating hormone. In an experiment analogous to that of Chart 10, this list was extended to include luteinizing hormone (tested at 5 μg/ml) and, in addition, vasopressin (1 μg/ml), oxytocin (1 unit/ml), and fibroblast growth factor (0.1 μg/ml), none of which was even partially effective in replacing the extract.
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Identification of the Enzyme Form Responsive to Hormonal Regulation

It is now well established that PA activity as usually measured is due to either one of 2 enzymes, or a mixture of both. These enzymes differ in molecular weight, antigenic structure (11, 22, 35, 47), and catalytic (23) properties; in rodent tissues, the 2 PA types migrate in sodium dodecyl sulfate gels (under nonreducing conditions) with apparent molecular weights of 48,000 and 75,000 to 80,000 (14). The M, 48,000 species comigrates with human urokinase. The second enzyme is homologous with human tissue PA, and we have not yet applied the other assays that lend itself well to detecting moderate changes in secretion of tissue PA. The homologue of human (HeLa cell) tissue PA was stimulated in parallel with urokinase-like PA in NMU-induced tumors exposed to pituitary extract (Fig. 1). For reasons that are not fully understood at present, the zymographic procedure does not lend itself well to detecting moderate changes in secretion of tissue PA, and we have not yet applied the other assays that are necessary to determine specific changes in this PA form in response to different regulatory signals.

DISCUSSION

The results described above should be considered from several points of view. The first concerns the difference in PA production between normal mammary tissue and mammary tumors. All of the observations bearing on this question indicated that enzyme production was greatly enhanced in neoplastic tissue. This was evident both in the large PA content of the tumors and in the much greater rates of enzyme secretion in organ culture. The increases were in the range 10- to 100-fold for tissue levels (Chart 2) and 10- to 1000-fold for rates of enzyme accumulation in culture (Chart 3). Although on the average somewhat larger than previously reported, these differ-

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Table 6

<table>
<thead>
<tr>
<th>Hormones</th>
<th>PA (milliunits urokinase/hr/0.1 mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor 1</td>
</tr>
<tr>
<td>Prolactin</td>
<td>5.6</td>
</tr>
<tr>
<td>Prolactin + pituitary extract</td>
<td>24.4</td>
</tr>
<tr>
<td>Prolactin + follicle-stimulating hormone</td>
<td>4.8</td>
</tr>
<tr>
<td>Prolactin + thyroid-stimulating hormone</td>
<td>6.0</td>
</tr>
<tr>
<td>Prolactin + adrenocorticotrophic hormone</td>
<td>7.2</td>
</tr>
</tbody>
</table>

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Fig. 1. Pituitary extract enhances production of both PAs. Conditioned media of NMU tumor organ cultures were electrophoresed, and a zymogram was developed as in “Materials and Methods.” Lane 1, 5 milliunits of urokinase; Lanes 2 and 3, 0.01 ml of conditioned medium from duplicate cultures treated with pituitary extract B (70 µg/ml) in the presence of prolactin (5 µg/ml); Lanes 4 and 5, 0.01 ml of conditioned medium from duplicate cultures treated with prolactin (5 µg/ml). TA, rat tissue activator, UK, rat urokinase-like PA.

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Concentrations of hormones in basal medium were as follows: prolactin, 5 µg/ml (2.2 x 10^-7 M); pituitary extract A, 30 µg/ml; follicle-stimulating hormone, thyroid-stimulating hormone, and adrenocorticotrophic hormone, 5 µg/ml each. Tumors were explanted into the indicated hormonal milieu and maintained for 4 days with daily medium changes. Enzyme secretion during the fourth day of culture is shown; results are expressed per 0.1 mg DNA. To convert to mg protein multiply by 10. Duplicate cultures of 50 explants were used for each determination.

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DISCUSSION

The results described above should be considered from several points of view. The first concerns the difference in PA production between normal mammary tissue and mammary tumors. All of the observations bearing on this question indicated that enzyme production was greatly enhanced in neoplastic tissue. This was evident both in the large PA content of the tumors and in the much greater rates of enzyme secretion in organ culture. The increases were in the range 10- to 100-fold for tissue levels (Chart 2) and 10- to 1000-fold for rates of enzyme accumulation in culture (Chart 3). Although on the average somewhat larger than previously reported, these differ-

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7 D. Belin, personal communication.
ences are both qualitatively and quantitatively in accord with a substantial body of information on the association between PA and neoplasia (28, 30, 31, 34). While this is the first systematic study of PA in primary, experimental rodent mammary tumors, Markus et al., using the azacoseinolytic PA assay, have previously reported similar findings for a large series of primary human mammary carcinomas (13) among others (7, 12, 22). These investigators showed in addition that 80% of the PA activity in extracts of human breast tumors was inhibited by antikeratinase antibody, against a 62% inhibition of the activity in extracts of normal breast tissue.

One aspect of interest is the wide range in the amounts of PA produced by tumors. The MTV-induced mouse tumors were the most homogeneous population in this respect, all tumors containing and secreting large quantities of enzyme. The relative heterogeneity of the rat tumors is not surprising and may reflect the correspondingly greater genetic and cellular heterogeneity to be expected from carcinogen-induced as compared with virus-induced tumors. It seems worthwhile to test for some correlation between the rates of tumor growth and enzyme production, and we have in fact made a few observations that are consistent with such a relationship, but the results are as yet too few to support any firm conclusions.

A second point of importance concerns the identity of the PA-producing cells. Because tumors have occasionally been reported to contain significant numbers of macrophages, it appeared possible that some of the observed PA might have been the product of macrophages or other nonmammary cells, especially in view of the repressible action of glucocorticoids, a well-known characteristic of macrophages (44). In spite of the similarity in glucocorticoid effects, several properties of the PA response to modulating agents clearly differentiate the mammary tumors from macrophages. Thus, choriola toxin and retinoic acid, which strongly inhibit macrophage PA synthesis (44), both stimulate tumor enzyme secretion (Tables 4 and 5). Pituitary extract provides a further example of contrasting PA responses in mammary tumors and macrophages. It is stimulatory to both DMBA- and NMU-induced rat tumors (Chart 7) but inhibitory to macrophages. In addition, 3 cell lines have been isolated by directly cloning cells released from organ cultures of NMU-induced tumors; all of these lines are clearly epithelial in morphology and in their ability to form epithelial sheets separating 2 fluid compartments ("domes" in monolayer culture), and they have preserved the pattern of PA synthesis modulation by glucocorticoids and pituitary extracts found in parent organ cultures. For these reasons, it seems safe to conclude that the enzyme we have measured is formed by mammary tumor cells. However, it cannot as yet be excluded that there is some contribution of the mesenchyme to the total observed PA, especially since some tumors appear to secrete 2 distinct forms of the enzyme.

Three findings of importance emerge from our data on the hormonal regulation of PA synthesis. One is the fact that normal and neoplastic mammary tissues are significantly different in their pattern of response to hormones and hormone-like agents: the respective responses are summarized in Table 7 where qualitative differences can be seen in the effects of prolactin, estrogen, and retinoic acid, apart from a quantitative difference in the case of hydrocortisone. It is of interest that no single pattern is common to all classes of tumors and further that there is some heterogeneity of response to most agents within each tumor category including even the virus-induced mouse tumor. This display of individuality in organ-specific primary tumors is reminiscent of human cancer although probably less conspicuous than the heterogeneity of tumors to be expected in a randomly selected human population.

The second significant result bears on the coordinate response of tumor growth (in vivo) and PA production (in vitro) to hormones. In Table 8 are summarized the PA data obtained in the present work, pertinent results from the literature, and one as yet unpublished finding. It is apparent that all of these results, taken together with those previously cited (see "Introduction"), support the proposition that PA production in malignant tumors is part of the biosynthetic program induced by hormones that stimulate growth in vivo. Although the information is not yet sufficient to permit any generalization, the correlations thus far observed are excellent and encourage further testing of the hypothesis that PA responses in organ culture may be useful in predicting tumor growth responses even in the vastly more complex physiological environment existing in vivo. It also appears worthwhile at this stage to explore the PA responses of

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Prostate</th>
<th>Breast</th>
<th>Liver</th>
<th>All other</th>
<th>Normal</th>
<th>Neoplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Hydrocortisone</td>
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<td>↑</td>
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</table>

* A. Horiuchi, personal communication.
* J. de la Harpe, unpublished observations.
human mammary tumors in the experimental system that we have applied to the rodent tumors.

The third point of endocrine interest concerns the stimulation of PA production by pituitary extract. We have already established that this effect is not due to any of the known pituitary hormones, and the phenomenon therefore appears to represent both a new pituitary factor and a new hormonal response of mammary tumor cells. Using the PA response of rat NMU-induced tumors in organ culture as an assay, we have purified the responsible peptide approximately 2000-fold, and its size and ion-exchange fractionation properties distinguish it from previously characterized products of the pituitary gland.11

In summary, our exploratory studies of PA synthesis in tumor organ culture suggest that the experimental system used here deserves further investigation because it may be of value in several respects: (a) it provides an approach defining some of the endocrine responses of individual tumors and thereby obtaining information that might be a valuable adjunct in designing appropriate therapeutic regimens for individual patients; (b) the PA response on which the system is based may have some predictive value in the context of endocrine therapy for tumors if the current patterns of correlation with growth in vivo are reinforced with additional observations; (c) the system can be applied both for detecting new hormonal responses and for identifying and purifying the responsible molecules.

ACKNOWLEDGMENTS

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REFERENCES


Modulation of Plasminogen Activator in Rodent Mammary Tumors by Hormones and Other Effectors

Rafael Mira-y-Lopez, E. Reich and Liliana Ossowski