Hormone-induced Ductal DNA Synthesis of Human Breast Tissues Maintained in the Athymic Nude Mouse

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ABSTRACT

Five biopsy specimens of morphologically normal human breast tissues, obtained from the margins of five benign human breast tumors, were processed into slices (4.0 x 4.0 x 0.1 mm) and transplanted s.c. dorsally (eight to ten slices/mouse) into forty-three 6- to 8-week-old female BALB/c athymic nude mice. Each individual human breast tissue specimen was transplanted into seven to ten mice. After 30 days, the mice were divided into four groups and treated for 30 days as follows: (a) controls receiving s.c. cholesterol pellets (38 mg); (b) estrogens that were administered in s.c. pellets containing 2 mg 17β-estradiol and 38 mg cholesterol and in drinking water containing 0.5 mg estrone per liter; (c) rat pituitary tumor (RPT), a cell suspension of MIT-W10 RPT that secretes large amounts of prolactin and growth hormone, injected dorsorostrally; and (d) RPT plus estrogens. Three to five human breast tissue grafts were removed from each mouse at the onset of treatment, and the remainder were removed at termination of treatment. DNA synthesis in the ductal epithelium was determined in pre- and posttreatment grafts by [³H]thymidine autoradiography after incubation of grafts for 4 hr in an isotope-enriched medium. The labeling index (LI), mean number of labeled epithelial cells per unit area of epithelial tissue, in pre- and posttreatment grafts was, respectively: (a) control, 7.6 ± 1.4 and 7.1 ± 1.4; (b) estrogens, 5.5 ± 0.6 and 17.9 ± 2.3; (c) RPT, 6.2 ± 0.7 and 8.0 ± 1.5; and (d) RPT plus estrogens, 6.3 ± 0.8 and 26.6 ± 2.5.

A significant increase in LI was observed after treatment with estrogens (p < 0.01) or RPT plus estrogen (p < 0.001). Mean LI after treatment with RPT plus estrogens was significantly greater (p < 0.02) than after estrogens alone. RPT alone did not significantly affect the LI. Thus, these results provide in vivo evidence that estrogens enhance DNA synthesis of the ductal epithelium of the normal human breast and that a growth factor (or factors) from RPT acts synergistically with estrogens to produce a more pronounced increase in DNA synthesis. RPT growth factors (perhaps prolactin and/or growth hormone) appear to require estrogens for DNA synthesis stimulation in normal human breast ductal epithelium.

INTRODUCTION

The role of pituitary and steroid hormones in the initiation and progression of human breast neoplasia cannot be defined accurately by any of the carefully documented murine mammary cancer models. In vitro organ and cell culture studies with primary normal and malignant human breast tissues and human breast cell lines have improved our knowledge in some areas, but the limitations of the in vitro system and conflicting results especially with regard to the action of estrogen on growth of human mammary epithelium have left many uncertainties (1, 5, 6, 11, 15, 18, 19, 21).

The immunodeficient athymic nude mouse is being used increasingly for "ex vivo-in vivo" studies to determine the growth-promoting properties of a variety of factors in normal, benign, and malignant human breast tissues (2, 4, 7, 8, 12, 13, 16). In our laboratory, a protocol has been developed to determine the responses of s.c. mammary xenografts to hormonal manipulation of the nude mouse host (8). The feasibility of such a tissue-host system was demonstrated clearly when significant growth (DNA synthesis) and differentiation (lactation) were induced in xenografts of mammary tissue from a pregnant cow by treatment of the nude mice with physiological doses of ovarian and adrenal steroids and bovine pituitary hormones (22). Subsequently, we have reported that the epithelium of human breast tissue grafts in nude mice responded to human placental lactogen treatment with increased DNA synthesis (8). In the present report, the effects of estrogen and pituitary factors on DNA synthesis and morphology of the epithelium in human breast tissue grafts in nude mice are described. Since purified pituitary hormones are very difficult to obtain in sufficient quantities for in vitro studies, pituitary growth factors were provided by the secretions of MIT-W10 RPT³ congruently growing in the host mice. This transplantable RPT is known to secrete very high levels of prolactin and growth hormone (3).

MATERIALS AND METHODS

Preparation of HBT. Five breast biopsy specimens (HBT 1 to HBT 5) were obtained at area hospitals from 5 women of ages 21 to 50 years. The breast tissue specimens were excised from the margins of benign tumors and were identified by pathologists as containing largely normal inter- and intralobular ductal epithelium with some ductal hyperplasia and/or cysts. The HBT's were processed aseptically in a laminar flow hood, according to the method described previously (7), into 90 to 100 slices (approximately 4.0 x 4.0 x 0.1 mm) per specimen for transplantation into nude mice and for 0-day [³H]thymidine autoradiographic analysis. Each HBT was transplanted to 7 to 10 mice, 8 to 10 slices per mouse. Any remaining tissue from each specimen was fixed in Bouin's fluid for histological examination.

Transplantation of HBT Slices Into Nude Mice. Forty-three female BALB/c athymic nude mice, 6 to 8 weeks old, purchased from Harlan Sprague-Dawley Division, Harlan Industries, Madison, Wis., were shipped in sealed containers that prevent en-route exposure of the

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mice to pathogens. The mice were continuously housed in aseptic conditions (enclosed overhead laminar flow hood and sterilized cages, bedding, food, and water). Surgical procedures for transplantation were also performed in aseptic conditions in the same laminar flow hood in which the HBT slices were prepared. The mice were anesthetized with sodium pentobarbital injected i.p., and HBT slices were transplanted s.c. in the dorsal area, 8 to 10 slices per mouse, 4 or 5 slices per side.

**MIT-W10 RPT Preparation.** MIT-W10 RPT was obtained by courtesy of Dr. U. Kim, Department of Pathology, Roswell Park Memorial Institute, Buffalo, N. Y. The RPT was maintained in our laboratory by serial s.c. transplantation to female Wistar-Furth rats. The donor rat was anesthetized, and the RPT was removed and processed aseptically in a separate laminar flow hood from the one in which nude mice are handled. The RPT cells were expressed and mixed with approximately equal volumes of Medium 199 (Hanks' base) (Grand Island Biological Co., Grand Island, N. Y.) and the pre- and posttreatment and the remainder were removed at termination of treatment. The RPT cells were expressed and mixed with approximately equal volumes of Medium 199 (Hanks' base) (Grand Island Biological Co., Grand Island, N. Y.) and the cell suspension was aspirated into a 3-mL syringe and injected via an 18-gauge needle into the nude mice dorsocaudally, 0.3 ml/mouse.

**Treatment of HBT-grafted Nude Mice.** Thirty days after grafting of HBT, the nude mice for each individual HBT specimen were divided into 4 groups of 2 or 3 mice per group and treated as follows: (a) controls, s.c., cholesterol pellet (38 mg cholesterol) inserted into the dorsocaudal area; (b) estrogens, 17β-estradiol pellet (2 mg 17β-estradiol:38 mg cholesterol) (Nutritional Biochemical Corp., Cleveland, Ohio) inserted s.c. dorsocaudally and estrone (Nutritional Biochemical Corp.) in the drinking water, 0.5 mg/liter; (c) RPT, injected s.c. dorsorosally plus cholesterol pellet; (d) RPT plus estrogens, as described for Groups 2 and 3. Because of an insufficient amount of biopsy specimen, only 3 groups (Group 1, 2, and 4) were used for HBT 5. The hormone treatment period was 30 days. At termination, the animals were weighed and killed by decapitation. Blood was collected and centrifuged, and the serum was stored at -20° for prolactin radioimmunoassay (National Institute of Arthritis, Metabolism, and Digestive Diseases rat prolactin radioimmunoassay kit). Ovaries, uterus, and inguinal mammary glands were excised. Uteri were weighed, and the ovaries and uteri were fixed in Bouin's fluid for histological examination. Mammary glands were fixed in 10% formalin and stained for whole-mount examination by a standard method (20). RPT were excised and weighed, and a portion of each tumor was fixed in Bouin's fluid and processed for histological examination.

**Preparation of HBT Slices and Grafts for [³H]Thymidine Autoradiographic Analysis.** Thirty days after transplantation, before hormonal treatment was initiated, 3 to 5 grafts were excised from each mouse, and the remainder were removed at termination of treatment. The HBT 0-day slices (5 to 10 slices per HBT) and the pre- and posttreatment grafts were incubated in 10- to 30-mm Falcon disposable Petri dishes containing 2.0 ml of Medium 199 (modified Earle's salts, 1250 mg NaHCO₃ per liter) (Grand Island Biological Co., Grand Island Biological Co.) to which was added 17β estradiol:38 mg cholesterol (Nutritional Biochemical Corp.) in the drinking water, 0.5 mg/liter; (c) RPT, injected s.c. dorsorosally plus cholesterol pellet; (d) RPT plus estrogens, as described for Groups 2 and 3. Because of an insufficient amount of biopsy specimen, only 3 groups (Group 1, 2, and 4) were used for HBT 5. The hormone treatment period was 30 days. At termination, the animals were weighed and killed by decapitation. Blood was collected and centrifuged, and the serum was stored at -20° for prolactin radioimmunoassay (National Institute of Arthritis, Metabolism, and Digestive Diseases rat prolactin radioimmunoassay kit). Ovaries, uterus, and inguinal mammary glands were excised. Uteri were weighed, and the ovaries and uteri were fixed in Bouin's fluid for histological examination. Mammary glands were fixed in 10% formalin and stained for whole-mount examination by a standard method (20). RPT were excised and weighed, and a portion of each tumor was fixed in Bouin's fluid and processed for histological examination.

**Results**

**Human Breast, Response to Hormones in Nude Mice**

**DNA Synthesis of HBT Grafted to Nude Mice.** The pre- and posttreatment LI's of grafts in individual nude mice for HBT 1 to HBT 5 are listed in Table 1, with the means and S.E.'s for 4 treatment groups. The mean differences in LI, pre- to posttreatment, of each group are illustrated in Chart 1. Analysis of variance on HBT 1 to HBT 4 (with 4 complete treatment groups) showed a significant effect of treatments (p < 0.01) but no significant interaction between HBT and treatments (p > 0.1). The mean LI's observed for posttreatment RPT plus estrogen and estrogen grafts were significantly greater (p < 0.001 and p < 0.01, respectively) than were pretreatment grafts and control grafts. RPT appeared to be synergistic with estrogen as the mean LI for RPT plus estrogen treatment was significantly higher than was the mean LI for estrogen alone (p > 0.02). Although analysis of variance did not indicate a significant interaction between HBT and treatments, there appeared to be some variation in response to estrogen alone among the 4 HBT's tested. There was little or no response to estrogen in HBT 2. On the other hand, HBT 3 response to estrogen was similar to that of RPT plus estrogen. RPT alone had no significant effect on the LI of HBT. Zero-day LI's for the 5 HBT's were, for HBT 1 to HBT 5, respectively, 5.4, 9.6, 1.8, 19.0, and 26.3. Donor ages of HBT 1 to HBT 5, respectively, were 21, 32, 41, 24, and 50 years. The high 0-day LI for HBT 5 was unusual, since we have not observed LI of over 10 previously in donors 50 years or over. We were unable to ascertain whether this woman was postmenopausal or premenopausal. It should be noted that neither donor age nor the initial LI of the breast tissue affected the response of HBT grafts to estrogen or RPT plus estrogen.

**Histology of HBT and Organ Weights.** There was a definite change in the histological appearance of the HBT epithelial cells in the RPT plus estrogen group (Figs. 2 and 3) and to a lesser degree in the estrogen group when compared to controls (Fig. 1). Epithelium in grafts of mice receiving RPT plus estrogen showed an apparent increase in cell size and general viability. In addition, mitotic figures were fairly frequently observed in the grafts of the RPT plus estrogen groups (Figs. 2 and 3), were less frequently observed in the estrogen treatment groups, and never observed in the controls or RPT groups. There was no apparent increase in graft size as a result of hormone treatment.

Mean body, uterine, and RPT weights are presented in Table 2. Body weights for RPT and RPT plus estrogen groups are given as the whole-body weight minus the RPT weight. The mean body weights in both RPT and RPT plus estrogen groups were significantly greater than those of controls or the estrogen group. All mice gained weight (p < 0.05) during the

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*4 M. J. McManus and C. W. Welsch, unpublished data.*
Chart 1. Mean change in $[^3H]$thymidine LI, pre- to posttreatment, of 5 HBT's in female athymic nude mice treated with estrogen, RPT, RPT plus estrogen, and controls. Posttreatment LI was significantly greater than pretreatment LI for estrogen ($p < 0.01$) and RPT plus estrogen ($p < 0.001$).

treatment period (mean weight gains ± S.E. were 1.7 ± 0.4, 4.0 ± 0.5, 8.6 ± 0.7, and 10.0 ± 2.1 g for control, estrogen, RPT, and RPT plus estrogen groups, respectively). The heavier weights and increased gains in RPT and RPT plus estrogen groups reflect the effects of pituitary growth hormone secretion from the RPT (3).

Estrogen treatment inhibited the growth rate of RPT; i.e., mean RPT weight in the RPT group was significantly greater than in the RPT plus estrogen group. Histologically, the RPT in estrogen-treated animals appeared compartmentalized into discrete areas surrounded by connective tissue stroma. There were less vascularity, few large vascular sinuses, and less necrosis in the RPT with estrogen than in the RPT without estrogen. RPT cells did not differ in appearance in the 2 groups. A marked dorsal dermal edema was present in all mice receiving RPT plus estrogen. The dorsal integument was up to 3 mm thick in these animals. This was not seen in the 2 other treatment groups or in controls. Histological examination of the integument of these mice showed no particular pathology except edema.

Uterine weights reflected the stimulatory effect of estrogen, being significantly enlarged in both estrogen-treated groups. Histological examination of uteri revealed a marked increase in glandular development in estrogen-treated mice, i.e., more glands and more secretion. The ovaries of all control mice contained CL and appeared normal. On the other hand, all treatments appeared to depress ovarian function, for there were CL in the ovaries of only 26, 58, and 23% of mice in the estrogen, RPT, and RPT plus estrogen groups, respectively. In both estrogen-treated groups, those ovaries with CL had only one or 2 CL, whereas there were multiple CL in most ovaries of
widely varied responses in mouse mammary glands. Some
development was 1.8, 1.6, 4.8, and 3.1 for controls, estrogen,
the control group and in the CL-containing ovaries of the RPT
On a scale of 1 to 6, the mean mouse mammary gland
development was 1.8, 1.6, 4.8, and 3.1 for controls, estrogen,
RPT, and RPT plus estrogen treatment, respectively. Mouse
mammary glands in mice with RPT alone had increased
numbers of ducts and extensive lobuloalveolar development
compared to controls. RPT plus estrogen treatment produced
widely varied responses in mouse mammary glands. Some
glands were fully developed (comparable to RPT alone), while
others had development only slightly greater than controls.
Estrogen treatment alone did not stimulate mouse mammary
gland development as analyzed by whole-mount evaluation.
Rat prolactin levels in the sera of mice bearing RPT, with or
without estrogen, were > 4000 ng/ml. Despite the smaller size
of RPT in estrogen-treated mice, these RPT cells secreted very
high levels of prolactin. The larger RPT in mice with no estrogen
treatment may still have produced more prolactin than RPT
with estrogen, but our assay system was not sensitive beyond
4000 ng/ml, and differences beyond these levels could not be
reliably detected.

**DISCUSSION**

These studies provide compelling evidence that the epithe-
lium of human breast tissues, transplanted s.c. to athymic nude
mice, can be induced to grow by the treatment of host mice
with estrogens and pituitary hormones. In all 5 breast biopsy
specimens transplanted to nude mice, the mean [3H]thymidine
LI was significantly increased by administration of 17β-estro-
diol and estrone and the presence of a congruently growing
RPT in the murine host. Estrogen treatment alone increased
the LI of grafts in 3 of 4 HBT’s. Mouse blood serum radioim-
unoassay revealed extremely high levels of rat prolactin in all
animals bearing RPT. The significantly larger body weight gains
in the RPT-bearing mice indicated that growth hormone was
also being produced by the RPT. Nevertheless, human breast
epithelium did not respond to the rat pituitary mammotropic
hormones except in the presence of administered estrogens.

It is conceivable that growth factors from RPT other than
prolactin and/or growth hormone are acting synergistically
with estrogen to stimulate human breast DNA synthesis. Leung
and Shiu (4) recently reported a similar synergism of RPT (GH₃)
and estrogen in the estrogen-dependent T-47D human breast
carcinoma cell line grown in nude mice. Again, RPT alone
produced no growth stimulation. The authors proposed several
theories for these results but favored the concept that estrogen
stimulates the production by RPT of a growth factor or factors
not normally present in the absence of estrogen. Mittra (9, 10)
has demonstrated recently that a posttranslational cleavage of
rat prolactin yields a polypeptide chain structure (M.W. 16,000)
that had a marked mitogenic effect on rat mammary epithelium,
whereas purified rat prolactin had no such effect. It is conceiv-
able that estrogen stimulation initiated the biochemical events
that would provide more of the prolactin-derived mitogenic
factor.

One breast biopsy specimen (HBT 2) did not respond to the
administration of estrogen, suggesting that estrogens may not
always promote cell division of human breast epithelium. Yet,
with added RPT growth factors, estrogen invariably induced an
increase in DNA synthesis. In one HBT (HBT 3), response to
estrogen alone was as great as for RPT plus estrogen. It is
possible then that, in some HBT, the presence of pituitary
factors is necessary to prime the human breast epithelium to
respond directly to estrogen. For example, RPT factors may
induce estrogen receptors in the human breast epithelium.
Shafie and Brooks (14) described in vitro studies with the MCF-
7 human breast carcinoma cell line where the presence of
ovine or human prolactin in the media increased the level of
estrogen receptors in the cultured breast carcinoma cells.

Paradoxically, RPT secretions alone were sufficient for max-
imum host mouse mammary gland development but did not
affect the LI of the grafted human breast epithelium. With
added estrogen, RPT did not consistently increase mouse
mammary gland development but induced a striking and con-
sistent increase in the LI of the human breast tissues. Estrogen
administration alone produced no apparent increase in mouse
mammary gland development but did increase the LI of 3 of 4
of the human breast specimens. These results, the first to
directly compare rodent and human mammarie in the same
experimental system, provide substantial evidence that human
breast tissue hormonal requirements for growth are consider-
ably different from those of the murine species. Alternatively,
the mouse mammary glands may be far more responsive to
rodent pituitary hormones than are the HBT’s.

In this communication, we have provided ex vivo-in vivo
evidence, heretofore not reported, that estrogen is mitogenic
to the human breast epithelium and that pituitary factors (pro-
lactin, growth hormone, and/or other unknown mammotropic
hormones) act synergistically with estrogen to enhance mito-
genesis. The nude mouse model we have proposed has proven
to be very suitable for in vivo studies of normal human breast
epithelium, and we anticipate enthusiastically further elucid-
tion of the complex hormonal regulation of human breast epi-
thelium and of its transformation to carcinoma.

**ACKNOWLEDGMENTS**

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LeBlanc for technical assistance in this study and the doctors of our area
hospitals for their cooperation in supplying the breast biopsy specimens.

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of nude mice</th>
<th>Body wt (g)</th>
<th>Uterine wt (mg)</th>
<th>RPT wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>26.7 ± 1.0^a</td>
<td>130.0 ± 19.6 (e)</td>
<td></td>
</tr>
<tr>
<td>Estrogen</td>
<td>8</td>
<td>26.0 ± 1.2</td>
<td>214.5 ± 34.1 (f)</td>
<td></td>
</tr>
<tr>
<td>RPT</td>
<td>12</td>
<td>32.7 ± 1.3</td>
<td>96.8 ± 16.9 (g)</td>
<td></td>
</tr>
<tr>
<td>RPT + estrogen</td>
<td>13</td>
<td>33.1 ± 1.2</td>
<td>189.9 ± 17.1 (h)</td>
<td></td>
</tr>
</tbody>
</table>

^a Mean ± S.E.  
^b c/d, d/e, f/g, g/h, i/j, p < 0.01; e/f, e/h, p < 0.05.
REFERENCES


Fig. 1. Normal lobular ducts in graft of HBT 2 from a control nude mouse 60 days after transplantation. H & E, x 240.

Fig. 2. Lobular ducts in a graft of HBT 2 from a nude mouse treated with RPT plus estrogen for 30 days. 60 days after transplantation. Note hypertrophic epithelial cells and mitotic figures (arrows). H & E, x 240.

Fig. 3. Large hyperplastic duct with multiple layers of epithelial cells in graft of HBT 2 from nude mouse treated with RPT plus estrogen for 30 days, 60 days after transplantation. Note, as for Fig. 2, hypertrophic cells and mitotic figure (arrows). H & E, x 240.

Fig. 4. Autoradiograph, same duct as for Fig. 3, 10 to 15 μm distant, showing numerous [3H]thymidine-labeled cells. H & E, x 240.
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