Response of Beagle Mammary Dysplasias to Various Hormone Supplements in Vitro

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SUMMARY

The canine mammary tumor system is receiving increasing attention as a model system for drug testing and for experimental study of basic mammary biology. In order to contribute to this model system, the hormone sensitivity of three types of canine preneoplastic lesions was identified morphologically on the basis of in vitro responses of organ-cultured explants to various hormone supplements. Dysplastic tissues were selected in vivo on the basis of their difference in hormone responsiveness. Differential hormone sensitivity of dysplastic from normal tissues persisted in vitro. Three types of tissues were studied: ductal connective tissue dysplasias, hyperplastic alveolar lobular dysplasias, and explants of an adenoma. All maintained their structure in vitro, independent of the hormone supplement provided. Secretion in alveolar lesions was hormone-independent. Secretion in the adenotibroma was hormone responsive, both to supplements that promote secretion in normal tissues of beagles (cortisol, mammotropin, and insulin; and estradiol, progesterone, cortisol, mammotropin, somatotropin, and insulin) and to two hormone combinations to which normal tissues are unresponsive (mammotropin, somatotropin, and insulin; and estradiol, progesterone, cortisol, and insulin).

Exploitation of in vitro techniques promises to be a valuable tool for exploring the progression from normal to neoplastic in canine mammary tissues.

INTRODUCTION

Preneoplastic mammary lesions have been described, and their biological behavior has been characterized in mice (12, 13) and rats (2). Dysplasias precede tumors in rodents and are more numerous in treatment groups in which tumors are most common (2, 12, 13). Murine preneoplastic lesions have provided a valuable system for the study of human disease. Additional nonrodent models for human disease have been sought for basic research and for drug testing. The canine mammary system also has many advantages in this regard (15).

Warner (23) documented that in bitches between 2 and 4 years of age dysplasias occur prior to the time of palpable tumor development (at 6 to 8 years of age). Connective tissue and epithelial components contributed jointly to these dysplasias, as they also do to the ensuing tumors (7).

Canine mammary tumors are said to be hormone sensitive, although they may persist throughout the hormone fluctuations characteristic of reproductive life (6, 9). It is possible to distinguish systemic from local tissue level influences on hormone-sensitive tissues by using organotypic culture in which responses may be characterized in a chemically defined environment in the presence of known amounts of test compounds.

In the present pilot experiments, we have explored the hormone sensitivity in organ culture of 3 morphological types of early dysplastic lesions of the canine mammary gland as a means of elucidating their departure from normal and as an attempt to contribute to understanding of the process of canine mammary neoplasia.

MATERIALS AND METHODS

Animals. Purebred beagles of known age and reproductive history were obtained from the facilities of the Radiobiology Laboratory, University of California at Davis, through the courtesy of Dr. A. C. Andersen. Dry kibbled dog food was fed each morning, plus a can of Smokey moist food twice each week. Water was available from Lixit self-waterers. Animals were housed in wooden barrels in fenced 3- x 6.1-m outdoor pens.

Necropsy. Stage of the estrous cycle was determined as described by Warner (22), and anestrous bitches in the 2nd or subsequent cycle were selected. Animals were killed by electrocution with 110 V for 15 to 30 sec (Electrocutor; Electronics Shop of the Radiobiology Laboratory, University of California, Davis, Calif.). Mammary glands were dissected from the body wall as described by Warner (23) and were examined under a dissection microscope. Dysplasias were distinguished from the normal regressed tissues which surrounded them by empirical means using a dissection microscope. Duct dysplasias formed dense, hard knots which surrounded them by empirical means using a dissection microscope.

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2 Present address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

3 M. R. Warner. Differentiation of Beagle Mammary Glands In Vitro: During Oestrus, submitted for publication.
Table 1
Response to various hormone combinations of 2 types of canine mammary dysplasias after 5 days of culture
Lesions were excised from regressed mammae of 5 bitches, 2 to 4 years old, and were organ cultured at 37° with Waymouth’s Medium MD 705/1 in 95% O₂:5% CO₂ for 5 days with subculture Day 2.5 to 3.

<table>
<thead>
<tr>
<th>Hormone supplement</th>
<th>No. of dysplasias</th>
<th>Overall maintenance grade</th>
<th>No. of dysplasias</th>
<th>Overall maintenance grade</th>
<th>Secretion grade</th>
</tr>
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<tbody>
<tr>
<td>Insulin</td>
<td>8</td>
<td>4*</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>MSI</td>
<td>20</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>17β-Estradiol-progesterone-insulin</td>
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<td>4</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Somatotropin-insulin</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FMI</td>
<td>11</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cortisol-somatotropin-insulin</td>
<td>2</td>
<td>4</td>
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<td>0</td>
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<tr>
<td>EPFI</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>EPFMSI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>4</td>
<td>14</td>
<td>4</td>
<td>4</td>
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</tbody>
</table>

* Four was the maximum response for maintenance of secretion on a scale of 0 to 4.

**Culture Method.** Explants were transferred to pieces of washed, sterile lens paper, supported by a stainless steel mesh raft in a prepared culture chamber (Bioquest, Cockeysville, Md.). The central well was filled with 1 ml of nutrient medium. Three ml of sterile, double-distilled water were used to moisten the absorbent outer ring. The maximum time from the initial excision of tissue until the chamber with medium and tissue was placed in the incubator was 1 hr. Dishes were incubated at 37° in 95% O₂:5% CO₂ saturated with water vapor. Gas flow was adjusted to maintain a pH of 7.4. The medium was changed after 60 to 70 hr of culture. Experiments ended on Day 5 of culture.

The basic medium was either Waymouth’s MB752/1 or Waymouth’s MD705/1 (Grand Island Biological Co., Grand Island, N. Y.). Stock solutions of hormones at 10-fold final concentration were prepared as described by Rivera (17). Final concentrations of hormones were: insulin, 5 μg/ml (Calbiochem, Los Angeles, Calif.); 17β-estradiol, 0.001 μg/ml (Calbiochem); progesterone, 1 μg/ml (Schering, Union, N. J.); cortisol, 1 μg/ml (Calbiochem); mammotropin 5 μg/ml (NIH-P-S8); somatotropin, 5 μg/ml (NIH-GH-BB). No antibiotics were used in final media.

Explants were cultured with various hormone supplements (Table 1; Fig. 1), and their responses were compared after culture. The basic medium was supplemented with the following hormone combinations: (a) insulin; (b) mammotropin-insulin; (c) somatotropin-insulin; (d) MSI; (e) estrogen-progesterone-insulin; (f) EPFI; (g) FMI; (h) cortisol-somatotropin-insulin; and (j) EPFMSI.

**Histology.** Explants were fixed in Tellyesniczky’s fluid for 48 hr and then transferred to 70% alcohol, dehydrated, embedded in paraffin, sectioned serially at 7 μm, and stained with a modification of Masson’s trichrome stain (11).

**Evaluation Criteria.** All tissues were evaluated on the basis of the appearance of serial histological sections. The entire area of the explant was considered, and an average grade value was assigned to the whole explant. Grades used were: 0, all tissue nonsecretory, or no tissue maintained; 1, viable (or secretory) tissue found in less than one-half of explant; 2 one-half of explant viable (or secretory); 3, more than one-half of explant viable (or secretory); 4, entire explant viable (or secretory, as relevant). Secretion was evaluated on the basis of stainable noncellular luminal content. When a trial sample of 3 sets of glands was rated on 3 occasions, the correlation coefficient for ratings agreed at the 0.001% level. For purposes of statistical analysis, the previous 5 major classes were further subdivided by plus-and-minus groupings, after separate gradings and a subsequent check. Special note was made of types and/or patterns of necrosis, abnormal patterns of development, growth into medium, staining properties, and morphological types of secretion. “Maintenance” was judged on the basis of tissue viability. Presence of pyknotic or degenerated nuclei and loss of cytoplasmic staining were considered.

**Statistical Methods.** Statistical significance was determined using the Mann-Whitney U test (19).

**RESULTS**

**Duct Dysplasias (Table 1; Figs. 2 and 3).** Fig. 3 shows histology of normal controls. A total of 44 lesions from 5 bitches 2 to 4 years of age was examined (Table 1). Maintenance was excellent with all hormone supplements tested.

**Hyperplastic Alveolar Dysplasias (Table 1; and Figs. 1 and 9).** A total of 14 lesions from 2 bitches 2 to 4 years of age was examined (Table 1). Both maintenance and secretion were excellent with all hormone supplements tested.

**Adenoma (Chart 1; Figs. 6 to 11).** A 4-year-old bitch developed the 4- x 6-cm adenoma used in this experiment. Explants cultured with insulin, mammotropin-insulin, somatotropin-insulin, 17β-estradiol-progesterone-insulin, EPFI, FMI, or EPFMSI were maintained at Grade 2 or 3 in all hormone supplements and did not differ significantly from the insulin group except after culture with MSI which de-
The number of alveolar dysplasias studied in the present series was too small to determine their response to individual hormone combinations; however, alveolar dysplasias remained secretory in media that did not support secretion in contiguous normal areas and thus displayed a reduced sensitivity to hormone deprivation.

A progression of hormone sensitivity has been affirmed in vitro for murine mammary tissues. Normal tissues are most dependent on the hormonal environment, hyperplastic tissues are intermediate in hormone sensitivity, and tumors tend to be least hormone dependent (1). Elias and Rivera (5) found that explants of murine adenocarcinomas, unlike explants of normal or dysplastic tissues, survived in the presence of toxic levels of progesterone (18) and in the absence of insulin. Tumor tissues were also less dependent on the presence of hormones for survival in vitro (5). Murine tumors did not require insulin in order to use glucose in vitro (14). According to the work of Turkington and Riddle (21), tumors are not able, as normal cells are, to differentiate to form casein, lactalbumin, or lactose synthetase A protein in vitro in response to FMI supplementation of the basic medium. DNA synthesis was stimulated by insulin but was inhibited by estrogen in both normal and tumor cells (20).

Hormone responsiveness was present, but it occurred at a reduced level in explants of a secretory canine mammary adenoma in the present study. A differentiated secretory state was maintained after culture with supplements that were subminimal for maintenance of differentiation in control metestrous tissues. However, adenoma explants still responded to differentiation-inducing hormone supplements (FMI or EPFMSI) by an increase in secretion.

Increase in secretion in canine tissue after culture with EPFI may reflect a decreased inhibitory effect of progesterone in dysplastic tissues such as that reported by Elias and Rivera (5) for murine hyperplastic alveolar nodules.

The malignant potential of the tissues studied in this paper has not been experimentally defined, so that the relevance of the present findings to breast cancer research is speculative.

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REFERENCES


Fig. 1. Gross dissection of the skin with attached mammary glands shows several lobular dysplasias (L); B, body wall. x 0.5.

Fig. 2. Mammary glands are pinned to a board, ready for fixation. Dense, mixed, connective tissue and epithelial dysplasias are shown (D). N, nipple. x 1.

Fig. 3. A spherical duct dysplasia. Morphology is similar in noncultured controls and after 5 days in culture with cortisol, mammatropin, and insulin. Note the connective tissue core with epithelium on its surface. Masson's trichrome, x 200.

Fig. 4. A lobular hyperplasia. Morphology is similar in noncultured controls and after 5 days of culture with insulin. Upper right, normal nonsecretory tissue. Masson's trichrome, x 200.

Figs. 5 to 11. Microscopic morphology of mammary adenoma explants from an 8-year-old bitch. Female pregnant in 6 of 8 estrous cycles. Culture for 5 days in 1972's medium MB 752/1, supplemented with various hormone combinations. Masson's trichrome.

Fig. 5. Histologic control for Figs. 6 to 11. Lobular structure is poor. Uniform secretion is present in most alveoli, although there are some clusters of nonepithelial cells. x 160.

Fig. 6. Medium supplemented with insulin. There is vacuolated secretion in some areas of the explant. Clumps of epithelium in other areas are maintained but not secretory. x 160.

Fig. 7. Magnification of Fig. 6. Details of the secretory process as well as irregularities of alveolar structure are illustrated. x 400.

Fig. 8. Medium supplemented with MSI. The tissue at the lower right stains like epithelium, with Biebrich Scarlet. These reorganized cells appear to be migrating into the medium. Both uniform and vacuolated secretion are present. Sloughed cells are present in lumina. Intraalobular connective tissue is sparse. x 160.

Fig. 9. Medium supplemented with EPFI. Secretion is rare. Many lumina contain sloughed cells and cell debris. Alveolar morphology is abnormal at the lower left. x 160.

Fig. 10. Medium supplemented with EPFMSI. Ducts and alveoli contain uniform secretion and cell debris. Intraalobular connective tissue is sparse. x 160.

Fig. 11. Magnification of Fig. 10, showing details of secretion. x 400.
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