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Research Article

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Growth Hormone mRNA in Mammary Gland Tumors of Dogs and Cats

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Abstract

We have shown recently that in the dog progestin administration results in mammary production of immunoreactive growth hormone (GH). At present we demonstrate the expression of the gene encoding GH in the mammary gland of dogs and cats using reverse-transcriptase PCR.

GH mRNA was found in the great majority of normal mammary tissues as well as benign and malignant mammary tumors of the dog and was associated with the presence of immunoreactive GH in cryostat sections. The mammary PCR product proved to be identical to that of the pituitary. The highest expression levels were found after prolonged treatment with progestins. In carcinomas GH mRNA was also found in progesterone receptor–negative tissue samples, indicating that after malignant transformation GH gene expression may become progestin independent. GH mRNA was also present in mammary tissues of cats with progestin-induced fibroadenomatous changes.

It is concluded that GH gene expression occurs in normal, hyperplastic, and neoplastic mammary tissue of the dog. The expression in normal tissue is stimulated by progestins and might mediate the progestin-stimulated development of canine mammary tumors. The demonstration of progestin-stimulated GH expression in mammary tissue of cats indicates that the phenomenon is more generalized among mammals. (*J. Clin. Invest.* 1995. 95:2028–2034.) Key words: cats • dogs • mammary tumor • progestins • growth hormone

Introduction

The role of progestins in the pathogenesis of human breast cancer has been highly debated (1–4). Although there is convincing evidence that ovarian hormones play a prominent role at all stages of mammary development, epidemiologic studies in general do not support an overall increased risk of breast cancer after progestin treatment. However, oral contraceptives or depot medroxyprogesterone acetate does not decrease the risk of female breast cancer, in contrast to inhibitory effect on the risk of developing endometrial cancer. Furthermore, several epidemiologic studies have linked the use of contraceptive agents during adolescence or before a full-term pregnancy to a higher risk of developing breast cancer at a young age (4–6). The question of whether this increased risk is attributable to the estrogen or the progestin content of the contraceptives has not been answered satisfactorily, but there is good reason to think that this increased risk may be due to the progestin component (7–9). This hypothesis is supported by the observations that the highest proliferation rates of mammary epithelium are found in the progesterone-dominated luteal phase of the menstrual cycle and in women receiving progestin-only formulations of contraceptives (3, 10), indicating a strong mitogenic action of progestins upon mammary epithelium.

In dogs and cats, as in humans, spontaneous forms of benign and malignant mammary gland tumors are found. These animals may therefore provide a model, in addition to the carcinogen-induced mammary tumors in rodents, for the study of comparative aspects of mammary tumor formation. In the dog many toxicity studies have been carried out with estrogens and progestins. Prolonged administration of estrogens does not increase the incidence of mammary tumors in the dog (11), but treatment of female dogs with progestins causes mammary tumor development in a dose-dependent manner (11–15). Many of the neoplastic changes are benign, but malignant tumors have also been encountered (14, 15).

Some 14 yr ago it was shown that exogenous (16–18) and endogenous (19) progestins may induce a syndrome of growth hormone (GH)[1] excess in the dog, leading to acromegalic features and glucose intolerance. The GH excess is reversible upon progestin withdrawal. Very recently we reported that this progestin-induced growth hormone excess originates from foci of hyperplastic ductular epithelium of the mammary gland (20).

In cats progestins also induce mammary hyperplasia, sometimes resulting in extensive fibroadenomatous changes (21). This proliferation of mammary duct epithelium and stroma occurs in the luteal phase of the estrous cycle, in the early stages of pregnancy, and after administration of progestins (22). In cats, however, administration of progestins does not result in increased circulating concentrations of GH (23).

In this study we investigated the sequence of the cDNA encoding mammary GH in the dog and compared this sequence with the cDNA encoding pituitary GH. The presence of the GH mRNA was investigated in normal mammary tissue and in benign and malignant mammary tumors of the dog. The presence of GH mRNA was also investigated in cats with fibroadenomatous changes and other proliferative lesions in the mammary gland.

Methods

Origin of mammary tissue. Eight mammary tissue samples were obtained from female experimental beagle dogs that had been treated with long-acting progestins after ovariohysterectomy. Full details of treat-

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1. *Abbreviations used in this paper:* ER, estrogen receptor; GH, growth hormone; MPA, medroxyprogesterone acetate; PR, progestin receptor; RT, reverse transcriptase.

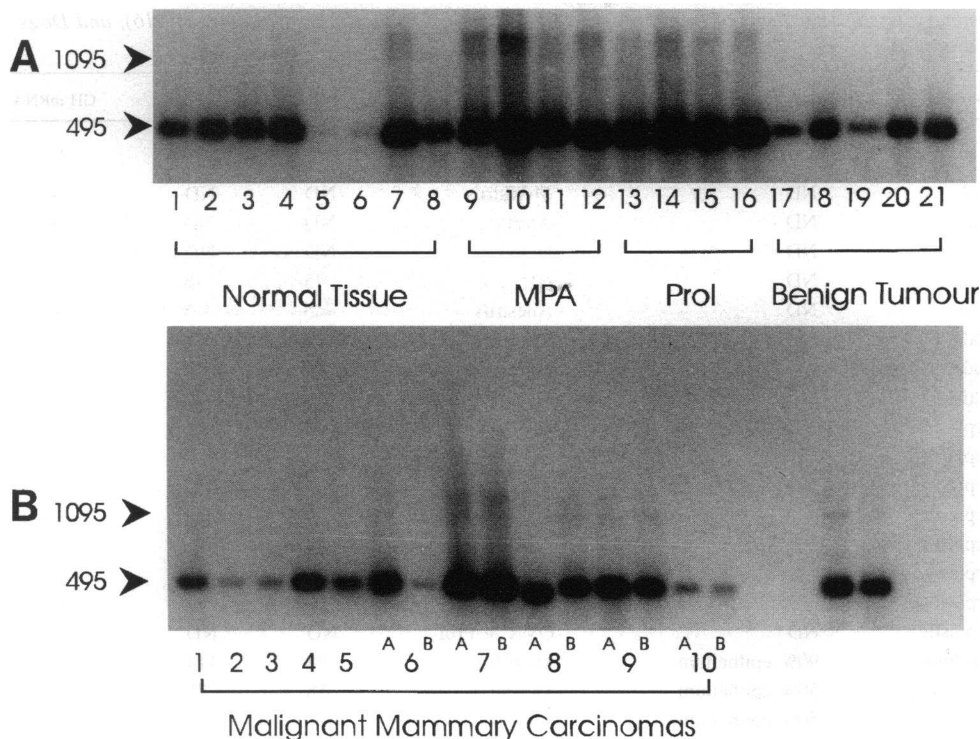

Figure 1. Size separation by electrophoresis on 1.5% agarose of the RT-PCR products obtained from 1 μg total RNA of canine mammary tissues using porcine specific GH primers. The RT-PCR products were hybridized after a Southern blot using a ^{32}P-labeled canine GH cDNA probe. The codes for the products correspond to the data for the tissue samples in Table I. (*A*) Samples 1–8 were obtained from female dogs at various stages of the reproductive cycle. Samples 9–16 were obtained after pretreatment with the synthetic progestins MPA or proligeston (*Prol*). Samples 17–21 were classified as benign mammary tumors. (*B*) Samples derived from malignant tumors.

ment and hormonal changes have been published previously (20). Briefly, four dogs were treated with medroxyprogesterone acetate (MPA; Upjohn, Ede, The Netherlands; 10 mg/kg body wt), and four dogs were treated with proligestone (Mycofarm, De Bilt, The Netherlands; 50 mg/kg body wt). The progestins were administered at 3-wk intervals for a total of 8 injections, stopped for 6 mo, and then resumed at the same doses and intervals for a total of 5 additional injections. Mammary glands were collected immediately after excision, frozen in liquid nitrogen, and stored at −70°C until analysis. The other mammary specimens were obtained in the same manner from privately owned dogs and cats that were referred to the Utrecht University Clinic for Companion Animals. Mammary tumors and unaffected mammary tissue were well separated from each other during collection at surgery or, in inoperable cases, at autopsy. Sections from all macroscopically tumorous and nontumorous specimens were processed for routine histopathology and classified as described (24).

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using the guanidinium isothiocyanate (GITC) method. Briefly, 1 g of tissue was homogenized in 10 ml denaturing mix (4 M GITC, 25 mM sodium citrate, 0.5% [wt/vol] sarcosyl, and 1% β-mercaptoethanol) by ultra-turrax (Janke & Kunkel, Staufen, Germany) for 30 s. To the homogenate was added 1 ml 2 M NaOAc (pH 4.5), 10 ml phenol, and 2 ml chloroform:iso-amylalcohol (24:1), and this was followed by incubation on ice for 15 min. From the upper phase, obtained after centrifugation for 15 min at 10,000 *g*, total RNA was precipitated with 10 ml isopropanol by incubation for 1 h at −70°C and centrifugation at 3,600 *g*. The pellet was redissolved in denaturing mix and RNA was again precipitated by isopropanol. After washing with 75% ethanol the RNA pellet was dried under vacuum and redissolved in 10 mM Tris (pH 8.0) containing 1 mM EDTA.

The RNA samples were analyzed by RT-PCR amplification. For the RTs, 1 μg total RNA was added to a 20 μl (final volume) reaction containing 1 × AMV buffer (Promega Corp., Madison, WI), 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 20 U RNase inhibitor (RNasin; Promega Corp.), 12 U AMV reverse transcriptase, and 2.5 μM Oligo d(T)$_{15}$ primer. The reaction mixture was overlaid with 50 μl of mineral oil and incubated at 42°C for 60 min, followed by heat denaturation at 99°C for 5 min and cooling at 5°C for 5 min. The total RT reaction was then subjected to PCR amplification using cDNA primers derived from the sequence of the porcine GH gene from the second to the fifth exon (upstream primer: 5′-TTCCCAGCCATGCCC-TTGTCC; downstream primer: 5′-CTTGAAGCAGGAGAGCAG-CCC). The upstream primer coincides with the start codon of the GH sequence, which is 60 bp downstream from the start codon of the preprohormone starting with a signal peptide. The downstream primer ends within the coding sequence of the fifth exon. To the RT mixture was added 69.5 μl sterile bidistilled water, 0.5 μl *Ampli Taq* DNA polymerase (5 U/μl; Promega Corp.), 8 μl 10 × *Taq* buffer, and 1 μl of each primer (10 pmol). After an initial denaturation for 5 min at 95°C the cDNA was amplified by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Samples were kept for 10 min at 72°C after the last amplification cycle.

10% of the PCR product was size-fractionated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The PCR products were then transferred by southern blotting to *Hybond-N*$^+$ nitrocellulose membranes (Amersham International, Buckinghamshire, United Kingdom) and hybridized with a ^{32}P–random-labeled canine GH probe purified for sequence analysis.

In an additional study a semi-quantitative RT-PCR was performed for the analysis of the progestin induction of mammary GH mRNA expression. Therefore the RT-PCR of GH mRNA was performed in the presence of primers directed against the mRNA encoding the housekeeping gene β-actin (upstream primer: 5′-GGCTGGGGTGTTGAA-GGTCTC; downstream primer: 5′-GATATCGCCGCGCTCGTCGTC).

Sequence analysis. The 495-bp fragment obtained from mammary tissue of experimental dogs treated with progestins was isolated from the gel and subjected to a second PCR amplification. The sequence of this product was determined by the dideoxy method (25). The 600-bp fragment found in mammary tumor tissue of dogs was also subjected to a second PCR amplification and partially sequenced. The sequences were analyzed with the *PC/GENE*® software package (IntelliGenetics, Inc./Betagen, Mountain View, CA). Homology comparisons were made with the EMBL sequence database of the CAOS/CAMM Centre (Nijmegen, The Netherlands).

Immunohistochemistry. The presence of GH in mammary tissue was shown by immunohistochemical analysis of 5-μm sections of frozen specimens of canine mammary tumors as described previously (20). A rabbit anti–porcine GH antiserum was used as the primary antibody.

Table I. Some Characteristics of Mammary Tissue Specimens of Healthy Control Dogs (1–8), Progestin-treated Dogs (9–16), and Dogs with Benign (17–21) or Malignant Tumors

Sample	Code	Histology	% Cells	Cycle	ER	PR	GH mRNA
Normal and hyperplastic tissues							
1	A7	Normal	ND	Metestrus	ND	ND	+
2	A11	Normal	ND	Anestrus	ND	ND	+
3	P206B	Normal	ND	ND	ND	ND	+
4	Et44B	Normal	ND	ND	45	78	+
5	Et42	Inactive	ND	Anestrus	<5	<5	−
6	8609	Normal	ND	Anestrus	19	18	−
7	P117R4	Normal	ND	Metestrus	ND	ND	++
8	P182L4	Normal	ND	MPA—5 mo	35	36	+
9	A2	Hyperplastic	ND	OVX + MPA	ND	ND	++
10	A3	Hyperplastic	ND	OVX + MPA	ND	ND	++
11	A4	Hyperplastic	ND	OVX + MPA	ND	ND	++
12	A5	Hyperplastic	ND	OVX + MPA	ND	ND	++
13	B1	Hyperplastic	ND	OVX + Prol	ND	ND	++
14	B4	Hyperplastic	ND	OVX + Prol	ND	ND	++
15	B6	Hyperplastic	ND	OVX + Prol	ND	ND	++
16	B7	Hyperplastic	ND	OVX + Prol	ND	ND	++
17	P154	Benign tumor	90% epithelium	Metestrus	98	114	+
18	P79	Benign tumor	50% epithelium	Metestrus	58	75	+
19	P51L4	Benign tumor	50% epithelium	MPA—3 mo	22	<5	+/−
20	P81R4	Benign tumor	50% epithelium	MPA—4 mo	25	45	+
21	P60	Benign tumor	50% epithelium	Metestrus	96	173	+
Malignant mammary carcinomas							
1	P78	Anaplastic	30% carcinoma	Metestrus	18	21	+
2	P157	Tubular	40% carcinoma	OVX—2 yr	<5	26	+/−
3	P159	Anaplastic	5% carcinoma	Prol—7 mo	ND	ND	+/−
4	P177	Solid	75% carcinoma	OVX—3 yr	6	<5	+
5	P191R4	Solid	40% carcinoma	OVX—5 yr	3	<5	+
6A	P170R5	Complex	90% carcinoma	Metestrus	42	147	+
6B	P170L4	Normal 6A	70% epithelium	Metestrus	119	40	+/−
7A	P221L4	Anaplastic	5–70% carcinoma	Prol—1 wk	ND	ND	++
7B	P221L5	Normal 7A	ND	Prol—1 wk	ND	ND	++
8A	P1881	Carcinoma	1% carcinoma	Lactating	ND	ND	+
8B	P188ln	Metastasis	40% carcinoma	Lactating	ND	ND	+
9A	P211L3	Carcinoma	70% carcinoma	MPA—2 mo	ND	ND	+
9B	P211ln	Metastasis	90% carcinoma	MPA—2 mo	ND	ND	+
10A	P217R4	Carcinoma	25–50% carcinoma	Lactating	4	12	+/−
10B	P217ln	Metastasis	50% carcinoma	Lactating	2	<3	+/−

Samples correspond to the numbers of Fig. 1. Histology, findings in formaldehyde-fixed tissue samples. Cycle, *OVX*, ovariohysterectomy; the time is the interval between intervention and tissue sampling; *Prol*, proligeston. ER/PR, receptor concentrations in fmol/mg protein in tissue cytosol. ND, not done. GH mRNA, ++, abundant; +, present; −, absent; +/−, faint band.

The antiserum was a generous gift of Dr. M. M. Bevers (Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University). A peroxidase-conjugated, goat anti-rabbit IgG antiserum was used as the second antibody. Specimens in which the first antiserum was omitted served as negative controls.

Steroid receptor analysis. Determination of estrogen (ER) and progestin receptors (PR) in frozen tissue specimens was performed by a multiconcentration dextran-coated charcoal assay (26).

Results

All experimental dogs treated with high doses of either MPA or proligestone had developed acromegalic features (20). Amplification of GH mRNA in nontumorous mammary tissue samples of these dogs by RT-PCR using porcine specific GH primers resulted in the presence of a 495-bp fragment in all samples (Fig. 1 *A*; Table I). Single strand conformation polymorphism analysis revealed no difference between the 495-bp products obtained from the pituitary and mammary tissue (not shown). The identity of the mammary and the pituitary products was confirmed by sequence analysis (Fig. 2). A very high homology was found with pituitary GH sequences of other species. The highest homology was found with mink and porcine GH, and lower homology was found with GH from artiodactyls and from rat and various human GH variant genes (Fig. 3).

```
         10        20        30        40        50        60
CAACGCCGTGCTCCGGGCCCAGCACCTGCACCAACTGGCTGCCGACACCTACAAAGAGTT
 AsnAlaValLeuArgAlaGlnHisLeuHisGlnLeuAlaAlaAspThrTyrLysGluPhe

         70        80        90       100       110       120
TGAGCGGGCGTACATCCCCGAGGGACAGAGGTACTCCATCCAGAACGCGCAGGCCGCCTT
 GluArgAlaTyrIleProGluGlyGlnArgTyrSerIleGlnAsnAlaGlnAlaAlaPhe

        130       140       150       160       170       180
CTGCTTCTCGGAGACCATCCCGGCCCCCACGGGCAAGGACGAGGCCCAGCAGCGATCCGA
 CysPheSerGluThrIleProAlaProThrGlyLysAspGluAlaGlnGlnArgSerAsp

        190       200       210       220       230       240
CGTGGAGCTGCTCCGCTTCTCCCTGCTGCTCATCCAGTCGTGGCTCGGGCCCGTGCAGTT
 ValGluLeuLeuArgPheSerLeuLeuLeuIleGlnSerTrpLeuGlyProValGlnPhe

        250       260       270       280       290       300
TCTCAGCAGGGTCTTCACCAACAGCCTGGTGTTCGGCACCTCAGACCGAGTCTACGAGAA
 LeuSerArgValPheThrAsnSerLeuValPheGlyThrSerAspArgValTyrGluLys

        310       320       330       340       350       360
GCTCAAGGACCTGGAGGAAGGCATCCAAGCCCTGATGCGGGAGCTGGAAGATGGCAGTCC
 LeuLysAspLeuGluGluGlyIleGlnAlaLeuMETArgGluLeuGluAspGlySerPro

        370       380       390       400       410       420
CCGGGCCGGGCAGATCCTGAAGCAGACCTACGACAAGTTTGACACGAACCTGCGCAGTGA
 ArgAlaGlyGlnIleLeuLysGlnThrTyrAspLysPheAspThrAsnLeuArgSerAsp

        430
CGATGCGCTGC
 AspAlaLeu
```

Figure 2. Nucleotide sequence of the 495-bp fragment amplified by RT-PCR from canine mammary gland RNA using porcine specific GH primers and the translation into protein. The product proved to be identical to the 495-bp fragment obtained by amplification of pituitary GH mRNA.

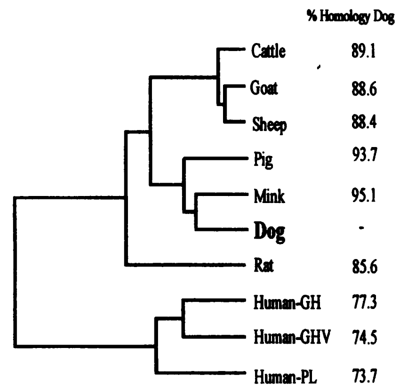

Figure 3. Dendrogram of the relationship of the canine 495-bp sequence encoding part of canine GH, with the corresponding part of well-known sequences of GH of different species. Data were obtained from the nucleic acid databank of the CAOS/CAMM Centre (Nijmegen, The Netherlands).

GH mRNA was expressed in most of the normal, benign, and malignant mammary tissues of dogs, listed in Table I (Fig. 1). The presence of GH mRNA corresponded to the immunohistochemical staining of tissue specimens with anti–porcine GH antiserum (Fig. 4). The GH immunoreactivity was predominantly localized in cells of hyperplastic ductular epithelium. Although the RT-PCR is not quantitative, the highest concentrations of the 495-bp fragment were obtained after amplification of cDNA of dogs treated with progestins. GH mRNA was absent in one sample of inactive tissue that was PR negative and in one tissue sample obtained from a dog in anestrus (Table I). Among the five benign tumors, the lowest amount of GH mRNA was found in a PR-negative tissue sample.

There was a large variation of GH mRNA concentrations in the malignant mammary carcinomas (Fig. 1 *B*). The highest levels were found in dogs that had received progestins recently. The expression in primary tumor tissue was higher (Fig. 1 *B*, sample *6*) or comparable (Fig. 1 *B*, sample *7*) to that in normal tissue or metastases (Fig. 1 *B*, samples *8–10*) obtained from the same dog. It is noteworthy that GH mRNA expression was found in PR-negative tissue of two solid tumors of dogs that had been ovariohysterectomized at least 3 yr before.

In female cats the highest expression of the 495-bp fragment was found in areas of fibroadenomatous changes induced by the administration of different progestins with or without estradiol (Table II, Fig. 5). The expression was also present in mammary tissue of a male cat that had been treated with the synthetic progestin delmadinone acetate (Fig. 5, sample *4*), for its androgen antagonistic properties. No expression was found in two cases of feline epitheliosis. Varying amounts of the 495-bp fragment were present in mammary tissue of cats with mammary carcinomas. The expression was most prominent in a cat receiving megestrol acetate continuously.

For a more quantitative analysis of the GH mRNA expression, the RT-PCR reaction was performed in the presence of primers against GH mRNA and the housekeeping gene β-actin (Fig. 6). Ethidium bromide staining of the agarose gels on which the combined PCR products of canine mammary RNA were separated shows an enhanced concentration of the 495-bp GH mRNA product in comparison with the concentration of the 383-bp product of β-actin after progestin treatment (Fig. 6). As shown, a 600-bp fragment is amplified in the RT-PCR. In other tissue samples, whether normal, hyperplastic, or neoplastic, a 600-bp fragment was also amplified. This fragment did not react with the canine GH probe on the Southern blot, indicating that this fragment was not related to the GH mRNA. Sequence analysis of the 600-bp fragment failed to demonstrate an unambiguous homology to well-known genes of other species. Translation of the 5′ side of the PCR product, using an open reading frame, showed motifs in the corresponding protein sequence of a glycosaminoglycan attachment site and an RGD structure pointing to a cell attachment sequence (Fig. 7).

Discussion

The demonstration of the expression of the gene encoding pituitary GH in canine and feline mammary tumors reveals a new aspect of the local endocrine environment of normal and tumorous mammary epithelium.

In the past decade, investigation of mechanisms involved in the growth regulation of breast cancer has changed from the concept of systemic endocrine stimulation of proliferation towards autocrine and paracrine processes. In the growth regulation of breast cancer cells, peptidergic growth factors play a prominent role. cDNA probes have revealed the expression of TGFα, IGF-II, and PDGF in breast cancer epithelial cells (27). In research on endocrine stimulation of breast cancer the main attention has been focused on estrogens and prolactin, which are also produced in the mammary gland (28, 29). It has been shown that in human breast cancer there is autonomous regulation of the tissue estradiol concentration (29), which does not depend on plasma levels. The primary role of estrogens may be mainly related to induction of the PR concentration (10). In the dog, prolonged treatment with estrogens does not increase the incidence of mammary tumors (11) and is also not a prerequisite for the induction of GH excess by progestins (18, 20). In our limited series of feline fibroadenomatous lesions, expression of GH mRNA was found in animals treated with progestins and appeared not to depend on treatment with estrogens. GH mRNA expression was even present in a male cat treated with a synthetic progestin.

Of the pituitary hormones both nonlactogenic and lactogenic forms of GH have been proved to be far more potent than prolactin in directly stimulating mammary gland differentiation

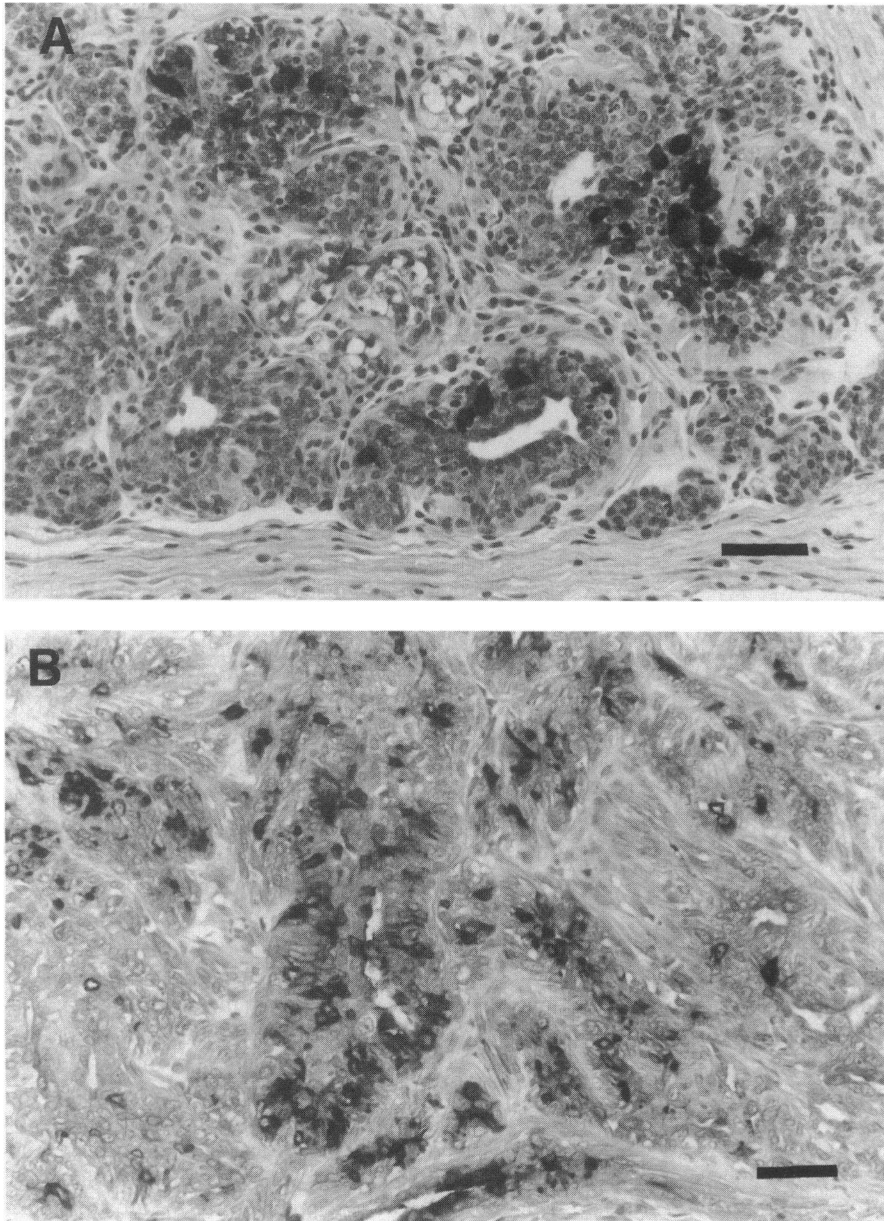

Figure 4. Immunochemical analysis of GH in canine mammary tissue on 5-μm slides obtained from paraffin-embedded, Bouin Hollande–fixated tissue specimen. (*A*) Mammary tissue obtained after prolonged treatment of ovariohysterectomized dogs with synthetic progestins. The immunoreactive GH is present in epithelial cells in areas of hyperplastic ductular epithelium. Positive cells are situated in a basal to intermediate position in these hyperplastic areas. The bar represents 15 μm. (*B*) Immunohistochemical staining for canine GH in a fibroadenoma, complex type. Immunoreactive cells are confined to tumorous secretory epithelium, myoepithelial proliferations are negative for GH. The bar represents 15 μm.

(30). This important regulator of cell growth and differentiation has now been proved to be produced locally in the mammary gland of the dog and the cat under the influence of progestins. Mammary GH may have a physiological role in mammary gland differentiation. This differentiation requires recruitment of stem cells that, after an early differentiation, may fully develop into ductular epithelium by clonal expansion under the influence of the IGFs. This process may be a generalized dual effector pathway of the action of GH and IGFs in various tissues, as hypothesized by Green et al. (31). Malignant transformation may occur during the recruitment phase, giving rise to mammary cancer originating from mammary stem cells in both humans (32) and dogs (33). Moreover, progestins may enhance the mammary production of IGF-II, as shown in the human breast cancer cell line T-47D (34). Taken together, the progestin-induced synthesis and release of GH and of IGF-II may generate an environment highly promoting proliferation of the mammary epithelium. In humans, administration of high doses of MPA increases plasma IGF-I levels (35), most probably via increased plasma GH levels. Whether this is caused by local mammary GH production needs to be clarified. In view of the finding of progestin-induced GH expression in the mammary gland and the well-known tumor-promoting effect of progestins in dogs and cats (11), additional studies are needed to determine whether the tumorigenic effect of the steroid hormones is mediated by GH and GH-stimulated growth factors.

Progestins may temporarily enhance sensitivity to carcinogens by creating a highly proliferative environment. It is uncertain whether GH induction in the mammary gland is involved in this proliferative effect and contributes to tumorigenesis, or whether instead the local GH effects promote differentiation of the gland and are thus protective against initiation of tumor development.

In the benign mammary tumors of dogs and cats, a definite relationship was found between the presence of GH mRNA and exposure to endogenous or exogenous progestins. Such a

Table II. Some Characteristics of Mammary Tissue Specimens Obtained from Cats

Sample	Code	Histology	Cycle	ER	PR	GH mRNA
1	K2R1	Fibroadenomatous change	MPA—10 wk	16	112	++
2	KMT56	Hyperplasia	MPA—2.5 mo	55	46	+
3	GRK28	Fibroadenoma	MA + EE—1 mo	6	12	+
4	GRK40	Fibroadenomatous change	DA—6 wk	0	67	+
5	GRK42	Fibroadenomatous change	MA—1 mo	15	71	++
6	GRK22	Epitheliosis	OVX—8 mo	21	22	−
7	GRK29	Epitheliosis	MA + EE—5 mo	13	5	−
8	GRK33	Metastasis	OVX—8 yr	1	3	−
9	GRK36	Carcinoma	MA—4 mo	6	4	−
10	GRK14	Carcinoma	MA (continuous)	<3	16	+
11	GRK25	Carcinoma	MA + EE—3 mo	41	32	+/−

Samples correspond to the numbers of Fig. 5. Code, tissues from female cats except GRK40 = male cat. Cycle, MA, megestrol acetate; EE, ethinyl estradiol; DA, delmadinone acetate; OVX, ovariohysterectomy; the time is the interval between intervention and tissue sampling. ER/PR, receptor concentrations in fmol/mg protein in tissue cytosol. GH mRNA, ++, abundant; +, present; −, absent; +/−, faint band.

relation was much less certain for the malignant mammary tumors. It cannot be excluded that in some of the latter tumors GH is produced constitutively even by PR-negative cells. Studies are needed to relate GH production to steroid receptor concentrations and biologic behavior of the tumor.

The expression of the 600-bp fragment was not related to GH mRNA expression or to histologic criteria of malignancy. The presence of a glycosaminoglycan attachment site and an RGD sequence suggests that this protein belongs to the family of proteoglycans. These proteins are important modifiers of the organization of the pericellular and extracellular matrices. Their functions range from linking proteins to hyaluronan to providing a binding site for fibroblast growth factor or TGFβ in the extracellular matrix (36, 37). The function of this canine protein in cell attachment and/or metastasis needs further characterization.

These data indicate that progestins stimulate benign proliferation of mammary tissue in the dog and the cat by the local induction of the GH gene. In the dog this results in acromegalic features due to high plasma levels of GH and IGF-I (20). Elevated plasma IGF-I levels may act in concert with the mam-

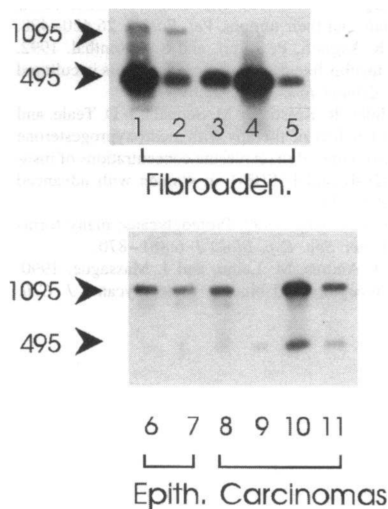

Figure 5. Southern blot hybridization of the RT-PCR products obtained from 1 μg total RNA of feline mammary tissues hybridized with a ^{32}P-labeled canine GH cDNA probe. The codes correspond to the data in Table II. Samples 1–5 are mammary RNA from cats with progestin-induced fibroadenomatous (*Fibroaden.*) changes. Samples 6 and 7 were obtained from cats with mammary epitheliosis (*Epith.*), while samples 8–11 were from malignant mammary carcinomas.

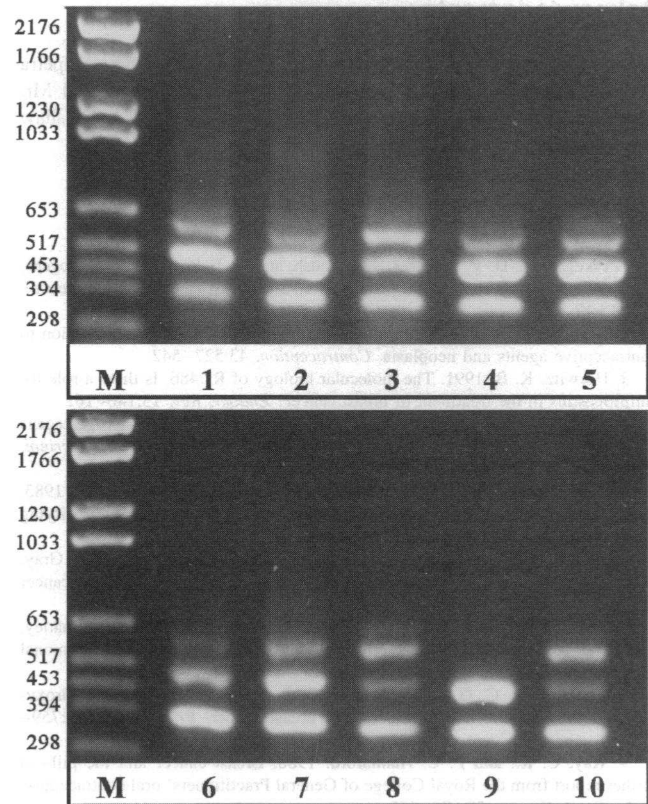

Figure 6. Size separation by electrophoresis on 1.5% agarose of RT-PCR products obtained from 1 μg total RNA of normal canine mammary tissue using porcine specific GH primers and human specific β-actin primers. After electrophoresis the gel was stained with ethidium bromide and photographed under ultraviolet illumination. The lower 383-bp band corresponds to β-actin. The band at 495 bp is obtained from GH mRNA while a 600-bp fragment is amplified of unknown origin. Normal mammary tissues were analyzed of dogs treated with progestins (*top*) or mammary tissue from control dogs (*bottom*). Sample 9 corresponds to the tissue sample with code P221L5 (Table I), which is normal mammary tissue of a dog treated with proligestone 1 wk before surgical removal of a malignant mammary carcinoma.

```
         10        20        30        40        50        60
TACTCCTGGAACCTGGGCAGGGGACCTTGTTTGCTGTTGTGCTGACATCAAGAAATTCTC
 Y  S  W  N  L  G  R  G  P  C  L  L  L  C  -  H  Q  E  I  L

         70        80        90       100       110       120
AAGATGTCATCCTGTATTAGCGGGGTGGGTCCAAATTCAGTGAGAAGTGTSSTTATAAAG
 K  M  S  S  C  I  S  G  V  G  P  N  S  V  R  S  X  X  I  K
                   ----------

        130       140       150       160       170       180
GACACAAGAGGAGACCCAGAGTCAGGGAAGCCACGTGAAAGAGGGGCGCACACATGGGAG
 D  T  R  G  D  P  E  S  G  K  P  R  E  R  G  A  H  T  W  E
       -------

        190       200
GGATGTTGGCCCCTGGCAAGTGCA
 G  C  W  P  L  A  S  A
```

Figure 7. 5′-Nucleotide sequence of the 600-bp fragment amplified by RT-PCR from canine mammary gland RNA using porcine specific GH primers and the translation into protein. There was no homology between this fragment and DNA and protein sequences in the EMBL sequence data bank. An RGD and a glysaminoglycan attachment site (SG × G) are given in bold.

mary GH to create a highly proliferative environment of the mammary epithelium. The GH mRNA is also found in malignant canine mammary tumors, irrespective of the presence of progesterone receptors.

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