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Expression of Growth Hormone in Canine Mammary Tissue and Mammary Tumors

Evidence for a Potential Autocrine/Paracrine Stimulatory Loop

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The role of progestins in the pathogenesis of breast cancer in women remains controversial. To advance this discussion, we report the demonstration and localization of progestin-induced biosynthesis of growth hormone (GH) in canine mammary gland tissue. Nontumorous mammary tissues and tumors, both benign and malignant, were obtained from private household dogs. Immunoreactive GH was localized in mammary epithelial cells and correlated with the presence of GH mRNA. Local synthesis of GH was also proven immunoelectron microscopically by demonstrating GH-containing secretory granules. Cellular GH production in nontumorous tissues was more extensive during the progesterone-dominated luteal phase of the ovarian cycle or during exposure to synthetic progestins than during anestrus. GH was also associated with areas of hyperplastic mammary epithelium, which may indicate that locally produced GH enhances proliferation, acting in an autocrine and/or paracrine manner. In 41 of 44 tumors, GH was present. Of 3 GH-negative tumor samples, 2 were from progestin-depleted, castrated bitches. In nonmalignant mammary tissues, GH production is stimulated by progesterone and synthetic progestins interacting with progesterone receptors. In some progesterone-receptor-negative malignant tumors, GH expression was found, indicating loss of this control. Progestin-induced GH probably participates in the cyclic development of the mammary gland but may promote mammary tumorigenesis by stimulating proliferation of susceptible, and sometimes transformed, mammary epithelial cells. (Am J Pathol 1997, 150:1037–1047)

Growth and development of both normal and tumorous mammary tissue is a complex and incompletely understood process. Growth-enhancing as well as growth-inhibiting mechanisms are likely to be implicated. Until now, many hormones, growth factors, and proto-oncogene products involved in these processes have been identified.[1–7] Although progestin-based oral contraceptives are widely used by women, there still is controversy in human medicine about the role of progestins in mammary gland tumorigenesis.[8–14]

In veterinary medicine, it has become clear that administration of progestins at high dosages to dogs induces a dose-dependent mammary tumor development.[15–19] Also, endogenous ovarian steroids appear to promote mammary tumorigenesis in dogs, as ovariectomy at an early age exerts a major protective effect.[20] However, whether these steroids exert their promotion directly or indirectly is still an unresolved question.

Recently, research from our group has demonstrated mammary growth hormone (GH) production in progestin-exposed beagle dogs by showing an arterio-venous GH gradient across the mammary glands.[21] In related work using reverse transcriptase polymerase chain reaction, GH mRNA has been detected in mammary gland homogenates in dogs and

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cats and in mammary tumors of dogs, cats,[22] and women.[23]

In this report, we present a potential molecular mechanism by which progesterone and progestins may contribute to mammary tumor development by inducing GH gene expression in the mammary gland. Progestin-induced mammary GH might function as an autocrine or paracrine growth factor, in view of the presence of the GH receptor that has recently been demonstrated in the normal rabbit mammary gland[24] and in mammary tumors of women.[25,26]

To further substantiate the (patho)biological significance of progestin-induced mammary GH, we describe the presence of immunoreactive GH in a range of canine mammary tissues obtained from domestic pet dogs. The examined samples included normal mammary tissues, benign mammary tumors, and malignant mammary tumors. To determine the origin of GH in these samples, mammary GH production was demonstrated using immunoelectron microscopy, and GH gene expression was localized using *in situ* hybridization (ISH).

Materials and Methods

Tissues

Formalin-fixed and paraffin-embedded canine mammary tissues were selected from our diagnostic pathology archives. Selection of cases was primarily based upon the availability of data concerning plasma progesterone concentrations or previous progestin administration and steroid receptor concentrations in mammary samples. In total, 68 tissues obtained from 43 different animals were selected. The bitches were presented to the university clinic because of single or multiple mammary nodules. At surgery, nodules and in many cases a sample of nontumorous mammary tissue were obtained. After excision, tissues were fixed in a 10% buffered formalin solution, processed, and embedded in paraffin. The cases were histologically evaluated independently by two veterinary pathologists (E. van Garderen and W. Misdorp) without knowledge about plasma progesterone or progesterone receptor concentrations. Lesions were diagnosed according to the World Health Organization nomenclature.[27] In a minority of malignant tumors, data on metastasis were available.

Immunohistochemistry

Immunohistochemistry was performed using a polyclonal rabbit anti-porcine GH antibody (generous gift of Dr. M. M. Bevers, Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University) in an indirect immunoperoxidase staining procedure, using the avidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Sections (5 μm) were mounted onto glasses coated with 0.01% poly-L-lysine (Sigma Chemical Co., St. Louis, MO). After deparaffinization and rehydration, antigen retrieval was performed by pretreatment of the slides with 0.1% Pronase for 10 minutes. Endogenous peroxidase was blocked by incubating the slides with 1% H_2O_2 in methanol for 30 minutes at room temperature. After rehydration and washing in phosphate-buffered saline (PBS, three times for 5 minutes each), slides were preincubated with normal goat serum in PBS (1:10) for 15 minutes at room temperature. The primary antibody was applied in a 1:5000 dilution in 10% normal goat serum, and incubation was overnight at 4°C. After incubation, sections were rinsed in PBS (three times for 5 minutes each) and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:100 in PBS and 1% normal goat serum for 30 minutes. After washing in PBS (three times for 5 minutes each), sections were incubated with avidin-biotin complex, freshly prepared according to the manufacturer's instructions. Immunoreactive GH was visualized using 0.3% H_2O_2 and 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma), diluted in 0.05 mol/L Tris/HCl buffer, during a 10-minute incubation step. After rinsing in distilled water for 2 minutes, sections were counterstained with Mayer's hematoxylin for 1 minute. Finally, slides were rinsed in water, dehydrated, sealed, and covered with coverslips.

A canine pituitary gland served as a positive control. Approximately 70% of the cells in the anterior pituitary gland stained positive for GH. Moreover, validation of the primary antibody included serial dilution and preabsorption of this antibody with increasing concentrations of canine GH. After preabsorption, immunoreactivity could not be detected anymore.

Distribution of immunoreactivity in mammary tissue samples was semiquantitatively scored, ranging from 0 to 3. A score of 0 represents absence of immunoreactivity. Mammary tissue in which 30% or less of the cells displayed immunoreactivity scored 1, tissue in which more than 30% but less than 70% of cells were positive scored 2, and finally a score of

3 was obtained when more than 70% of cells showed immunoreactivity.

In Situ *Hybridization*

cDNA Probe

GH gene expression was demonstrated by ISH using a digoxigenin-labeled cDNA probe. This probe was obtained by reverse transcriptase polymerase chain reaction of mammary total RNA. After reverse transcription, the PCR amplification was performed with primers derived from the sequence of the porcine GH gene from the second to the fifth exon. Primer sequences and details of probe validation are given elsewhere.[22] These primers generate a GH-specific 495-bp DNA fragment. As the selected mammary tissues were archival specimens that had been stored for up to 12 years, we anticipated decreased mRNA contents in the tissues. Therefore, digoxigenin labeling of the probe was performed during the PCR according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). This procedure yields a probe with maximal label content.

Hybridization Procedure

Basically, the protocol published by Ehrlein[30] was used, with modifications aimed at the use of a cDNA probe. Hybridization was performed under RNAse-free conditions. All solutions were treated with 0.1% di-ethylpyrocarbonate (Sigma) and autoclaved. Glassware was baked at 180°C for 6 hours.

Sections (5 μm) were cut and mounted on glass slides coated with 0.01% poly-L-lysine. After deparaffinization and rehydration, they were digested with 20 μg/ml proteinase K (Sigma) in Tris buffer (100 mmol/L Tris/HCl, 50 mmol/L EDTA, pH 8.0) for 30 minutes at 37°C, rinsed in PBS, and postfixed in 4% paraformaldehyde in PBS for 5 minutes at 4°C. Again they were rinsed in PBS and acetylated in 0.25% acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 10 minutes. The acetic anhydride was removed by washing in PBS.

Prehybridization followed in 50% deionized formamide in 2 × standard saline citrate (20 × SSC is 3 mol/L NaCL, 0.3 mol/L sodium citrate, pH 7.0) for 15 to 30 minutes at 37°C. The hybridization mix consisted of 50% deionized formamide, 0.1% sodium dodecyl sulfate, 250 μg/ml herring sperm DNA, and 5% dextran sulfate in 2 × SSC. Probe concentration was 5 ng/μl. Slides were covered with a coverslip and left to hybridize overnight at 60°C in a humidified chamber. The coverslips were gently removed by incubation in 4 × SSC at 37°C. Post-hybridization washing was carried out at 37°C for 30 minutes per step, once in 2 × SSC, twice in 1 × SSC, and twice in 0.5 × SSC. The digoxigenin label was detected with sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim), according to the instructions of the manufacturer. Nonspecific binding of the antibody was reduced by the addition of 10% normal sheep serum to the preincubation mix and 5% normal sheep serum to the antibody solution. Color development took place overnight by incubating the slides in a substrate that consisted of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) according to the manufacturer's instructions.

Specificity for ISH was confirmed using a formalin-fixed canine pituitary gland as a positive control. Strong hybridization was observed in approximately 75% of adenohypophyseal (pars distalis) cells. Negative controls included incubation of the pituitary gland in the absence of probe, pretreatment of the sections with 40 μg/ml RNAse A (Boehringer Mannheim) in 10 mmol/L Tris/HCl (pH 8.0), 0.5 mol/L NaCl, 1 mmol/L EDTA for 30 minutes at 37°C, and incubation with a noncoding digoxigenin-labeled probe. No hybridization was observed in these negative controls.

Electron Microscopy

Freeze Substitution and Low Temperature Embedding

Aldehyde-fixed nontumorous hyperplastic mammary tissue obtained from a dog that had been exposed to progestins was cut in small blocks of approximately 1 mm^3, infiltrated with 2.3 mol/L sucrose, and frozen in liquid nitrogen. Samples were stored in liquid nitrogen until use. Frozen mammary tissue was transferred (under liquid nitrogen) into a Reichert Cs-auto freeze-substitution unit (Leica Aktiengesellschaft, Vienna, Austria). Freeze substitution was carried out at −90°C in methanol supplemented with 0.5% uranyl acetate for at least 36 hours. After raising the temperature to −45°C at a rate of 5°C/hour and washing several times with pure methanol, the samples were infiltrated with Lowicryl HM20. Infiltration was achieved in the following graded series of Lowicryl/methanol mixtures: 1:1 for 2 hours, 2:1 for 2 hours, pure Lowicryl for 2 hours, and pure Lowicryl overnight. The samples were transferred, inside the Reichert Cs-auto, to a flat embedding mold filled with

pure Lowicryl and polymerized by ultraviolet light at −45°C for 2 days. Ultrathin Lowicryl sections of 50 nm were cut on a Reichert UltracutS (Leica Aktiengesellschaft).

Immunogold Labeling of Lowicryl Sections

Ultrathin Lowicryl sections of mammary tissue were placed on formvar-carbon-coated nickel grids and labeled according to the following procedure. Nonspecific binding sites were blocked by incubating the sections for 30 minutes on block buffer containing 5% bovine serum albumin and 0.1% cold water fish gelatin in PBS, pH 7.4. Sections were incubated at room temperature with primary antibody (rabbit anti-porcine GH diluted 1:5000) in 0.1% BSA-C (Aurion, Wageningen, The Netherlands) in PBS, pH 7.4 (BSA-C/PBS) for 1 hour. Sections were rinsed three times for 10 minutes with BSA-C/PBS and immunoreactivity was visualized by incubation with 10-nm protein A-gold particles prepared by the tannic acid/citrate method.[28] Finally, immunolabeled sections were rinsed three times for 10 minutes with BSA-C/PBS, four times for 5 minutes with distilled water, and stained for 2 minutes on a drop of Reynolds lead citrate.[29] Immunogold-labeled sections were examined and photographed in a Philips CM10 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 80 kV.

Plasma Progesterone Concentration

Progesterone concentrations were determined in dogs considered not to be exposed to exogenous progestins, using a radioimmunoassay.[31] In dogs that had received long-acting synthetic progestins, levels were interpreted as + (last injection <5.5 months before removal of mammary tissue) or ± (last injection between 5.5 and 7 months before removal of tissue), or − (last injection >7 months before tissue removal).

Steroid Receptor Analysis

After surgical removal of mammary tumors, samples were divided in two pieces. One sample was quickly frozen in liquid nitrogen, and the other one was fixed in formalin. Estrogen receptors (ERs) and progesterone receptors (PRs) were determined in the frozen tissue specimen by a multiconcentration dextran-coated charcoal assay.[31] Adjacent formalin-fixed tumor samples were histologically examined for the presence of nontumorous tissue, which may influence tumor-associated receptor concentrations. For the receptor concentrations in Tables 2 and 3, such cases are indicated.

Results

Histology and Immunohistochemistry

Nontumorous Mammary Tissue (n = 24; Table 1)

The histological characteristics of the glandular structures of nontumorous mammary tissues reflected the stage of the ovarian cycle (endogenous progesterone) or the effect of progestin administration. Tissue samples characterized by the presence of ductal structures with quiescent epithelium and remnants of lobules were classified as anestrus (n = 2). In the luteal or metestrus phase, which in the dog is characterized by prolonged persistence of corpora lutea irrespective of pregnancy, proliferation and differentiation occurred resulting in lobulo-alveolar structures. In early metestrus (n = 2), the tissue displayed moderate stromal proliferation and multifocal proliferation of ductal epithelium resulting in budding structures. Although these buds can essentially be divided into terminal end buds and alveolar buds in three-dimensional preparations,[32] in routine histology (two-dimensional) clear distinction between these two types is not possible. Therefore, we use the term budding structures. During metestrus, budding structures further proliferated and ultimately differentiated into alveoli, arranged in a lobular pattern. In middle metestrus, in several lobules alveolar secretion occurred (n = 9), whereas in late metestrus, alveolar involution started (n = 8). Three samples demonstrated complete lobulo-alveolar differentiation with abundant secretion. These changes are typical for canine pseudo-lactation that may occur during the metestrus.

Confirmation of the morphological assessment was attempted with reference to data on progestin levels, ER and PR concentrations, and distribution of immunoreactive GH in epithelial cells. From the results summarized in Table 1 it can be concluded that the histologically defined development stage of canine mammary gland tissue correlated with endogenous plasma progesterone levels. The results of the effect of exogenous administered progestins on mammary tissue appeared to vary considerably, ranging from atrophic glandular tissue to complete lobulo-alveolar development with secretion (pseudo-lactation). However, in most cases, progestin administration resulted in hyperplastic changes of ductal epithelium (mid-metestrus phase).

Table 1. *Summary of Data on Nontumorous Mammary Tissues*

Functional stage of mammary gland	Number	Progestin levels		ER/PR concentrations (fmol/mg protein)	Distribution of i-GH in epithelial cells	
		Endogenous (nmol/L)	Exogenous		Ducti	Alveoli/buds*
Anestrus	2		+	56/44	0	0
		7.6		<5/<5	1	2
Early metestrus	2	32.9		86/57	2	2
		53.2		56/70	1	3
Mid-metestrus	9	113.1		58/32	2	2
		9.5		189/90	2	3
			+	76/137	2	2
			+	nd/nd	1	2
			+	39/39	2	2
			+	47/35	2	2
			+	45/40	2	2
		14.0		97/23	1	2
			+	28/57	2	2
Late metestrus	8	ND		nd/nd	1	0
		1.3		15/40	1	1
		7.0		27/70	1	1
		7.0		13/46	1	1
		2.5		23/25	2	0
		0.6		88/64	1	1
		0.6		18/49	1	3
			+	35/36	2	2
Pseudo-lactation	3	19.1		42/33	0	1
			+	88/12	0	0
			+	82/26	1	0

For exogenous progestin concentration, + indicates injection of long-acting progestins less than 5.5 months before removal of the mammary tissue. Semiquantitative score of GH distribution was as follows: 1, less than 30% of epithelial cells positive; 2, more than 30% but less than 70% of epithelial cells positive; 3, more than 70% of epithelial cells positive. ND, not determined.
*Depending on functional stage of mammary gland.

Immunohistochemical examination of the samples demonstrated heterogenous immunoreactivity for GH both in terms of distribution patterns and staining intensity. Immunoreactivity was located only within the cytoplasm of ductal (Figure 1A) and alveolar epithelial cells. Myoepithelial cells and stromal cells were negative. Clustered immunoreactivity was observed in hyperplastic budding structures in early and mid-metestrus (Figure 1B), which persisted in a heterogeneous way in differentiated alveolar epithelium in late metestrus. Proliferation of epithelial cells occurs predominantly in early and mid-metestrus, in contrast to nonproliferation in anestrus, late metestrus, and pseudo-lactation. Therefore, we examined whether proliferation was correlated with GH expression. Indeed, GH expression was higher both in ductal and alveolar epithelial cells in the proliferation phase ($P < 0.05$, Mann-Whitney test). Thus, the distribution pattern of immunoreactivity paralleled the cyclic development pattern of the mammary gland.

Benign Mammary Tumors (n = 25; Table 2)

In female dogs, complex adenoma is the predominant benign mammary tumor. By definition, complex tumors are composed of both tumorous epithelial cells and spindle cells.[27] Immunohistochemical examination revealed that, with the exception of two complex adenomas, immunoreactive GH was detected in benign mammary tumors. Interestingly, immunoreactivity was located mainly in tumorous epithelium and rarely in spindle cells. Only in 4 of 19 tumors comprising spindle cell proliferations (complex adenomas and fibroadenomas) was focal slight immunoreactivity observed in spindle cells, located in the vicinity of immunoreactive epithelial cells.

Malignant Mammary Tumors (n = 19; Table 3; Figure 1C)

Several malignant tumor types could be identified, with solid carcinomas being the predominant type.

Immunohistochemical investigation revealed the presence of GH in 18 of 19 malignant mammary tumors. Immunoreactivity in carcinomatous epithelial cells was heterogeneous both in terms of distribution and intensity within one tumor and between the tumors. In one simple-type adenocarcinoma, derived from a castrated bitch, immunoreactive GH could not be detected. Immunoreactivity of spindle cell proliferations in the complex-type adenocarcinomas was

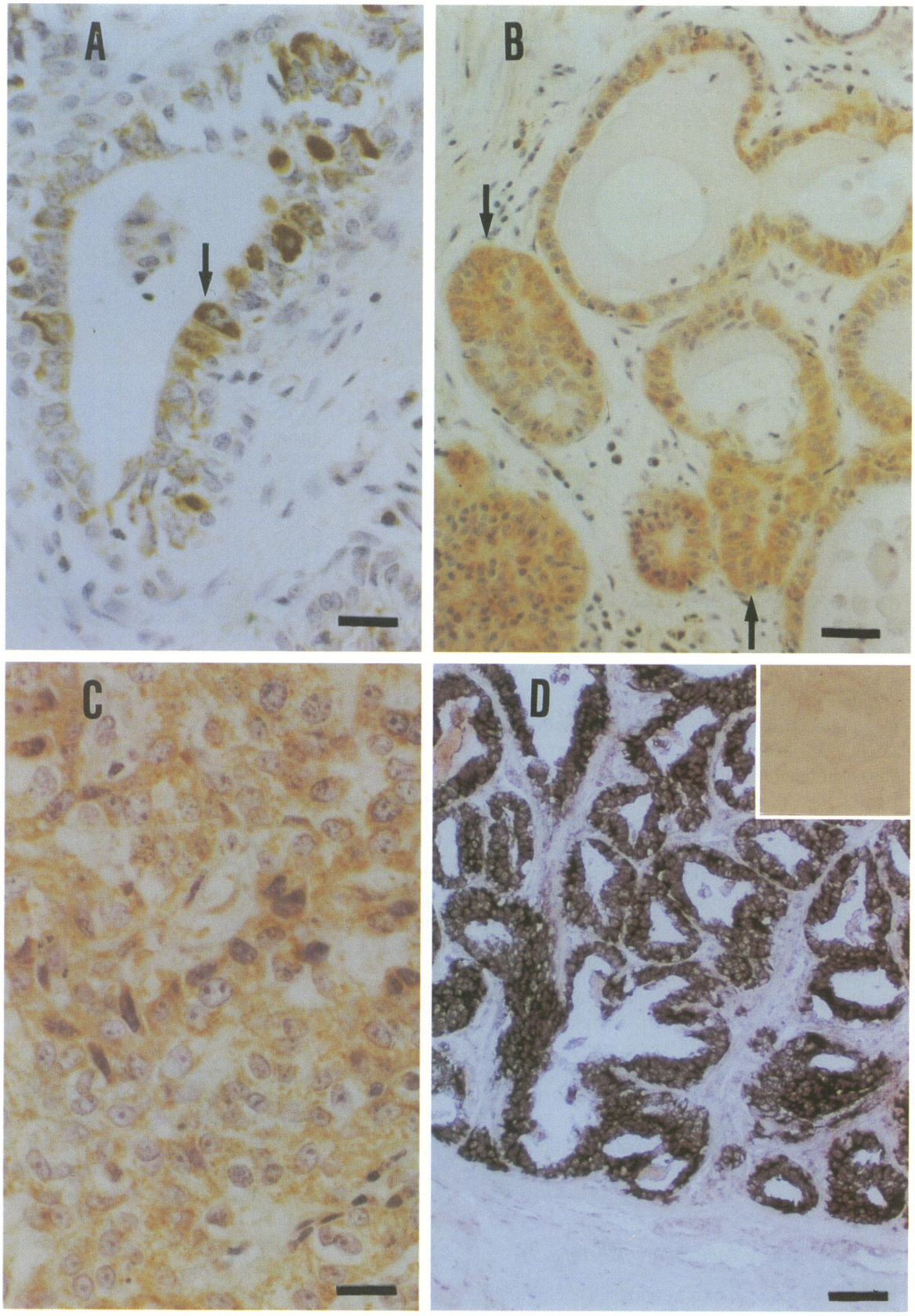
A
B
C
D

Table 2. *Summary of Data on Benign Mammary Tumors*

Histology	Number	Progestin levels		ER/PR concentrations (fmol/mg protein)	Distribution of i-GH in tumorous epithelial cells
		Endogenous (nmol/L)	Exogenous		
Simple adenoma, tubular type	2	1.3		39/79	2
			+	88/79	3
Complex adenoma	20		+	66/65	3
			±	60/167	3
		0.6		31/80	3
			+	80/370	3
			+	72/371	2
		4.1		ND/ND	0
			+	102/259	3
		68.6		96/173	3
			+	75/53	1
			+	187/423	1
			+	98/156	2
		4.5		58/106	1
		1.6		123/546	2
			+	98/133	1
		OVX		25/60	0
		68.6		58/21	2
			+	52/65	2
			+	39/50	2
		68.6		94/177	1
		8.6		66/22	3
Fibroadenoma	3		±	36/58	3
			+	100/107	3
			±	41/41	3

For exogenous progestin concentration, + indicates depot injection of progestins administered less than 5.5 months before removal of the mammary tissue and ± indicates depot injection of progestins administered between 5.5 and 7 months before removal of the mammary tissue. OVX, ovariohysterectomy; ND, not determined.

in most cases absent. However, in two cases, a faint multifocal reaction was noticed in this cellular compartment. In both malignant mixed tumors, the malignant spindle cell proliferations were faintly to moderately positive for GH in the majority of cells.

In Situ *Hybridization (Figure 1D)*

ISH for GH mRNA was performed on randomly chosen cases. Eight nontumorous mammary tissues, eleven benign tumors, and thirteen malignant tumors were examined. In most cases, ISH confirmed the mammary origin of GH. Results of this technique were not always consistent with the results of immunohistochemistry. A positive hybridization was demonstrated in twenty-four samples, and in eight samples the result was negative. In one nontumorous mammary gland sample that was histologically characterized by pseudo-lactation, a positive hybridization was noticed although GH immunoreactivity was absent. In seven cases, comprising two nontumorous mammary samples, three benign mammary tumors, and two malignant tumors, no hybridization was noticed, although these samples displayed immunoreactivity for GH. One tubular-type adenocarcinoma, derived from a castrated bitch, was negative in both immunohistochemical and ISH tests.

In nontumorous mammary gland, hybridization occurred in ductal and alveolar epithelial cells. Myoepithelial and stromal cells were negative. In the benign tumor group, in which the majority of tumors were composed of tumorous proliferations of both epithelial and spindle cells (complex adenomas and fibroadenomas), consistent hybridization was noticed in the epithelial component.

Generally, no hybridization was noticed in spindle cell proliferations. However, in agreement with immunohistochemical findings, in a few cases, focal hybridization was observed in spindle cell areas localized in the vicinity of tumorous epithelial cells. In the

Figure 1. *Growth hormone in mammary epithelial cells.* **A:** *Nontumorous mammary tissue in early metestrus phase. Immunoreactive GH is present in ductal epithelial cells* (**arrow**), *whereas stromal cells are negative. Bar, 20 μm.* **B:** *Nontumorous mammary tissue in mid-metestrus phase. The tissue is characterized by the presence of budding structures. Epithelial cells in ducti and buds* (**arrow**) *demonstrate immunoreactive GH homogenously. Bar, 30 μm.* **C:** *High-power magnification of a solid mammary carcinoma. Nearly all tumor cells are positive for GH. Bar, 15 μm.* **D:** *ISH for GH mRNA in a tubular adenoma. The presence of GH mRNA is restricted to epithelial tumor cells. The inlay represents a negative control in which the tissue was treated with RNAse before the hybridization. Bar, 55 μm.*

Table 3. *Summary of Data on Malignant Mammary Tumors*

Histology	Number	Progestin levels		ER/PR concentrations (fmol/mg protein)	Distribution of i-GH in tumor cells
		Endogenous (nmol/L)*	Exogenous		
Adenocarcinoma, simple type	2	OVX		<3/<3	0
			+	14/10	3
Adenocarcinoma, complex type	2		+	ND /205†	2
			+	59/56	3
Solid carcinoma, simple type	8		+	<5/<5	3
			+	16/28	2
		2.2		5/3†	3
		ND		ND/ND	1
			+	6/<5	1
		0.3		ND/ND	2
		0.6		<3/<3	3
		4.1		12/3	2
Solid carcinoma, complex type	1		+	8/21	3
Anaplastic carcinoma (or anaplastic carcinoma cells in ducti)	4		+	17/3†	3
			+	<5/<5	3
			+	88/12†	3
			+	ND/ND	3
Malignant mixed tumors	2	ND‡		ND/ND	3
		16.8		6/19	3

OVX, ovariohysterectomy; ND, not determined.
*Normal plasma concentration of endogenous progesterone during anestrus <3 nmol/l and during metestrus, 3 to 100 nmol/L.
†Receptor concentrations may not be representative for the tumor as the samples also contained nontumorous tissue
‡Multiple corpora lutea in ovaries.

malignant mammary tumors, findings were comparable with the results in benign tumors, including conflicting results in spindle cells.

Electron Microscopy

Using immunogold-based transmission electron microscopy, immunoreactive epithelial cells in hyperplastic mammary tissue revealed the presence of GH in secretory granules in these cells. In addition, gold particles were observed in the Golgi system, demonstrating the local biosynthesis of GH (Figure 2). Apparently, adjacent epithelial cells in which no GH was detected did not differ ultrastructurally from GH-positive cells.

Discussion

It is a well known phenomenon that female dogs can develop symptoms of GH excess during the luteal phase of the ovarian cycle or after administration of long-acting synthetic progestins to prevent estrus. Symptoms of GH excess include glucose intolerance and acromegaly. Also, female dogs not demonstrating clinical symptoms of GH excess experience a small but significant elevation of GH levels during metestrus or after progestin administration.[33] Previous experiments have demonstrated that this progestin-induced GH excess in dogs originated in the mammary gland and not in the pituitary gland.[21] In an attempt to further investigate the biological significance of this mammary GH, in this report we describe the presence of GH in (tumorous) mammary tissues obtained from domestic pet dogs. The local biosynthesis of GH is proven by ISH and immunoelectron microscopy.

Compared with the presence of immunoreactive GH, in 7 of 30 samples, a negative result with ISH was obtained. This discrepancy is probably inherent to the nature of fixed archival tissues and can be explained by variations in tissue handling, fixation conditions, and fixation time after surgery. Cross-linking effects in tissue induced by fixatives can diminish probe penetration and moreover can mask mRNA, resulting in diminished probe-target interaction. In addition, mRNA is notorious for rapid degradation under suboptimal fixation conditions.[34] Alternatively, the possibility remains that in these cases immunoreactivity reflected plasma-derived GH bound to its receptor. In one mammary sample with

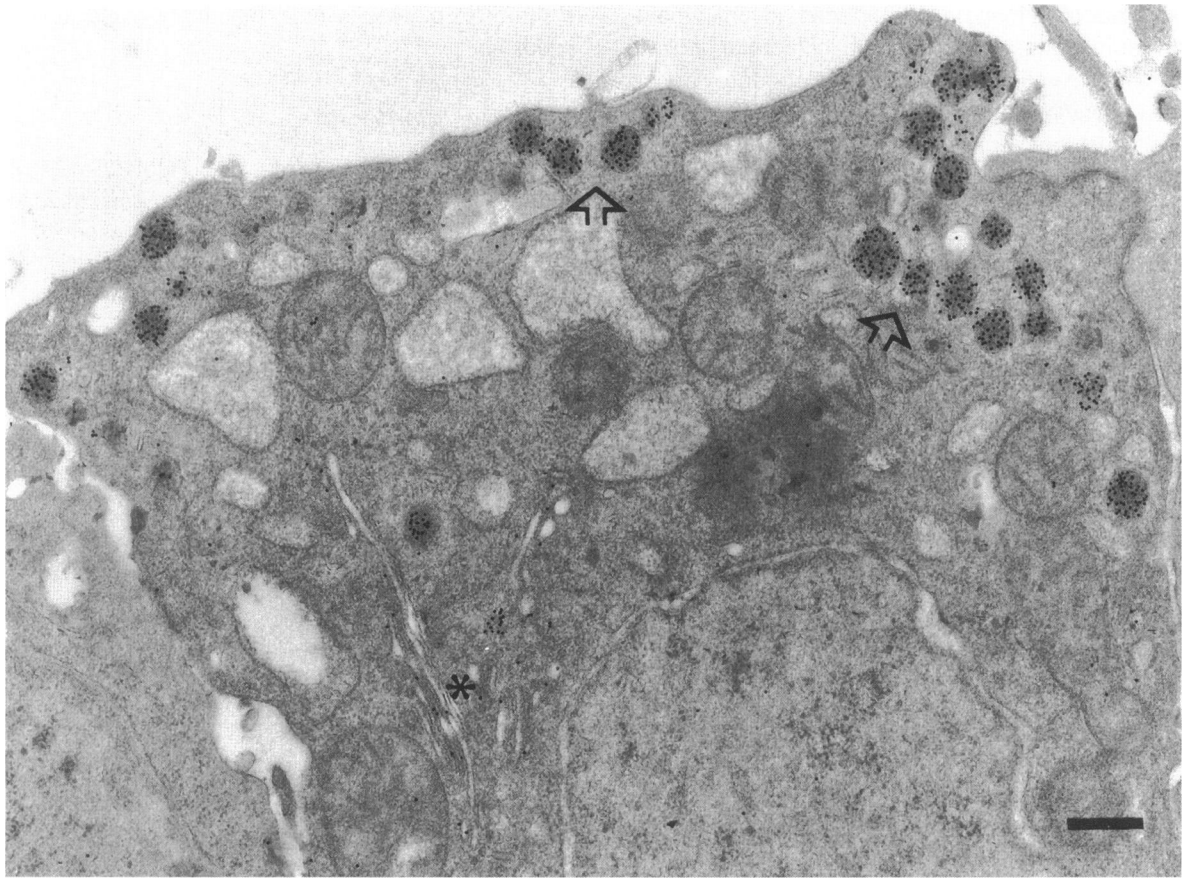

Figure 2. *Immunoelectron microscopic aspect of a nontumorous mammary epithelial cell. The tissue was obtained from a dog exposed to progestins. Gold particles representing GH are located in secretory granules* (arrow) *and in the Golgi system* (*). *Bar, 400 nm.*

pseudo-lactation, hybridization occurred although immunoreactive GH could not be detected. As GH has been detected in canine colostrum (I. Schoenmakers, submitted), in this particular case, epithelial cells may have secreted GH resulting in cytoplasmic GH contents that were under the immunohistochemical detection limit.

Based upon an inhibitory effect on the risk of developing endometrial tumors in women, it was initially thought that progesterone or progestins had an antiproliferative effect. However, it appears that this concept is too restricted and is not valid in other target tissues. Recent research has demonstrated a growth-promoting effect of progesterone or progestins in uterine leiomyomas in women.[35] In addition, in the mammary gland, progesterone and progestins have a prominent proliferative effect upon ductal epithelium both in female human beings[36] and several other mammalian species, including laboratory rodents,[37,38] dogs,[15,39,40] and cats.[41,42] As we observed in nontumorous mammary samples, this proliferative effect was associated with the formation of ductal outgrowths that are microscopically encountered as budding structures. After proliferation, these outgrowths finally differentiate into lobulo-alveolar glandular tissue during the luteal phase of the ovarian cycle. One of our most interesting findings in nontumorous mammary tissue is that epithelial cells in budding structures diffusely produce GH followed by a notable decrease of GH production in the differentiation phase of the mammary gland. Therefore, mammary-derived GH is possibly an autocrine or paracrine growth factor and may have a physiological function in the cyclic development of the mammary gland.

Epidemiological studies have provided circumstantial evidence that progestin administration results in dose-dependent mammary tumor induction in dogs and cats.[15,19,42] Therefore, as both mammary GH production and mammary tumor induction can be a sequel of progestin administration, we examined whether GH production also occurred in canine mammary tumors. Indeed, GH production was encountered in the great majority of benign and malignant mammary tumors. The significance of GH production for tumor growth is not clear yet. In fact,

GH production may be an epiphenomenon in mammary tumorigenesis by reflecting the cellular origin of mammary tumors. In rats, there is strong evidence that experimentally induced mammary tumors exclusively arise from epithelial cells in terminal end buds, terminal ducts, or alveolar buds.[32] If canine mammary tumors also preferentially originate in these structures, in which we frequently detected GH diffusely, then it can be explained that these tumors have the potential to produce GH. In this perspective, relatively few canine mammary tumors would originate in other parts of the glandular tree. Theoretically, the three GH-negative mammary tumor samples might represent the latter.

From a mechanistic point of view, research has to be focused on the link between progesterone/progestins and mammary GH production. A crucial question is whether a significant plasma progesterone concentration combined with the presence of PRs in (tumorous) mammary epithelial cells triggers mammary GH production. Although we have strong indications for this mechanism in nontumorous tissue, in benign and especially in malignant mammary tumors this is far from clear. On the one hand, we observed that in both tumor groups mammary tumors of castrated bitches were negative for GH; on the other hand, in several malignant tumors with very low PR concentrations, GH was diffusely present in tumor cells.

It is generally accepted that normal PR functions are dependent on the interaction of the ligand with the receptor. However, Cohen-Solal et al[43] demonstrated that a PR mutant with the complete steroid-binding domain deleted remained biologically active. As far as we know, until now only one report has documented the presence of a truncated PR in human breast tumors.[44] Although there are no data available about the presence of truncated PRs in canine mammary tumors, the presence of such receptors might explain negative findings in the ligand-binding assay, although they retain the ability to initiate GH gene expression.

Although the complete mechanism is not entirely clear, we conclude that progesterone/progestin-induced hyperplastic epithelial changes in the mammary gland may be mediated by locally produced GH. As proliferating cells are sensitive to carcinogenic insults, progesterone/progestins may indirectly stimulate mammary tumorigenesis. Although our results may have an impact on veterinary oncological research, we believe that, in view of the widely used progestin-based oral contraceptives by women, our results warrant a thorough re-evaluation of the role of progestins in mammary gland tumorigenesis.

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References

1. Rillema JA: Development of the mammary gland and lactation. Trends Endocrinol Metab 1994, 5:149–154
2. Fernig DG, Smith JA, Rudland PS: Relationship of growth factors and differentiation in normal and neoplastic development of the mammary gland. Cancer Treat Res 1991, 53:47–78
3. Cullen KJ, Allison A, Martire I, Ellis M, Singer C: Insulin-like growth factor expression in breast cancer epithelium and stroma. Breast Cancer Res Treat 1992, 22:21–29
4. Cullen KJ, Smith HS, Hill S, Rosen N, Lippman ME: Growth factor messenger RNA expression by human breast fibroblasts from benign and malignant lesions. Cancer Res 1991, 51:4978–4985
5. McAndrew J, Fernig DG, Rudland PS, Smith JA: Secretion of transforming growth factor-α and expression of its receptor in human mammary cell lines. Growth Factors 1994, 10:281–287
6. Bacus SS, Zelnick CR, Plowman G, Yarden Y: Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers: implication for tumor biology and clinical behavior. Am J Clin Pathol 1994, 102:S13–S24
7. Pierce DF, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ, Moses HL: Mammary tumor suppression by transforming growth factor-β1 transgene expression. Proc Natl Acad Sci USA 1995, 92:4254–4258
8. Pike MC, Spicer DV, Dahmoush L, Press MF: Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev 1993, 15:17–35
9. King RJB: Biology of female sex hormone action in relation to contraceptive agents and neoplasia. Contraception 1991, 43:527–542
10. Horwitz KB: The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer? Endocr Rev 1991, 13:146–162
11. van Leeuwen FE: Epidemiologic aspects of exogenous progestagens in relation to their role in pathogenesis of human breast cancer. Acta Endocrinol (Copenh) 1991, 125:13–26
12. Pike MC, Henderson BE, Krailo MD, Duke A, Roy S: Breast cancer in young women and use of oral contraceptives: possibly modifying effect of formulation and age at use. Lancet 1983, 2:926–930
13. Pike MC, Henderson BE, Casagrande JT, Rosario I, Gray GE: Oral contraceptive use and early abortion as risk factors for breast cancer in young women. Br J Cancer 1981, 43:72–76

14. Rookus MA, van Leeuwen FE: Oral contraceptives and risk of breast cancer in women aged 20–54 years. Lancet 1994, 344:844–851
15. Rutteman GR: Contraceptive steroids and the mammary gland: Is there a hazard? Insights from animal studies. Breast Cancer Res Treat 1992, 23:29–41
16. Casey HW, Giles RC, Kwapien RP: Mammary neoplasia in animals: pathologic aspects and the effects of contraceptive steroids. Recent Results Cancer Res 1979, 66:129–160
17. El Etreby MF, Graf KJ: Effect of contraceptive steroids on mammary gland of female dogs and its relevance to human carcinogenicity. Pharmacol Ther 1979, 5:369–402
18. Owen LN, Briggs MH: Contraceptive steroid toxicology in the beagle dog and its relevance to human carcinogenicity. Curr Med Res Opin 1976, 4:309–329
19. Misdorp W: Progestagens and mammary tumours in dogs and cats. Acta Endocrinol (Copenh) 1991, 125: 27–31
20. Schneider R, Dorn CR, Taylor DON: Factors influencing canine mammary cancer development and post-surgical survival. J Natl Cancer Inst 1969, 43:1249–1261
21. Selman PJ, Mol JA, Rutteman GR, van Garderen E, Rijnberk A: Progestin-induced growth hormone excess in the dog originates in the mammary gland. Endocrinology 1994, 134:287–292
22. Mol JA, van Garderen E, Selman PJ, Wolfswinkel J, Rijnberk A, Rutteman GR: Growth hormone mRNA in mammary gland tumours of dogs and cats. J Clin Invest 1995, 95:2028–2034
23. Mol JA, Henzen-Logmans SC, Hageman P, Misdorp W, Blankenstein MA, Rijnberk A: Expression of the gene encoding growth hormone in the human mammary gland. J Clin Endocrinol Metab 1995, 80:3094–3096
24. Jammes H, Gaye P, Belair L, Djiane J: Identification and characterization of growth hormone receptor mRNA in the mammary gland. Mol Cell Endocrinol 1991, 75:27–35
25. Jammes H, Decouvelaere C, Bonneterre J, Fournier J, Djiane J, Peyrat JP: Expression of growth hormone receptor in human breast cancers. Bull Cancer (Paris) 1994, 81:938–939
26. Decouvelaere C, Peyrat JP, Bonneterre J, Djiane J, Jammes H: Presence of the two growth hormone receptor messenger RNA isoforms in human breast cancer. Cell Growth Differ 1995, 6:477–483
27. Hampe JF, Misdorp W: Tumours and dysplasias of the mammary gland. Bull WHO 1974, 50:111–133
28. Slot JW, Geuze HJ: A new method of preparing gold probes for multiple labeling cytochemistry. Eur J Cell Biol 1985, 38:87–93
29. Reynolds ES: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 1963, 17:208–212
30. Ehrlein J, Wanke R, Weis S, Brem G, Hermanns W: Sensitive detection of human growth hormone mRNA in routinely formalin-fixed, paraffin-embedded transgenic mouse tissues by non-isotopic *in situ* hybridization. Histochemistry 1994, 102:145–152
31. Rutteman GR, Misdorp W, Blankenstein MA, van den Brom WE: Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: different receptor profile in non-malignant and malignant states. Br J Cancer 1988, 58:594–599
32. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ: Biology of disease: comparative study of human and rat mammary tumorigenesis. Lab Invest 1990, 62:244–277
33. Selman PJ, Mol JA, Rutteman GR, Rijnberk A: Progestins and growth hormone excess in the dog. Acta Endocrinol (Copenh) 1991, 125:42–47
34. Terenghi G, Fallon RA: Techniques and applications of *in situ* hybridisation. Curr Top Pathol 1990, 82:290–337
35. Rein MS, Barbieri RL, Friedman AJTI: Progesterone: a critical role in the pathogenesis of uterine myomas. Am J Obstet Gynecol 1995, 172:14–18
36. Shi YE, Liu YLE, Lippman ME, Dickson RB: Progestins and antiprogestins in mammary tumour growth and metastasis. Hum Reprod 1994, 9:162–173
37. Wang S, Counterman LJ, Haslam SZ: Progesterone action in normal mouse mammary gland. Endocrinology 1990, 127:2183–2189
38. Haslam SZ: Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. Endocrinology 1988, 122:464–470
39. Nelson LW, Kelly WA: Changes in canine mammary gland histology during the estrous cycle. Toxicol Appl Pharmacol 1974, 27:113–122
40. Nelson LW, Kelly WA: Progestogen-related gross and microscopic changes in female beagles. Vet Pathol 1976, 13:143–156
41. Hayden DW, Barnes DM, Johnson KH: Morphologic changes in the mammary gland of megestrol acetate-treated and untreated cats: a retrospective study. Vet Pathol 1989, 26:104–113
42. Misdorp W, Romijn A, Hart AAM: Feline mammary tumours: a case-control study of hormonal factors. Anticancer Res 1991, 11:1793–1798
43. Cohen-Solal K, Bailly A, Rauch C, Quesne M, Milgrom ETI: Specific binding of progesterone receptor to progesterone-responsive elements does not require prior dimerization. Eur J Biochem 1993, 214:189–195
44. Lemoisson E, Cren H, Goussard J: Chromatographic separation of eight progesterone receptor isoforms in human breast tumors, and detection by radioligand and monoclonal antibodies: association with hsp90 and hsp70 heat shock proteins. Ann Biol Clin Paris 1994, 52:433–442