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Mammary steroid metabolizing enzymes in relation to hyperplasia and tumorigenesis in the dog

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Abstract

Progesterone and estradiol play a crucial role in the control of mammary gland proliferation and tumour formation in the dog. However, little is known whether steroid metabolizing enzymes are present within the canine mammary gland that may play a modulating role in the bioavailability of progesterone and estrogen.

In this study we investigated the expression of the steroid metabolizing enzymes 5α-reductase (type I and type II) and aromatase in relation to hyperplasia or tumorigenesis in the canine mammary tissue. The relative mRNA concentrations were examined by a semi-quantitative reverse-transcriptase PCR analysis (RT-PCR). In addition the affinity of dihydroprogesterone (5α-reduced metabolite of progesterone) for canine progesterone receptors was investigated.

Quantification of the RT-PCR products revealed that in mammary tumours a significantly higher expression of aromatase is present in comparison to normal mammary tissue. Furthermore, significant decrease in expression of both aromatase and 5α-reductase type II enzymes was found in hyperplasic mammary tissue compared to tumours. The changes in expression of type II 5α-reductase and aromatase were highly correlated. 5α-Reduction of progesterone to dihydroprogesterone resulted in a six-fold less affinity for the canine progesterone receptor.

It is concluded that hyperplasia is associated with a decreased expression of type II 5α-reductase and aromatase enzymes, whereas in tumours the opposite situation is found.

Keywords: Dog; Steroid metabolism; Progestins; Mammary gland; Hyperplasia; Tumorigenesis

1. Introduction

In healthy animals and woman the ovaries are the primary source of female steroid hormones. However, it is now more evident that peripheral conversion in steroid hormone-dependent target tissues and neoplasms is important in various biological conditions [1]. In various species, the mammary gland tissue has been shown in vivo and in vitro to have complicate enzymatic pathways, which allow in situ synthesis or inactivation of steroid hormones [2–10]. Furthermore, there is evidence for the synthesis of a variety of locally produced peptides under steroid hormone control by the normal mammary gland in a number of species [11,12]. Because in normal cycling animals the contribution of steroids necessary for mammary gland function is assured from the gonadal secretion, it is unlikely that the local steroidogenesis could replace or integrate the systemic action. Consequently, the steroidogenic capabilities of the mammary gland tissue can be interpreted as a protective mechanism by inactivation of steroids or, alternatively, as a subtle local regulation of mammary physiology.

Also various breast cancers and mammary tumour cell lines have steroidogenic capabilities [4,7–9,13,14]. For instance normal human breast tissue and its neoplasms can produce 17β-estradiol, a biologically potent estrogen, in vitro

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and in vivo [13,14]. It has been demonstrated [15] that in situ synthesis of estrogen predominates over uptake from plasma to maintain local mammary estradiol concentrations after menopause in women. This intratumoral 17β-estradiol production by in situ aromatisation of estradiol precursors may function as an autocrine growth and mitogenic factor and give a growth advantage to these cells, regardless of serum estrogen concentrations. This is further substantiated by the fact that in human breast cancer an increased activity and/or expression of the aromatase enzyme is found associated with a malignant phenotype [16–19], supporting the use of aromatase inhibitors for postoperative adjuvant endocrine treatment of patients with breast carcinoma [20,21].

In veterinary medicine mammary cancer represents nearly 50% of all tumours in female dogs [22]. Evidence for involvement of steroid hormones in breast cancer development in bitches comes from the observation that bilateral oophorectomy at an early age markedly reduced risks of subsequent development of mammary cancer [23,24]; the earlier oophorectomy is done, the greater the risk reduction [23,25], as observed in women [26]. In intact dogs the incidence rate of mammary cancer continues to increase up to old age [23], probably due to continuation of ovarian activity throughout all the life in this species.

Progesterone plays a key role in the proliferation of canine mammary tissue. Prolonged exposure to exogenous progestins, given for oestrus prevention, or to endogenous progesterone during the luteal phase of the sexual cycle results in proliferation of mammary epithelium [27,28]. This is accompanied by increased biosynthesis of growth hormone (GH) in foci of hyperplastic mammary epithelium [29]. Thereafter, depending on the duration, the dose and the frequency of the administration, progestins can induce neoplastic changes in the mammary gland in bitches [25,27,30]. According to this findings, progesterone-induced hyperplasia appear to have significant premalignant potential in the canine mammary tissue.

Progesterone is subject to metabolisation within the mammary gland [7–10,31]. One of the assumed key enzymes of its metabolism is the steroid 5α-reductase to which progesterone has been shown to be a more efficient substrate than testosterone [32]. The 5α-reduction is a local regulatory mechanisms of sex steroids action either as a hormonally activating step, like in 5α-dihydrotestosterone synthesis, or as a catabolic step leading to the excretion of C21 and C19 inactive steroids. 5α-Reductase activity is enhanced in neoplastic mammary tissue as compared to normal mammary tissue [8,31,9] and one of the principal progesterone metabolites 5α-pregnan-3,20-dione (also referred to as allopregnanedione or dihydroprogesterone; DHP) [8–10,31] may stimulate breast cell proliferation and detachment in vitro [31]. Furthermore, a relationship between 5α-reductase and aromatase activity has been documented in human breast cancer cell lines and their assessment seems to be helpful in predicting hormone sensitivity in human breast tumours [33].

The aim of the present study was to investigate the expression of the steroid metabolizing enzymes 5α-reductase (type I and type II) and aromatase in the canine mammary gland in relation to hyperplasia or tumorigenesis and to establish the affinity of 5α-pregnan-3,20-dione to the canine progesterone receptor.

2. Materials and methods

2.1. Tissues

Mammary gland tissue samples were collected from three different groups of female dogs. Hyperplastic mammary tissue samples were obtained from six experimental beagle dogs that had been treated with long-acting progestins after ovariohysterectomy. Full details of treatments and hormonal changes have been published previously [34]. Briefly, dogs were treated with depot preparations of either 10 mg/kg body weight medroxyprogesterone acetate (MPA; Upjohn, Ede, The Netherlands) or 50 mg/kg body weight proligestone (PROL; Mycofarm, De Bilt, The Netherlands) each 3 weeks for a total of eight injections. Afterward, the treatments were stopped for 6 months and then resumed at the same doses and intervals for a total of five additional injections. Mammary tumour tissues ($n = 9$) were obtained from privately owned dogs referred to Utrecht University Clinic of Companion Animals because of a single or multiple mammary nodules. Mammary tumours were collected at surgery or, in inoperable cases, at autopsy, and sections processed for routine histopathological classification [35]. Normal mammary tissue samples as controls ($n = 5$) and normal uterus ($n = 3$) for the receptor study were collected from five intact young adult beagles sacrificed for reasons unrelated to mammary, uterine or endocrine disorders and without known history of progestin treatment.

After excision, all tissue samples were immediately frozen in liquid nitrogen and stored at −70 °C for RNA isolation and receptor analysis.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Starting from 50 mg of frozen mammary tissue total RNA isolation was performed using the total RNA isolation system kit (Promega Corporation, Madison, WI, USA). The quality of the isolated total RNA was verified by agarose gel electrophoresis [36] before further processing.

Reverse transcription (RT) of the poly $(A)^+$ RNA was performed with a reverse transcription system (Promega Corporation, Madison, WI, USA). One microgram of total RNA was used in a mixture of 2 μl 10× AMV-RT reaction buffer, 8 μl 25 mM $MgCl_2$, 2 μl 10 mM dNTPs, 20 U recombinant RNasin, 12 U Avian Myeloblastosis Virus reverse transcriptase, 0.5 μg Oligo $d(T)_{15}$ Primer and nuclease-free water to a final volume of 20 μl. The reaction occurred for 60 min at 42 °C followed by 5 min at 95 °C. For the PCR reaction

Table 1
Primers sequences

Primer	Size	Sense	Antisense
ARO	419	CCCACTTCAGGTTCC-TCTGGATGG	TGTTAGAGGTGTCC-AGCATG
5α-I	213	CTGAGGAATCTCCG-AAAAC	TCTCAAGGTACCACC-GGTGAT
5α-II	250	TCACTAGAGGGAGGCC-TTTTC	ACAAGCCACCTTGTG-GAATC

ARO: p450-aromatase; 5α-I: type I 5α-reductase; 5α-II: type II 5α-reductase.

10 μl of RT mixture were combined with 4 μl thermophillic DNA poly 10× buffer, 0.25 μl thermophillic DNA polymerase (5 U/μl; Promega Corporation, Madison, WI, USA), 1 μl (10 pmol) of each primer and nuclease-free water to a final volume of 50 μl. Primers for aromatase, 5α-reductase type I and type II (Table 1) were used in different reactions. Expression of β-actin mRNA was measured as control and a PCR on isolated RNA without reverse transcriptase reaction was used as negative control.

The numbers of PCR cycles were chosen in such a way that the reactions were in the linear part of amplification, enabling a semiquantitative evaluation of the results. For this purpose several tests were carried out with 20, 25, 30, 35 and 40 cycles to estimate the linear section. PCR was performed in a Perkin-Elmer Cetus thermal cycler using at first one step of denaturation (94 °C, 5 min) followed by proper numbers of cycles of denaturation (94 °C, 1 min), annealing (60 °C, 1 min) and extension (72 °C, 1 min) and a final incubation of 10 min at 72 °C for linearization (Table 2).

Ten microlitres of all PCR products were separated on 2% agarose gels containing 25 ng ethidium bromide/ml and photographed under UV illumination using a CCD camera. The DNA molecular weight marker VI (Boehringer, Mannheim, Germany) was used as a size marker. Per pair of primers all samples were amplified together during the same PCR session and separated on one gel, each gel containing a negative control.

The photographs were scanned and band intensities were measured using the Molecular Analyst (BioRad Laboratories, Veenendaal, The Netherlands) after negative control was subtracted as background. PCR products were sequenced with a DNA sequencing kit (Big Dye Terminator Cycle Sequencing kit, Perkin-Elmer, Foster City, CA, USA) using a genetic analyser (ABI Prism 310, Perkin-Elmer, Foster City, CA, USA). Sequences were matched with the GenBank database of the American National Centre of Biotechnology Information to be certain that the correct products were identified.

Table 2
PCR schedule

Primer	Annealing temperature (°C)	Cycles	Magnesium concentration (mM)
ARO	55	35	2
5α-I	58–50	30	2
5α-II	58–50	40	3

ARO: p450-aromatase; 5α-I: type I 5α-reductase; 5α-II: type II 5α-reductase.

2.3. Statistical analysis

Expression levels of the enzymes in normal, hyperplastic and neoplastic mammary tissues were analysed by a one-way analysis of variance (ANOVA) followed by a Bonferroni adjusted multiple comparison among experimental groups. The degrees of interaction among enzymes expression were evaluated statistically using Pearson's correlation coefficient. $p < 0.05$ was considered significant.

2.4. Progesterone receptor affinity study

All procedures involving tissues and cytosol were performed at 4 °C or below. About 250 mg of frozen uterus specimens was minced on a cooled worktop, transferred to a liquid nitrogen-cooled container and ground thoroughly with a dismembrator (Braun Biotech Int., Melsungen, Germany) for 45 s. Tissue powder was decanted in 2 ml receptor buffer (0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, 1 mM EDTA, 3 mM NaN_3, 2 ml monothioglycerol, 10% glycerol adjusted to pH 7.5) and after 1 h centrifuged at 100,000 × *g* for 30 min. The supernatant (cytosol fraction) was kept at −70 °C and used for receptor analysis.

Saturation binding analysis of canine progestin receptor (PR) in uterine cytosol was performed by a multiconcentration dextran-coated charcoal assay as described previously [37]. For displacement study aliquots of 50 μl of uterine cytosol were incubated with 1 mM [^{3}H]ORG 2058 (specific activity 42.1 Ci/mmol, Amersham International, Amersham, UK) and increasing concentrations of unlabeled competing steroids to a total volume of 100 μl. The competing steroids were progesterone (Sigma–Aldrich Corporation, St. Louis, USA) or 5α-dihydroprogesterone (Steraloid Inc., Newport, USA), which were added in amount ranging from 0.025 to 2 μM (final concentrations). Incubation was at 4 °C for 18–22 h and all preparations were analysed in triplicate. After incubation, the cytosols were treated with a 100 μl suspension of 0.25% (w/v) activated charcoal, 0.025% (w/v) dextran in receptor buffer and 15 min later the preparations centrifuged at 1000 × *g* for 10 min. Aliquots of the supernatants were counted in a liquid scintillation counter (1212 Rachbeta, LKB, Wallac). The apparent dissociation constant (K_d) was calculated by Scatchard analysis [38] and data of multiple experiments were processed simultaneously using the LIGAND program [39] as modified by McPherson.

3. Results

3.1. Gene expression

Summary of data on mammary tissue specimens are reported in Table 3. Amplification of 5α-reductase type I

Table 3
Summary of data on mammary tissue specimens of healthy control dogs (normal, 1–5), progestin-treated dogs (hyperplastic, 6–11) and dogs with tumours (tumour, 12–20)

Sample	Code	Histology	Tumours cells (%)	Treatment	PR	ARO mRNA	5α-I mRNA	5α-II mRNA
1	Et44B	Normal	–	–	78	±	+	–
2	P117R4	Normal	–	–	nd	±	+	+
3	Dog 4	Normal	–	–	24	±	+	+
4	Dog 10	Normal	–	–	48	±	+	+
5	N1	Normal	–	–	nd	±	+	+
6	A2	Hyperplastic	–	OVX + MPA	nd	–	+	–
7	A3	Hyperplastic	–	OVX + MPA	nd	–	+	+
8	A4	Hyperplastic	–	OVX + MPA	nd	–	+	+
9	B1	Hyperplastic	–	OVX + Prol	nd	–	+	+
10	A5	Hyperplastic	–	OVX + MPA	nd	±	+	–
11	Donna	Hyperplastic	–	OVX + MPA	nd	–	+	+
12	P191R4	Solid	40	OVX: 5 years	<5	+	+	+
13	P221L4	Anaplastic	5–70	Prol: 1 week	nd	+	+	+
14	P157	Tubular	40	OVX: 2 years	26	+	+	+
15	P170R5	Complex	90	–	147	+	+	+
16	P208L5	Solid	35	OVX	nd	+	+	+
17	P187L2	Anaplastic	25	OVX	4	+	+	+
18	P182L2.1	Adenoma	70	MPA: 5 m	nd	+	+	+
19	P210L5.1	Solid	60	Prol	27	+	+	+
20	P233R3	Anaplastic	60	MPA	nd	+	+	+

Samples are in the same order as in Fig. 1. Histology, findings in formaldehyde-fixed tissue samples. Treatment: OVX: ovariohysterectomy; Prol: proligeston; MPA: medroxyprogesterone acetate; the time is the interval between treatment and tissue sampling. PR: progesterone receptor concentrations in fmol/mg protein in tissue cytosol; nd: not done; 5α-I: type I 5α-reductase isoenzyme; 5α-II: type II 5α-reductase isoenzyme; ARO: p450 cytochrome aromatase enzyme; (+) mRNA present; (−) mRNA absent; (±) faint band of mRNA present.

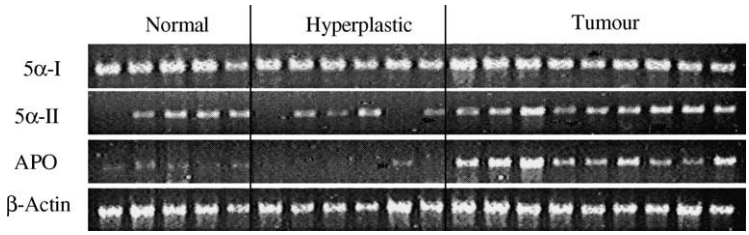

Fig. 1. Analysis of the RT-PCR products of mammary gland samples from young intact female dogs (normal), progestin-treated dogs (hyperplastic) and canine mammary gland tumours (tumour). 5α-I: type I 5α-reductase; 5α-II: type II 5α-reductase; ARO: p450-aromatase.

mRNA resulted in the presence of a 213 bp fragment, in all samples. Aromatase mRNA and 5α-reductase type II were expressed in most of the mammary tissue samples as a fragment of 419 and 250 bp, respectively (Table 3, Fig. 1). Sequence analysis of the mammary RT-PCR products confirmed that 5α-reductase type I and type II and aromatase were amplified. Analysis of the relative densities of the resulting PCR products revealed differences in expression levels of the enzymes in normal, hyperplastic and neoplastic mammary tissues (Table 4).

Hyperplasia is associated with 45% decrease and tumours had a 65% increase in the expression of isoenzyme type II compared to the normal mammary tissues (Table 4). The highest change was found in expression of aromatase as 61% decrease in hyperplastic tissues and 389% increase in mammary neoplasms (Table 4). Significant differences

Table 4
Intensity of PCR products expressed as volume counts/mm^2 (mean ± S.D.) and percent of control (normal)

Factor	Normal		Hyperplastic		Tumour	
	Counts (mm^2)	Percentage	Counts (mm^2)	Percentage	Counts (mm^2)	Percentage
β-Actin	33.8 ± 3.1^{A}	100.0	31.3 ± 2.5^{A}	92.7	35.3 ± 1.3^{A}	104.5
5α-I	34.4 ± 2.7^{A}	100.0	31.5 ± 1.7^{A}	91.6	35.4 ± 1.2^{A}	103.0
5α-II	17.8 ± 4.9^{AB}	100.0	9.7 ± 4.1^{A}	54.3	29.3 ± 3.5^{B}	164.8
ARO	3.0 ± 0.3^{A}	100.0	1.2 ± 0.4^{A}	38.9	14.7 ± 2.7^{B}	489.0

Different letters on the same line indicates significant differences among means (Bonferroni; $p < 0.01$). ARO: p450-aromatase; 5α-I: type I 5α-reductase; 5α-II: type II 5α-reductase.

among means (Bonferroni; $p < 0.01$) were present between hyperplastic and tumour samples for type II 5α-reductase expression and tumours and the other two groups for aromatase. No difference in enzyme expressions was found between tumour samples obtained from bitches with or without progestins treatment history (Table 3, Fig. 1). The changes in expression of type II 5α-reductase and aromatase mRNA were highly correlated ($p = 0.009$). The expression of β-actin was identical in all samples and no changes were found in the expression of 5α-reductase type I isoenzyme (Table 4).

3.2. Progesterone receptor affinity study

Saturation binding experiments using [^{3}H]ORG 2058 in canine uterus cytosol revealed high affinity, low capacity receptor for progestins and Scatchard analyses of these experiments resulted in a mean apparent dissociation constant (K_d) of 7.1 ± 0.5 nM of [^{3}H]ORG 2058 for the canine PR. In the displacement studies inhibition constants (K_i) of steroids competing with [^{3}H]ORG 2058 for binding to the PR were 100.3 ± 8.2 and 617.5 ± 126.3 nM for progesterone and dihydroprogesterone, respectively.

4. Discussion

In this study we demonstrated that in the mammary gland of dogs, hyperplasia is associated with a low expression of the steroid converting enzymes type II 5α-reductase and aromatase, whereas in tumours the opposite situation is found.

The role of progesterone in the regulation of the growth of mammary epithelium is controversial. Although the highest mitotic indexes have been found in the mammary gland during the progesterone-dominated phase of the sexual cycle and during pregnancy, high doses of progestins can be used to reduce mitotic figures in mammary cancer and inhibit growth of mammary carcinomas in humans [40]. This discrepancy in effect of progesterone on cell proliferation is attributed to a biphasic response of cells on progesterone treatment [41]. The initial growth stimulating effect could prime cells to growth-stimulation by peptide growth factors thereby switching from a steroid hormone driven proliferation towards a peptide hormone driven proliferation. This may contribute, in part, to the development of steroid hormone resistance during breast cancer progression. On the other hand, the prolonged exposure to progesterone would stimulate the expression of inhibitors of cell cycle progression such as p21 and p27 [41]. Another explanation for the high dose progestin-induced inhibition of tumour progression is the fact that many synthetic progestins used for breast cancer treatment have intrinsic androgenic and glucocorticoid activities [42,43]. Due to this glucocorticoid activity progestin treatment results in suppression of plasma cortisol concentrations in humans [44,45], dogs [46–48] and cats [49,50] and reduces the plasma concentrations of sex steroids in postmenopausal breast cancer patients [51–53]. In vitro progestins inhibit the biosynthesis of DHP and estradiol in rat granulosa cells [54,55] and of estradiol in breast cancer cell lines [56]. The beneficial effects on tumour progression of medroxyprogesterone acetate show also a better correlation with androgen receptor content than progesterone receptor content of mammary carcinomas [42].

The lower aromatase and type II 5α-reductase gene expression in hyperplastic mammary tissue induced by treatment with progestins compared to tumour should be seen against this background. A reduced aromatase activity would result in a lower conversion of androgens into estrone, the precursor of 17β-estradiol within the mammary gland. A lower activity of type II 5α-reductase activity can be seen as inhibitory towards the activation of testosterone into the biologically more potent dihydrotestosterone, but also a reduced conversion of progesterone into the metabolite dihydroprogesterone that has a reduced affinity for the progesterone receptor as shown in the present study. The effects of this reduced metabolism of steroids within the mammary gland on cell proliferation/differentiation is difficult to predict. The lower local biosynthesis of estrogens is expected to be beneficial to reduce estrogen-stimulated proliferation of mammary epithelium. A reduced activation of androgens may have an opposite effect in facilitating cell growth.

The low conversion of progesterone is even more difficult to interpret. A higher bioavailability of progesterone can stimulate mammary proliferation and promote carcinogenesis in the dog [25,27,30]. In this respect 5α-reduction of progesterone in DHP could represent in this tissue a protective mechanism. In line with this is the result of the displacement studies that showed that dihydroprogesterone has a six-fold lower affinity for the canine progesterone receptor than progesterone. However, a recent study conducted on breast cell lines [31], has proved that 5α-pregnanes, especially DHP, stimulates cell proliferation and reduces cell adhesion both facts facilitating carcinogenesis and tumour progression. The DHP binds weakly to the progesterone receptor but in human breast cancer cell lines a specific plasma membrane receptor has been identified, which can be induced by estradiol [57]. The role of this metabolite in the mammary gland physiology and breast carcinogenesis needs further studies concerning its possible biological effects in this tissue. Finally, a high correlation was found between aromatase and 5α-reductase activity. Both activities have been shown to be present in the stromal compartment of the mammary gland, and may indicate a general reduced activity of the stromal tissue [58].

Canine mammary tumours have shown the opposite situation with increased expression of both type II 5α-reductase and aromatase enzymes. To the best of our knowledge, aromatase activity or expression has never been documented previously in mammary tissue of the bitch, while its role is under intensive study in human breast cancer maintenance and progression. The higher expression of the aromatase enzyme could represent an advantage for neoplastic cells since many studies have provide evidence that estrogens play an important role in cell proliferation in breast cancer [59]. For

the increased expression of the type II 5α-reductase enzyme a growth promoting effect will be present in case the formed DHP stimulates cell proliferation as discussed previously.

It can be concluded that, in contrast with progestin-induced proliferation of mammary epithelium, in mammary cancer specimens a relative high activity of stromal cells is found with regard to the expression of steroid converting enzymes. The nature of the activating signals from tumour tissue are yet unknown as far as we know. The enhanced expression of these enzymes could result in a growth advantage of the tumour cells, and consequently the low expression of these enzymes in hyperplastic tissue can be regarded as a mechanism to protect against too rigorous growth stimulation by progestins. The role of the progesterone metabolite DHP awaits further investigations.

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