Characterization and cell of origin of prostate carcinoma in the dog

Chen-Li Lai

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Karakterisering en cel van oorsprong
van prostaatkanker bij de hond

(met een samenvatting in het Nederlands)

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Chen-Li Lai

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Promotor:	Prof. dr J.E. van Dijk
Co-promotoren:	Dr. E. Teske Dr. R. van den Ham

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CHAPTER 1

General Introduction

SCOPE OF THE THESIS

The prostate is a male accessory reproductive gland present in all domestic species. It is an exocrine gland with a tubuloalveolar structure and is located just caudally of the bladder and surrounding the urethra. The function of the prostate is to store and secrete a slightly alkaline fluid that, together with the sperm cells, makes up the major part of semen.

The prostate in dogs and humans is prone to several disorders, among which are prostatitis, abscesses and cysts, hyperplasia and cancer. To validate the information obtained from in vitro studies and to further elucidate the pathogenesis of prostate diseases, in vivo studies are inevitable and finding an appropriate animal model becomes an urgent demand. In biomedical research, rodents are usually chosen as animal models, because they are easy to handle, economic and many historical data are available. For prostate cancer, numerous transgenic and drug-induced models have indeed been described in mice and rats.[15,138] The cancer studies usually start with inducing or implanting tumours in the rodent models, followed by the designed therapeutic experiment on these animals.

The dog may offer an alternative option as a spontaneous animal model for human prostate diseases, and the aging male dog has for example been used as a spontaneous model for human benign prostatic hyperplasia (BPH)[10,99] In addition, canine spontaneous prostate carcinoma has been argued to be an interesting model for late stage human prostate cancer [113,131,132,178,180] Just as human late stage prostate cancer (hPC) is canine prostate cancer (cPC) most commonly found in elderly patients.[179] In addition, in both species is invasive growth of tumour cells into and outside the prostate capsule common, and are regional lymph nodes, (pelvic) bone and lungs common sites where metastases are found.[33,128,180] Just as human late stage prostate cancer is cPC non-responsive to androgen ablation therapy.

Thus, cPC may serve as a precious model for advanced human prostate cancer, filling the gap between the rodent model studies and human clinical research. In order to evaluate cPC to fulfil this role, the similarities and differences between the normal prostates of the two species should be investigated. In addition, a histological, cellular and molecular characterization of cPC in relation to its human counterpart is necessary.

In this introduction, an outline is given on the comparative histological, cellular and molecular organization of human and canine prostates. In addition, the current understanding of prostate cancer development in humans and dogs is presented. Finally, the aim of this thesis is introduced.

THE HUMAN PROSTATE

Anatomy

The prostate gland is a walnut shaped structure situated in front of the rectum, just below the bladder and surrounding the beginning of the urethra. Another pair of accessory reproductive glands, called the seminal vesicles, is located between the rectum and the bladder and attached to the prostate. The prostatic urethra descends vertically through a somewhat elongated prostate and shows the prostatic utricle opening on to its posterior wall. Both the urethra and the ejaculatory ducts traverse the prostate gland.

Histology

The slim connective tissue capsule of the prostate gland is composed of a richly vascularized, dense irregular collagenous connective tissue, interspersed with smooth muscle cells. The stroma of the gland is derived from this capsule and is therefore, in addition to the usual connective tissue cells, also enriched with smooth muscle fibres. The prostate epithelium consists of a conglomeration of many individual tubuloalveolar glands. Each tubuloalveolar gland ends in a duct to deliver its secretory product to the urethra. The glands of the prostate comprise a simple to pseudostratified columnar epithelium, which cells are well endowed with organelles that are responsible for the synthesis and packaging of the secreted proteins.

The human prostate is (patho)histologically often characterized by the presence of different zones (the peripheral zone, the transition zone, and the central zone). Each zone has a characteristic histology. In addition, each zone has a predisposition to developing a certain prostatic disease.[103,104] Glands of the peripheral zone have a simple, rounded shape, with gentle ripples of the luminal borders. Of the carcinomas, 70–75% arise in this zone. Glands of the transition zone are similar to that of the peripheral zone, and about 15–20% of prostate carcinomas arise in this area, while it is the main source of BPH. Glands of the central zone are larger and often arranged in lobules with luminal ridges and papillary foldings. The epithelium of the central zone usually has a granular cytoplasm. Only about 10% of carcinomas arise in the central zone.

The human prostate epithelium is morphologically composed of two cell layers. The secretory luminal cells are cuboidal to columnar and secrete proteins such as PSA into the glandular lumina. Depending on their localisation they may appear as single or multilayered. The basal layer consists of flat to triangular cells that are regarded as the progenitor cells of the luminal epithelium. The basal layer surrounds the secretory cells. Basal cells, which commonly touch several secretory cells, are often difficult to recognise by routine light microscopy.

Development

Growth and development of the prostate already begin during embryonic life. In the embryo the urogenital sinus, composed of an endodermally derived epithelial layer surrounded by a mesodermally derived mesenchymal layer, is found just caudal to the neck of the developing bladder. Here, prostate development is initiated and promoted by circulating testosterone (a major form of androgen) produced by the foetal testes. Testosterone excites the epithelial-mesenchymal interaction in the urogenital sinus and initiates its development into prostate tissue. In addition to androgens, prostatic development is very sensitive to levels of estrogenic compounds; early exposure to estrogens accelerates the advance of prostatic development.[73,123,164]

According to studies in rodents, the solid prostatic epithelial buds emanate into the urogenital sinus mesenchyme and branch.[163] The solid epithelial cords then become tubules, starting from the urethral terminus toward the ductal tips.[148] Androgens, the products of androgen receptor activated genes and epithelial-mesenchymal interactions are involved in the further process of prostate development.[39,53,152] At puberty, the serum testosterone level rises significantly and the prostate weight increases rapidly. The development of the prostate is complete at sexual maturity. In the adult, the size of the prostate gland is maintained by balanced cell proliferation and cell death. This balance largely depends on testosterone.

Cellular and molecular regulation of the prostate epithelium

Cell division is a physiological process that occurs under many circumstances in almost all tissues. To ensure the integrity of organs and tissues, a process called programmed cell death or apoptosis, balances this proliferation. In normal tissues, both processes are under tight regulation. In most if not all epithelia, slowly dividing stem cells divide and give rise to an intermediate type of cells that proliferate more actively. These intermediate type, or differentiating cells then terminally differentiate to fully functionalized, or differentiated cells. These differentiated cells finally leave the epithelium via apoptosis.

The concept of tissue specific stem cells has been discussed since about 1900.[27,133] However, it was not until 1963 that the first quantitative description of the self-renewal activities of transplanted mouse bone marrow cells were documented by the Canadian scientists Ernest A McCulloch and James E Till[102,161,162] Since then, the presence of stem cells was reported in several tissues that have a rapid cell turnover, such as skin[35,49] and the gastrointestinal tract[63,130] Later, it was recognized that the epithelium of most solid organs is able to continuously replace itself. Hence, stem cells were thought to be present in these organs as well .[78,125,140,146,156]

Stem cells have characteristics that distinguish them from other types of cells. In general, stem cells can be defined by two features: pluripotency and expandability.[76,126,170,183] Pluripotency

is defined by the ability of the stem cells to produce daughter cells of more than one fate, and expandability means the ability to produce a large number of differentiating progeny.[101,112a,126,170,] Stem cells have a high capacity of proliferation and self-renewal, slow cycling rates and their progeny can differentiate further under certain physiologic or experimental conditions.[101] As written in Reid's review[126], the maturational process results in cells with a gradient in expression of phenotypic markers which were dictated in part by restriction of genetic potential through physical and biochemical changes in the chromatin and, secondarily, by signal transduction mechanisms activated by gradients of hormonal and extracellular matrix signals. So far, the only recognized stem cells with the capacity to produce all known cell types (omnipotent stem cells) are the zygote and germ cells. During embryogenesis, omnipotency gets restricted in somatic stem cells to a pluripotent and unipotent state in determined tissue derived stem cells; cells that have the ability to generate daughter cells maturing into all the cell types for subsequently several or one specific tissue(s). The pluripotent and unipotent stem cells produce lineages of daughter cells that undergo a unidirectional, and terminal differentiation process.[126] As described by Morrison and Kimble in their review article[110], the prevailing strategy that stem cells use for self-renewal is asymmetric cell division. With a single division, a stem cell divides to generate one daughter cell with a stem-cell fate and the other daughter cell that is destined to differentiate further. The differentiating progeny, or transient proliferating cells, may rapidly divide and continue to differentiate and become mature cells.[110] Stem cells can also use symmetric divisions to generate two daughters that both have a stem cell fate or that both will further differentiate. Some mammalian stem cells seem to switch between symmetric and asymmetric cell divisions, but the mechanism of switching between these two modes is not yet clear.[110]

In rapidly renewing tissues such as the intestine and skin, terminally differentiated cells often form stratified layers whereas the stem cells distribute basally, underneath the maturing and mature cell layers. Isaacs and Coffey in 1989 postulated a similar stem cell driven hierarchical arrangement of the epithelium for the more slowly renewing adult prostate.[70] The arrangement of prostatic epithelium has been observed for decades on the basis of localization, morphology and the expression of keratins, which enabled the discrimination of basal, luminal and putatively intermediate or transiently proliferating cells.[174] The compartment of the prostatic epithelium in humans can be depicted as follows. The basal cells are located on the basal membrane. These cells, that can be characterized by the expression of the cytokeratins (CK) 5 and 14, likely encompass the slowly proliferating stem cells and give rise to a more rapidly cycling intermediate/transient population of cells (expressing CK5 and CK18 and one or more of CK8, CK18 and CK19). These intermediate cells finally differentiate toward the lumen into the luminal/secretory cells (expressing CK8 and CK18). The fully differentiated luminal cells express, among others, PSA that is excreted into the prostatic duct and ultimately drained to the prostatic urethra.[54,67,177]

Hormonal regulation of prostatic integrity

Studies in rodents and human show that both prostate development and maintenance largely depend on male hormones. The secretion of these androgens is part of the complex hypothalamic-pituitary-testicular axis. The hypothalamus first secretes locally acting luteinising hormone releasing hormone (LHRH), also known as gonadotrophin releasing hormone (GnRH), and corticotrophin releasing hormone (CRH). These polypeptides act on the pituitary gland to release luteinising hormone (LH), follicle stimulating hormone (FSH) and adrenocorticotrophin (ACTH). These hormones then enter the circulation and act distantly on their target organs such as the testes and adrenal glands. LH acts on the Leydig cells in the testes to stimulate the production of androgens. A small proportion of the circulating androgens is also produced by the adrenal gland in response to ACTH.[25,26]

Androgens play a critical role in regulating the growth, differentiation and survival of the epithelial cells in the normal prostate. Studies in individuals who have a low circulating testosterone level show an underdeveloped prostate.[129] In addition, male rat fetuses that are treated with 5-α reductase inhibitors (inhibiting the conversion of testosterone to the more potent dihydrotestosterone) during the critical period of sexual differentiation in utero reveal an impaired prostate growth and development.[40,69] Such rodent studies have led to the concept that androgens function as survival factors in the adult prostate.[68,124] Following castration, the prostate gland of adult male rats regresses due to a massive loss of cells.[31,44,88] Histologically, the acinar secretory epithelial layer is lost, but the basal epithelial cells and the basement membrane remain intact, suggesting that the secretory epithelial cells require androgens directly and/or indirectly for their survival whereas the basal epithelial cells do not. When the castrated males were treated with testosterone, their regressive prostate was restored to its normal weight through the formation of new acini from the remaining tubules .[147]

The epithelial-stromal interactions are essential for a functional androgen-androgen receptor (AR) axis in the development and differentiation of the normal prostate. Cunha and his co-workers isolated the murine embryonic urogenital sinus (UGS), from which the prostate develops, and separated it into its mesenchymal and epithelial components.[38] Subsequently, they conducted tissue recombination experiments using urogenital sinus stroma and epithelium from testicular feminized embryos (in which the gene encoding the androgen receptor (AR) was mutated and AR was not expressed) and wild type embryos. With the results from the different combinations, they demonstrated that a functional androgen receptor, expressed in the UGS mesenchyme, is the key component for the normal growth and differentiation of prostate epithelial cells.[1,37,79,93] Moreover, Habib and co-workers showed that when adult human normal prostate epithelial cells (including basal cells) were cultured without stromal cells, these cells lost AR and PSA expression and 5-alpha-reductase activity and did not even grow under testosterone influence.[59] In contrast, when such cells were co-cultivated with prostate stromal cells that were physically separated by a

microporous membrane, the epithelial cells retained AR and PSA expression and 5α-reductase activity, and their growth became androgen sensitive.[59] A possible explanation for these results is that androgens regulate the proliferation and differentiation of prostate epithelial cells (basal/stem cells) by stimulating the expression of mediators such as andromedins in the prostate stroma, which stimulate, via a paracrine interaction, the AR expression in the epithelial cells to respond directly to androgens and trigger the expression of downstream androgen-responsive genes.

The AR belongs to the superfamily of nuclear receptors that act as ligand-inducible transcription factors (Charestal factor).[45,55] ARs are located in the stromal cells of the prostate as well as in the epithelial cells. The basal cells of the prostatic epithelium, however, do not seem to contain AR and are androgen-independent.[42] The effective ligands binding to AR are androgens, in which testosterone is the major form of serum androgen. After testosterone from the plasma has entered the prostatic cell through diffusion, it is metabolized into other steroids by a series of enzymes.[18,90] In the cytoplasm 5α-reductase converts over 95% of testosterone into dihydrotestosterone (DHT), a more potent form of androgen. The enzyme 5α-reductase exists in two isoforms, types 1 and 2.[120] Type 1 distributes wildly in most tissues of the body like skin, liver, sebaceous glands and prostate while type 2 is exclusively found in the prostate and genital tissue.[60,74,159] Immunohistochemical studies using 5α-reductase type 2-specific antibodies have demonstrated that this enzyme is primarily localized in the stroma of the gland.[142] The newly formed DHT (and some of the testosteron) then binds to the activated androgen receptor that subsequently homodimerizes.[168] This then enables the interaction of the DHT/AR complex with co-activators and the translocation of the complex to the nucleus, where it binds to androgen responsive elements in the DNA to activate the transcription of androgen-dependent genes such as PSA.[7,23,58,65]

Besides its direct effect on prostatic cells, the DHT/AR complex activates in the prostate stroma the secretion of peptide growth factors known as andromedins, among others FGF7.[94] These andromedins diffuse across the basement membrane into the epithelial compartment. There, the andromedins bind to specific plasma membrane receptors to initiate signalling for proliferation and epithelial cell survival.[71]

HUMAN PROSTATE CANCER

Histological changes during prostate cancer progression

PIN (Prostatic Intraepithelial Neoplasia) lesions are assumed to be the first recognizable precursor of carcinoma.[3,61,84,105] The term PIN was first proposed by Bostwick and Brawer in 1987.[16] However, before the term PIN was proposed, non-invasive epithelial lesions (eg, intraductal hyperplasia, hyperplasia with malignant change, large acinar atypical hyperplasia, marked atypia,

ductal-acinar dysplasia) were already described in the prostate that shared some of the cytologic and architectural features of adenocarcinoma,[77] but at an international conference in 1989, the term PIN was accepted as a replacement for the various other terms.[15a,40a] Intraductal dysplasia was also used once to describe this lesion, and the authors proposed a three-tier system to grade that lesion.[105] However, those at the 1989 consensus conference agreed that only the terms low-grade PIN (LGPIN) and high-grade PIN (HGPIN) would be used. PIN are described as large acinar atypical hyperplasia displaying malignant changes and duct-acinar dysplasia. PIN is usually multifocal, involves a gland either partially or completely, and has four main architectural patterns, from a simple flat epithelium to a tufting, micropapillary or cribriform pattern.[6] These patterns often merge with each other. Other than diagnostic utility, these architectural patterns have no known clinical significance.

The histological appearance of PIN changes along with the malignant progression. In low grade PIN atypical cells show a prominent increase in nuclear size or anisokaryosis (increased size variability), cell crowding accompanied by irregularity of nuclear spacing, cytoplasmic eosinophilia, occasional dark filiform nuclei, and a few small but prominent nucleoli. With malignant progression an increased density of chromatin staining is seen in addition to the nuclear enlargement, anisokaryosis, and irregular cellular spacing. Chromatin is finely granular and condensed, and nucleoli are more numerous. In high grade PIN marked nuclear enlargement with large, prominent eosinophilic nucleoli are seen in the majority of cells. Nuclear hyperchromatism and chromatin margination are more prominent in most nuclei. The degree of nuclear crowding can be quite variable. In some areas of severe crowding, bridges of epithelial cells extend across glandular lumina, producing a trabecular pattern. This is all about PIN lesions. In humans, the majority (up to 95%) of prostate cancer is adenocarcinoma, mainly characterized by an acinar differentiation. There are still some variants such as sarcomatoid carcinoma, adenosquamous carcinoma, ductal carcinoma, and transitional cell carcinoma. Next to the adenocarcinomas, poorly differentiated carcinomas were recognized, of which the tumour cells are not confined to the acini. Differences in pathomorphological appearance of androgen-sensitive and androgen-refractory prostate cancer have also been reported in human prostate cancer. Kondo et al. classified the glandular (including large and/or small simple glands), micro-glandular, and cribriform growth patterns as androgen sensitive, while fused glands, medullary/solid and columnar/trabecular patterns were classified as androgen refractory components.[83] They also reported that metastases tend to have androgen refractory components. In addition, Shah et al. studied the characteristics of metastatic, hormone-refractory prostate cancer and concluded that androgen-independent prostate cancer is a heterogeneous group of diseases.[135] The majority of cases showed a mixture of high grade of Gleason classification. This mixture of growth patterns seen in human androgen refractory prostate cancer remarkably resembles the mixture of growth pattern we have seen in canine prostate cancer.

Application of the stem cell hypothesis to human prostate cancer development

Leenders and co-workers found that only CK5 and CK18 expressing cells were detected in androgen sensitive as well as late stage prostate cancer, metastases and cell lines.[173] No CK14 expressing cells were detected by these authors in these specimens. They hypothesized that, if the normal scheme of prostatic epithelial differentiation also applies to cancerous cells, CK5 expressing cells are the earliest cells in the lineage of differentiation found in prostate cancer and that human prostate cancer likely originates from these intermediate type, transiently proliferating cells.[173]

Along a similar line of reasoning: cells that are at the origin of prostate cancer will be able to give rise to the full compendium of cell types that are seen in prostate cancer. Such cells are called cancer stem cells. Several (overlapping) populations of cells have shown to be able to reconstitute the different epithelial cell types of the normal prostate in vitro or in vivo when isolated from the normal prostate, or those commonly found in prostate tumours when isolated from prostate tumour specimens. CD44 is considered to be a marker of basal cells[95,157] and CD44(+) PCa (Prostatic Carcinoma) cells are more proliferative, clonogenic, tumourigenic, and metastatic than the isogenic CD44(-) PCa cells[119]. In addition, CD44(+) PCa cells express higher levels of several 'stemness' genes such as Oct-3/4, Bmi, β-catenin, and SMO. Finally, CD44(+) PCa cells, which are AR(-), can generate CD44(-) cells and differentiate into AR(+) tumour cells. Also basal cells that have a high expression of α2β1-integrin or that are CD133(+) subsequently mark smaller cell populations that have a greater ability to form colonies in vitro than the total basal population, and are able to reconstitute a normal epithelium in vivo.[29,127] Tumour cells with a CD44(+)/α2β1hi/CD133(+) phenotype approximately make up 0.1% of the cells in any tumour regardless of Gleason grade or metastatic state.[28] Sca-1 expressing cells in the proximal regions of the murine prostate have a higher capacity of reconstituting prostate tissue in vivo compare to Sca-1 depleted prostate cells, as well as Sca-1 expressing cells from other regions of the prostate (transient amplifying cells).[20] Genetic perturbation of PTEN/AKT signalling in these cells subsequently leads to tumour formation in an in vivo model.[184]

How these Sca-1(+), CD44(+), α2β3 integrin(hi) or CD133(+) cells relate to the CK5 expressing cells as indicated by van Leenders and/or to earlier type of (CK14 expressing) cells remains however unclear. Furthermore, it has been shown in human leukaemia that certain chromosomal aberrations can at least partially convert the expression of progenitor cells to that of stem cells and can confer self-renewal activity in committed progenitors.[85] Therefore, a definite answer to the question which cell population gives rise to prostate cancer cannot yet be given.

Androgen dependence

Since the first observation by Huggins and Hodges in 1941 that prostate cancer is androgen-ablation responsive, supportive results were shown in several studies.[5,56] Approximately 70-80% of human prostate cancer patients treated by androgen ablation respond favourably at first instance[116], reflecting an androgen dependence of tumour cell proliferation in the early stages of tumour development. In this point of view, prostate cancer cells have characteristics resembling the secretory epithelium of the normal prostate. Subsequent to successful androgen ablation treatment however, prostate cancer often relapses and progresses to a more aggressive and hormone-refractory stage in which cancer cell proliferate rapidly, infiltrate and metastasize, despite low concentrations of serum androgen.

Only a few years ago, Tomlins and co-workers discovered an important clue around the androgen sensitivity of a large portion of human prostate tumours.[166,167] They showed that gene fusions involving the untranslated region of the androgen-regulated gene TMPRSS2 and the ETS (E26 transformation-specific) family of transcription factors ERG, ETV1 or ETV4 were abundantly present in prostate cancer specimens. This has subsequently been confirmed by several other investigators and it seems that ~50% of human prostate tumours harbour the TMPRSS2-ERG fusion product, whereas 1-10% of tumours contain the TMPRSS2-ETV1 fusion gene.[106,109,111,182] As research progressed, several other gene fusions were discovered such as SLC45A3-ETV1, HERVK-ETV1, 22q11.3-ETV1, HNRPA2B1-ETV1 and C15ORF21-ETV1 fusions, with differences with respect to the extent of androgen regulation.[165] Primary prostate tumours appear to be heterogeneous with respect to the occurrence of these gene fusions, reflecting the multi-clonal nature of human prostate cancer.[107] However, prostate cancer metastases within individual patients are generally uniform in their expression of fusion products, pointing to an evolution of metastases from a single clone.

Perner et al identified the presence of the TMPRSS2:ERG fusion in 48.5% of clinically localized prostate cancers, 30% of hormone-naive metastases, 33% of hormone-refractory metastases, and in 19% of high-grade PIN lesions that were always found intermingling with cancer foci.[121] The generation of TMPRSS2-ERG of TMPRSS2-ETV1 fusion gene transgenic mice resulted in the formation of mouse PIN.[82] Finally, over-expression of TMPRSS2-ERG gene fusion in an immortalized benign tumour cell line induced an increased invasive capacity in these cells.[82] Taken these results together, the group of Chinnaiyan hypothesized that TMPRSS2-ETS gene fusions are involved in, but are not sufficient for the transition from PIN lesions to adenocarcinoma.[109] Likely, additional genetic aberrations such as single-copy loss of the tumour suppressors PTEN or NKX3-1 are necessary for this.

The androgen insensitivity in hormone resistant/refractory prostate cancer is likely to be caused by clonal selection, the selection of cells that are adapted to an environment without or with reduced concentrations of androgens.[143,151,160] Several adaptation mechanisms have been

reported for this, including AR amplification, AR mutation, altered expression of AR co-activator and co-repressor proteins, and activation of other pathways that can enhance AR function.[7] In humans, AR amplification at the DNA and RNA level has been reported in about 20-30% of hormone-refractory prostate cancer[43,46,47], whereas mutations in the AR gene have been reported in about 10-20% of prostate cancer specimens, with higher percentages in hormone refractory and metastatic tumours.[51,149,150153,154] Intriguingly, this phenomenon of hormone-refractory reoccurrence is supportive to the concept of a stem cell origin of prostate cancer, because the basal cells, harbouring the putative stem cells, are not dependent of androgen, are therefore likely to remain present after androgen ablation therapy, and thus may be responsible for the re-development of hormone depletion resistant prostate cancer after androgen ablation therapy. In a xenograft mouse model, Hermans et al found that in three out of four androgen independent prostate tumours a TMPRSS2-ETS fusion gene was present, but that none of these tumours expressed the fusion product. Notably, expression in these androgen independent specimens appeared to be bypassed by over-expression of wild type ETV4 or FLI1.[62]

The influence of inflammation, microenvironment and aging on prostate carcinogenesis

As stated before the microenvironment of the prostate gland consists of several distinct cellular components, including epithelium, smooth muscle, fibroblasts, vascular structures, extravasated blood cells, as well as insoluble matrix and circulating soluble factors, all interacting in the context of an extracellular matrix (ECM). This ECM plays an important role in the process of inflammation. The link between inflammation and cancers was noticed as early as in 1863 when Virchow indicated that cancers tend to occur at sites of chronic inflammation.[8] Evidence showed that about 20% of all human cancer in adults results from chronic inflammatory states and/or chronic inflammation.[2,36] Accumulated epidemiologic studies also support the notion that chronic inflammatory diseases are frequently associated with an increased risk of cancer development.[36,72,122,134] Moreover, several studies demonstrated that cancer incidence can be reduced by certain anti-inflammatory agents.[41,108] Cordon-Cardo and Prives investigated chronic inflammatory lesions and suggested that chronic inflammation is characterized by sustained tissue damage, damage-induced cellular proliferation, and tissue repair.[32] Cell proliferation in this context is often correlated with metaplasia, a reversible change in cell type.[32] To date, it is realized that the development of cancers by inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which together establish an inflammatory microenvironment.[36] Although this host response may suppress tumours, it may also facilitate cancer development via multiple signalling pathways.[185] The chronic inflammatory microenvironment is predominated by macrophages. Together with other leukocytes, those macro-

phages generate high levels of reactive oxygen and nitrogen species.[36,97,112] In a setting of continuous tissue damage and cellular proliferation, the persistence of these infection-fighting agents may produce mutagenic agents such as peroxynitrite that induce DNA mutations in proliferating epithelial and stromal cells .[50,97]

Tuxhorn et al reported that a reactive stroma exhibiting features associated with wound healing is capable of stimulating prostate epithelial proliferation in vivo.[172] The microenvironment is increasingly recognized as an important contributor to the development of epithelial cancers.[37,87,155] Prostate fibroblasts derived from regions harbouring invasive cancer for example, but not fibroblasts adjacent to normal epithelium, can promote the invasive phenotype of initiated prostate epithelial cells.[37] As we know that prostate cancer is a disease related to aging, the senescent prostatic stroma might contribute to tumour promotion. To identify senescence- or ageing-associated changes in stroma-derived factors that have potential to influence epithelial phenotypes, Begley et al used microarrays to identify transcript alterations that specifically associated with differences in the age of cells comprising human prostate stroma.[12] Fifty-four unique transcripts were found to be differentially expressed with ageing, of which 41 were up-regulated and 13 were down-regulated. Of the up-regulated transcripts, CXCL12 (SDF-1) was the most highly up-regulated gene. Subsequent experiments confirmed that the age-associated increases of CXCL12 signalling increased epithelial proliferation.[12] In their following study, Begley et al demonstrated that co-cultures of prostate epithelium with senescent fibroblasts produced significantly higher epithelial cell proliferation rates when compared with pre-senescent fibroblasts. Using only conditioned medium from the senescent fibroblasts, they showed that a large component of the growth promoting effect was due to paracrine-acting factors.[11] These studies showed that genes related to cell communication, extracellular matrix structures, immune and inflammatory responses, and insulin-like growth factor binding were active. As reviewed by Dean and Nelson, many of these up-regulated genes could potentially contribute to the effects of senescent stroma on epithelial cell behaviour. One set of such genes is that of the secreted autocrine- or paracrine-acting growth factors including amphiregulin, hepatocyte growth factor, bone morphogenic protein 1, macrophage-inhibitory cytokine 1 (MIC-1/ PLAB/GDF15), connective tissue growth factor, and vascular endothelial growth factor (VEGF). A second category of genes upregulated in senescent fibroblasts includes chemokines and cytokines; CXCL12, CXCL1, CCL11, CCL13, CCL20, C17, IL6, and IL8. A third set of upregulated genes in senescent stroma with the potential to modulate prostate carcinogenesis includes the extracellular matrix proteins, proteases, and protease inhibitors, that can modify paracrine-acting proteins and remodel structural components of the microenvironment. Notably, many of the genes mentioned here related to senescence and aging also play an important role in (chronic) inflammatory processes.

Genetic risk of prostate tumorigenesis

Prostate cancer risk is strongly influenced by familial history, and several susceptibility loci, such as Hereditary Prostate Cancer 1 (HPC1), located at chromosomal region 1q24-25, have been reported.[22,115,176] Men with BRCA1 germ line mutations have an estimated 3.33 higher risk of developing prostate cancer than those without mutations in this gene.[48]

Several genetic polymorphisms also contribute to the risk of developing prostate cancer. For example, PSA gene promoter polymorphisms and a homozygous PSA genotype are believed to be associated with the risk of prostate cancer. The length of a polymorphic polyglutamine (CAG) repeat in the androgen receptor gene has been reported to correlate with the risk of prostate cancer.[66,145,175] Humans with shorter CAG repeat length are at higher risk of developing prostate cancer compared to those with longer variants. Moreover, the coexistence of the PSA (GG) genotype combined with the short AR CAG repeat sequence is reported to increase the risk of prostate cancer development.[117]

THE CANINE PROSTATE

Development

The development of canine prostate is generally similar to the process in human. As Leav and co-workers revealed in their study, a developing canine prostate in the perinatal dog, age 2-8 weeks, showed a series of long main ducts that radiated from the prostatic urethra to the periphery of the gland.[91] The majority of these ducts contained a lumen but a few were still solid structures. Numerous small branches were seen to emanate from the main ducts as they extended toward the periphery. Epithelial cells, destined to form acini, were found as solid aggregates that surrounded the small ductal branches. At this stage of development, the intervening stroma was abundant, highly cellular, and separated the duct/acinar structures into lobules. At sexual maturity (15 months), the gland had a well-developed duct/acinar system. The papillary infoldings of the acinar lining were seen to project into the lumen. With increased age (4-8 years) these infolds became progressively more branched.[91]

Anatomy

Different from humans, the prostate is the only accessory sex gland in the male dog. It completely encircles the proximal portion of the urethra at the neck of the bladder, and is bordered dorsally by the rectum, and ventrally by the symphysis pubis and the ventral abdominal wall.

Histology

The prostate is surrounded by a fibromuscular capsule and divided into two lobes by a median septum. Each lobe is further divided into lobules by capsular trabeculae The stroma consists of fibroblasts and smooth muscle cells enmeshed in collagen fibres, with blood vessels and nerves. The acinic structures in the dog are much more prominent in comparison to the human prostate, while there is comparatively less stromal tissue. The dog prostate lacks the zonal differences based on epithelial cell morphology as seen in man, but rather displays a uniform morphology along its longitudinal axis. The canine prostate epithelium is organized as ductal structures that surround the urethra from the peri-urethral area to the periphery of the gland, ending into secretory acini. Similar to the human prostate, the tubuloalveolar structures excrete their secretory products into the prostatic ducts that deliver the secretion into the prostatic urethra. In addition, the duct system of the prostate possesses saccular dilations in which secretory material may be stored.

The epithelial cells are of two types: secretory cells and basal cells. The tubules and alveoli of the prostate are lined by a simple cuboidal or columnar secretory epithelium, interspersed by occasional basal cells. The simple epithelium changes to stratified columnar or transitional epithelium toward the terminal portions of the ducts. Some of the epithelial cells give a positive mucous reaction, but most of them contain proteinaceous secretory granules. The types of epithelial cells can be discriminated by immunochemistry. Several investigators have used high molecular weight cytokeratins (HMWCK) or CK5 to identify the basal cells, and CK18 to indicate the secretory cells.[89,91,99]

Hormonal regulation

The hormonal regulation for canine prostate is generally similar to that of the human prostate. Mahapokai and co-workers reported that an inflammatory response was induced in the canine prostate when animals were treated with androgens.[98] Furthermore, hormonal treatment with androgens plus estrogens induced hyperplasia in the canine prostate, followed by extensive multifocal mononuclear inflammation. This inflammatory response was more pronounced than that in dogs treated with androgens alone. For this, it may be relevant that estrogen plays a role in stimulating prolactin (PRL) synthesis and secretion, and that PRL plays a significant role in the regulation of the humoral and cellular immune responses as well as in autoimmune reactions.[24,52,186]

Canine Non-Tumorous Prostatic disorders

Prostatitis

Both acute and chronic infections occur in the canine prostate gland, usually as a result of ascending normal aerobic urethral bacteria (including mycoplasma) into a benign hypertrophic prostate. Escherichia coli is the most common bacterial organism identified in dogs with bacterial prostatitis.[81]

Hyperplasia

Benign prostatic hyperplasia (BPH) is found in aging intact male dogs. It may occur as benign glandular hyperplasia or as benign complex hyperplasia (or cystic hyperplasia). Glandular hyperplasia may begin at an age as early as 2.5 years in some dogs; by 6 years of age it is estimated that 60% of intact males have BPH and 95% by 9 years of age.[14,17] The etiology of BPH is still unclear. Administration of androgens in combination with estrogens to castrated dogs induced prostatic hyperplasia, with an increased number of transiently proliferating (TP)/amplifying cells and a hyperplastic luminal epithelium.[99] This relative increase in TP/amplifying cells in hormonally induced BPH in the dog is thought to be in line with a stem-cell-derived proliferation. Mohapokai reasoned that the androgen independent basal cells and TP/amplifying cells are much more abundant in the prostate of older dogs than in younger dogs and this may contribute to the enhanced risk of development of BPH with increasing age .[99]

BPH in the dog is quite uniform and diffuse. It involves the glandular epithelial cells in the entire gland, while the stromal involvement is less outspoken. This is in contrast to humans where BPH is nodular and complex, with involvement of both the stroma and the epithelium. Several authors reported that an absolute increase in the stromal elements of the prostate, rather than glandular hyperplasia, was the most important morphological finding in human BPH.[9,34,136,139] This difference has been explained by a much higher expression of 5-α reductase (converting testosterone to dihydrotestosterone) in the prostatic epithelium of the dog than in the prostatic epithelium of men, when compared to the expression in stroma. This difference in the localization of 5-α reductase compared to humans, may explain why the hyperplasia is mainly epithelial in dogs and stromal in humans.[171] However, there is another hypothesis suggesting that estrogen induces the stromal growth in human BPH.[30,64] The difference in estrogen levels and/or its effects on the prostate between humans and dogs may also provide an explanation for the difference in the histomorphology of the prostate between the two species.

Although BPH may be found in dogs with prostatic carcinoma, there does not appear to be a relationship between the two pathologic entities, nor with the pre-malignant HG-PIN lesions.[96]

CANINE PROSTATE CANCER

Pathology

Similar epithelial lesions as the precursor lesions of human prostate cancer, the high-grade PINs, have also been found in the majority (55%) of elderly sexually intact male dogs without clinical evidence of prostate cancer.[178] However, in a later study using military intact dogs, only 5% of the dogs were demonstrated to contain HG-PIN lesions.[4] The existence of PIN lesions in the canine prostate is still controversial and more thorough studies are needed. PIN lesions have been found to be precursor lesions of human prostate cancer and, given the (potentially) different pathogenesis of canine prostate cancer, they may not necessarily be precursors of canine prostate cancer (van Leenders, personal communication). This is probably also illustrated by the study of Waters et al in which castrated dogs only had HG-PINs in 8% of the cases[178]. Canine prostate cancer, however, is seen more often in castrated dogs. Thus, if PIN lesions do exist in the canine prostate, it seems at present not very likely that they play any role in the pathogenesis of cPC.

Canine prostate cancer is heterogeneous in morphology.[13,33,57] The World Health Organization (WHO) has classified cPC into adenocarcinoma and poorly differentiated carcinoma. The group of adenocarcinoma was further subdivided into an intra-alveolar and an acinar form. Bell et al. classified cPC in five groups, namely, intra-alveolar, small acinar, syncitial, discrete epithelial and poorly differentiated.[13] In their study, the small acinar type was the most frequent type in intact dogs, but in castrated dogs the only subtypes seen were intra-alveolar and poorly differentiated.[13] In the classification of Cornell et al. tumours were classified as either adeno-carcinoma, urethelial carcinoma, squamous cell carcinoma or mixed morphology (including two or more types of differentiation: glandular, urothelial, squamoid, or sarcomatoid). In their study adenocarcinoma was the most common subtype (36%), although more than half of the cPC exhibited intratumoural heterogeneity. In many cases, primary tumours showed a mixed morphology, characterized by two or more growth patterns.[33]

There is still debate about whether canine prostate cancer is incorrectly classified as primary prostate adenocarcinoma since a substantial part of the prostate cancers express markers of urothelial origin[144] and some authors argue canine prostate cancer to be in fact urothelial carcinomas arising in the prostatic urethra or in the periurethral ducts.[118] This debate reveals the complexity of the aetiology and classification of canine prostate cancer.[118] The origin of canine prostate cancer could possibly be elucidated by studies on the marker profiles (cytokeratins and others) that are expressed by the different tumour cells.

Development

Prostate cancer is one of the diseases that occur in aging dogs (mean age 10 years). Strikingly, when physiologic ages are compared prostate cancer occurs at the same age in both dog and human.[179] The prevalence of prostate cancer in the dog has been estimated to be between 0.29 and 0.6% in necropsy studies.[181] Although the prevalence is low, canine prostate cancer represents an insidious, highly malignant disease.[13]

Hormonal regulation

Canine prostate cancer was found in sexually intact and neutered dogs, and reports indicated a relatively higher risk of prostate cancer in neutered dogs.[13,144,158] The role of androgens and their receptor in canine prostate cancer is not yet clear. According to some authors the immunoreactive androgen receptor (AR) appeared to be abolished in most of the cPC and this might explain the resistance of cPC to androgen withdrawal therapy.[91] However, in a pilot study we could identify AR staining in most of the tumours. In several cases, a cytoplasmic staining pattern was seen, not only in castrated animals but also in intact dogs. This finding may indicate that hormone unresponsiveness is caused by a disturbed handling and/or localization of a possibly abnormal AR, for example due to mutations in the AR coding DNA. This would suggest a situation much more similar to human prostate cancer, in which the AR gene is quite often mutated and human hormone refractory prostate cancers often have an increased expression of AR.[92]

Inflammation

Canine prostate cancer is often infiltrated by inflammatory cells, as is benign prostatic hyperplasia. There is some evidence in humans that this inflammation contributes to the development and progression of prostate cancer[114] and that the inducible form of cyclooxygenase (COX-2) and its product prostaglandin E2 (PGE2) may play a role in this process.[75,80] Also in cPC COX-2 expression has been found, whereas it is not expressed in the normal prostate.[169] A possible correlation between COX-2 expression and histological classification of cPC or presence of inflammation has not yet been examined and little is known about the mechanisms regulating the expression of COX-2 in tumour cells.

Genetic risk

Several breeds, including Bouvier des Flandres, Doberman, and German Pointer dogs, have been found to have an increased risk for prostate cancer.[19,158] The canine androgen receptor sequence also contains polymorphic polyglutamine microsatellites.[137] It is unknown, however, whether this polymorphism of the canine AR gene can be related to the development of prostate cancer in dogs.

AIM OF THE STUDY

The aim of this thesis is to obtain basic information of spontaneous canine prostate cancers and to compare that to human prostate cancer, in order to evaluate the suitability of the dog as an animal model for prostate cancer studies. With the help of our results we will try to elucidate the cellular origin of canine prostate cancer. With the help of immunohistochemistry, we will examine the expression pattern and localization of several cytokeratins, the androgen receptor and specific prostate and urothelial markers in the normal prostate of the dog (chapter II). In chapter III, we will classify the different morphological types of canine prostate cancer and characterize cPC with various markers by immunohistochemistry. In chapter IV, we will examine the genomic sequence of exon 2 of the AR gene, that encodes the major part of the DNA binding region. This to see if any mutations in this region may interfere with the binding between DNA and the androgen receptor. In chapter V, we will investigate the association of a CAG repeat polymorphisms in exon 1 of the androgen receptor gene with prostate cancer risk. In chapter VI, the association of COX-2 expression with the presence of inflammation will be evaluated and in an in vitro study we will examine the regulation of COX-2 expression by several cytokines and growth factors.

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CHAPTER 2 *The Prostate, 68, 498-507, 2008*

Comparative characterization of the canine normal prostate in intact and castrated animals

Chen-Li Lai[1,2], René van den Ham[1], Geert van Leenders[3], Jaco van der Lugt[2], and Erik Teske[1]

[1]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.154, 3508 TD Utrecht, The Netherlands
[2]Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[3]Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands

Abstract

BACKGROUND. Prostate diseases in the dog are generally regarded as representative for their human counterparts. We characterized the normal canine prostate in comparison to the normal human prostate.

METHODS. Prostates of dogs were examined histomorphologically and by immunohistochemical detection of the markers CK14, HMWCK, CK5, CK18 , CK7, UPIII, PSA and PSMA.

RESULTS. Histomorphologically, the canine prostate lacks the human zonal differentiation, has much more prominent acini, while comprising less stromal tissue. In general, the canine prostate epithelium displayed a highly differentiated character, with no cells expressing CK14, minimal amounts of cells expressing HMWCK/CK5 and the vast majority of cells expressing CK18 and PSA. After castration, the prostate epithelium regressed, and the remaining tubules were largely populated by cells showing a ductal phenotype (HMWCK+/CK5+/CK18+/CK7+).

CONCLUSIONS. The human and canine prostate are histologically differently organized. The general scheme of cellular differentiation of the prostate epithelium may however be applicable to both species.

KEYWORDS: morphology; cytokeratins; uroplakin; PSA; PSMA; animal model

Introduction

The dog is widely regarded as a spontaneous model for prostate diseases associated with aging, such as benign prostatic hyperplasia (BPH) and prostate cancer (1,2). In fact, the dog is the only species other than man that naturally develops prostate cancer regularly (3-5). Therefore, the dog has been used extensively as a model to study the biological behavior of these diseases as well as to develop effective treatments (1-3,6-10). However, although the diseases in both species have several clinical aspects in common, not much is known about their comparative histology or the expression of markers like cytokeratins (CKs), Prostate Specific Antigen (PSA), Prostate Specific Membrane Antigen (PSMA) and Uroplakin III (UPIII) in the canine prostate. In order to make better use of the dog as a model for human prostate diseases, the similarities and differences between the normal prostates of the two species should first be investigated.

CKs are widely used to characterize and indicate several populations of cells within the human prostate. While basal cells are characterized by the expression of CK5 and CK14, the luminal epithelium highly expresses CK8 and CK18 (11,12). Based on the expression pattern of these CKs, at least two intermediate cell populations have been identified. The first population is localized in the basal compartment and identified by the expression of CK5 in the absence of CK14. The second population is part of the luminal cell layer and expresses CK5 as well as CK8 and CK18 (12). Besides the expression of CKs, luminal cells also show the presence of PSA and PSMA (13,14), while UPIII and CK7 have been used to differentiate between cancer of urothelial origin and prostatic origin (15,16).

In the dog, expression of CKs and PSA have mainly been analyzed with respect to prostatic disease (1,8,17-19). As in man, Leav et al. (1) and Mahapokai et al. (8) used high molecular weight cytokeratin (HMWCK: CK1, CK5, CK10, CK14) stainings to identify the basal cells, and CK5 and CK18 to specify intermediate and secretory cells respectively. Similarly, Landry et al. (20) used, among others, HMWCK expression to discriminate between the basal and secretory epithelial cells in hyperplastic dog prostates, whereas Grieco et al. (19) reported the expression of various cytokeratins in the neoplastic canine prostate. LeRoy et al. (17) used CK7 expression to differentiate between an urothelial or prostatic origin of canine prostate carcinomas. Finally, Sorenmo et al. (21) used the expression of PSA to determine the prostatic origin of cells in canine prostate carcinoma, while Anidjar et al. (22) used PSMA to characterize the canine prostate cancer cell line DPC-1.

As indicated, the aforementioned studies discuss mainly the pathological conditions of the canine prostate and use normal dog prostate tissue only as a reference. This limits the use of these markers in the characterization of the canine prostatic pathologies as suitable models for their human equivalents, as it is not obvious whether the normal canine prostate is similar to the

normal human one. Therefore, in this study we specifically examine the morphology as well as the expression of PSA, PSMA, UPIII, and the cytokeratins CK5, CK7, CK14, and CK18 in normal prostate epithelia in sexually intact as well as castrated dogs, and emphasize the similarities and differences in comparison to the morphology and expression of these markers in the human prostate.

Materials and Methods

Tissues

Prostate tissue was collected from 8 intact (ages from 22 months to 9 years) and 3 castrated adult dogs (ages from 26 months to 12 years and 10 months), which were sacrificed for reasons not related to prostate disorders. Tissues were fixed in 10% formalin and processed through paraffin. Four μm sections were cut and stained with haematoxylin and eosin for histological examination. From each tissue block at least 10 consecutive sections of 3 μm were cut for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using an indirect avidin-biotin-peroxidase staining procedure. The antibodies used are described in Table I. All incubations were performed at room temperature unless specifically indicated. Following deparaffinization and rehydration of the sections, several antigen retrieval methods were used. For the antibody 34βE12 recognizing HMWCK, sections were incubated with 0.1% pronase (w/v in distilled water) (Roche Diagnostics, Almere, The Netherlands; catalog # 11459643001) at 37°C for 10 min. For the antibodies recognizing PSMA, CK14, CK5 and CK18, antigen retrieval was achieved by submerging the sections in pre-heated 0.1M sodium citrate (pH 6) and subsequent further heating in a microwave oven (700W, near boiling) for 10 min and cooling for 20 min. For the antibodies UPIII and CK7, sections were incubated with ready-to-use proteinase K (DAKO Corporation, Carpinteria, USA; catalog # S3020) at room temperature for 10 and 15 min, respectively. For the polyclonal antibody recognizing PSA, no antigen retrieval was necessary. Endogenous peroxidase was neutralized by submersion of the slides in 0.3% H_2O_2 in 40% methanol-PBS for 30 min. After a short rinse with PBS the sections were pre-incubated with 10% normal goat serum (for anti-PSA) or normal horse serum (all other antibodies) for 15 min. Sections were then incubated with the primary antibodies at 4°C overnight, using the antibody concentrations in PBS or PBS with 10% normal goat serum (PSA staining) as indicated (Table I). After washing the slides three times for 5 min in PBS/0.05% Tween, sections were incubated with biotinylated secondary antibody in PBS (for PSA: goat-anti-rabbit diluted

Table I. Monoclonal antibodies used to stain the canine prostates

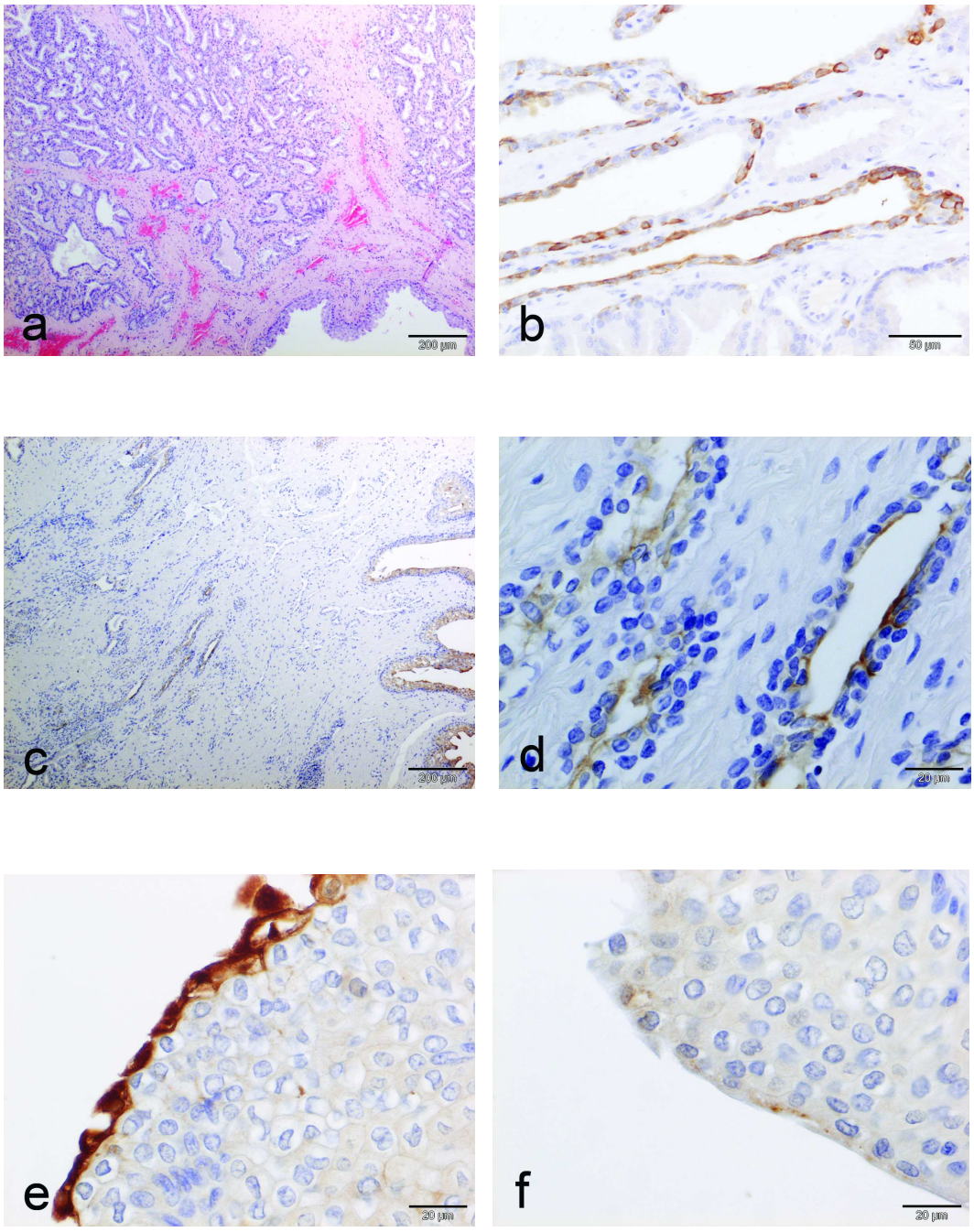
Antigens	Clone #	Dilution	Manufacturer
Cytokeratins 1,5,10,14	34βE12	1:50	DAKO, Carpinteria, USA
Cytokeratin 5	RCK103	1:5	Monosan, Uden, Netherlands
Cytokeratin 7	OV-TL12/30	1:40	BioGenex, San Ramon, USA
Cytokeratin 14	LL002	1:50	BioGenex, San Ramon, USA
Cytokeratin 18	DC-04	1:200	Abcam, Cambridge, UK
Uroplakin III	AU-1	1:10	Progen, Heidelberg, Germany
PSA	polyclonal	1:150	DAKO, Carpinteria, USA
PSMA	Y/PSMA1	1:40	Biodesign, Saco, USA

1:250, E0432, DAKO corporation; for all other antibodies: horse-anti-mouse diluted 1:125, BA-200, Vector Laboratories, Inc., Burlingame, USA) for 30 min. Slides were washed three times for 5 min in PBS/0.05% Tween and incubated with peroxidase coupled AB complex (ABC Kit, Vector Laboratories) for 30 min as indicated by the manufacturer, and washed three times for 5 min in PBS. Peroxidase activity was visualized by incubation with 3,3'diaminobenzidine (0.5mg/ml in 0.05M Tris (pH 7.6)/0.3% H_2O_2, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) for 10 min in the dark. Slides were then washed two times for 5 min in MilliQ, counterstained with haematoxylin, dehydrated, and mounted. As a negative control, primary antibodies were substituted with PBS. To evaluate the specificity of the antibodies, known positive tissues were used as controls. Canine skin was used as a positive control for the antibodies recognizing HMWCK, CK5 and CK14, while canine intestine was used as a positive control for CK18. For CK7 and UPIII, reactivity of normal urinary bladder epithelium was assessed, whereas human prostate tissues were used as positive controls for PSA and PSMA.

Results

Intact dogs

The canine prostate has a uniform morphology along the longitudinal axis and lacks a zonal differentiation based on glandular differentiation and epithelial cell morphology as in humans. Closely packed acini containing secretory epithelium constitute the major part of the canine prostate, from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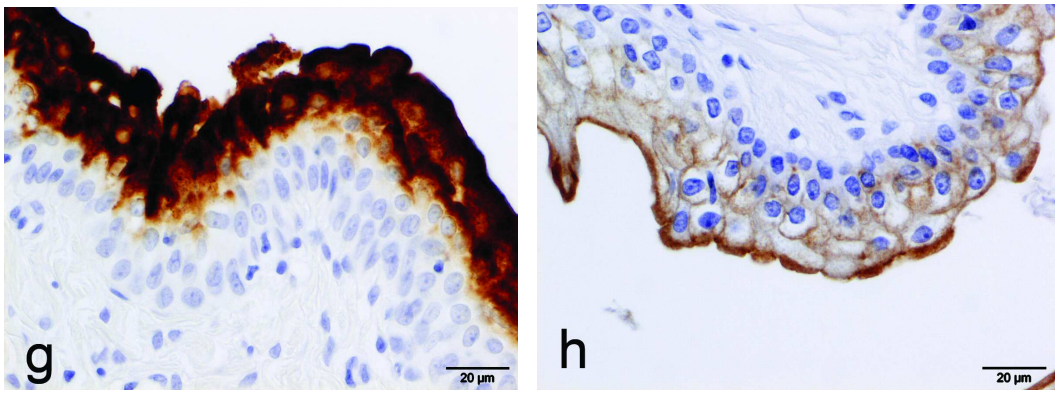


Figure 1

1A, Histology of a normal prostate of an intact dog (original objective: 4X, HE); 1B, CK5 stained the duct cells at the periphery of the gland (original objective: 20X); 1C, CK7 stained the cells of periurethral duct (original objective: 4X); 1D, high power view at the periurethral ducts of 1C (original objective: 40X, HE); 1E PSA stained the transitional cells of the prostatic urethra (original objective: 40X); 1F, PSMA stained scattered transitional cells of the prostatic urethra (original objective: 40X); 1G, UPIII stained the apical surface of the transitional cells (original objective: 40X); 1H, CK7 stained the transitional cells of the prostatic urethra (original objective: 40X).

the periphery to the peri-urethral area. Here, several end ductal structures are embedded within the stroma (Figure 1A). Although present, ductal structures can hardly be discerned in the periphery of the prostate when no specific staining is applied. The peri-urethral stroma extends dorsally and ventrally toward the outer boundary of the prostate and several less broad strings laterally, hereby forming several lobules of acinar epithelia. The urethra crosses the prostate just dorsal from the longitudinal central axis.

The secretory acini consist mainly of columnar epithelium with only few basally located stretched cells in the peripheral acini. The columnar cells of the prostatic acini gradually change to a single lining of cubic epithelial cells within the ductal structures. The cells of the urethra are variably sized, cubic or columnar cells, arranged as simple or stratified epithelia.

In the acini, strong PSA positive staining was observed in the luminal cells of all 8 animals. No expression of either PSMA or UPIII could be detected in acinar cells, whereas weak expression of CK7 in this area could be detected in the luminal cells of 2 animals. CK14 expression was not observed in the acini, whereas positive staining of HMWCK and CK5 was observed in scattered basal cells of a few acini in 2 and 5 prostates, respectively (Figure 1B). CK18 expression

Table II. Immunohistochemistry results for each marker in the prostate from 8 intact dogs.

Marker	Urethra		Peri-urethral	Peripheral	Acini	
	Basal	Luminal	duct	duct	Basal	Luminal
PSA	-	++(6/8)	++(6/8)	++(6/8)	-	++(8/8)
PSMA	-	+/-(4/8)	-	-	-	-
UPIII	-	++(8/8)	+(1/8)	-	-	-
CK7	-	+(6/8)	+(4/8)	+(1/8) -	+/-[3](2/8)	
CK14	+(1/8)	-	-	-	-	-
HMWCK	++(6/8)	+[1](3/8)	++(3/8)	-	+[2] (2/8)	-
CK5	++(8/8)	+[1](8/8)	++(6/8)	+(4/8)	+ (5/8)	-
CK18	-	+(7/8)	+(8/8)	+(7/8) -	++ (8/8)	

Intensity: +/-, weak staining; +, positive; ++, strong staining
[1] the staining intensity decrease gradually from the basal side toward the luminal side
[2]discontinuous basal layer
[3]50% of acinar secretory cells revealed mild positive in one prostate

was observed extensively in the luminal acinar cells of all 8 prostates. Although ductal structures are hard to detect at the periphery of the prostate when no or other immunostaining procedures were used, prostatic ducts in this area could in half of the prostates be identified when stained with the CK5 antibody.

In the periurethral area, PSA positive ductal cells were present in 6 prostates whereas no expression of PSMA staining could be detected in the prostatic ducts of any animal. Staining for UPIII was observed in scattered cells of the peri-uretheral ducts in 1 prostate, which was also one of the four cases positive for CK7 in the peri-urethral ducts (Figure 1C and 1D), and the only case positive for CK7 in the peripheral ducts. CK14 staining was never observed in the prostatic ducts. The antibody recognizing HMWCK stained the periurethral ductal cells of 3 prostates, while the CK5 immunostaining was seen in 6 out of 8 animals. CK18 staining was detected in the ductal cells of all 8 prostates.

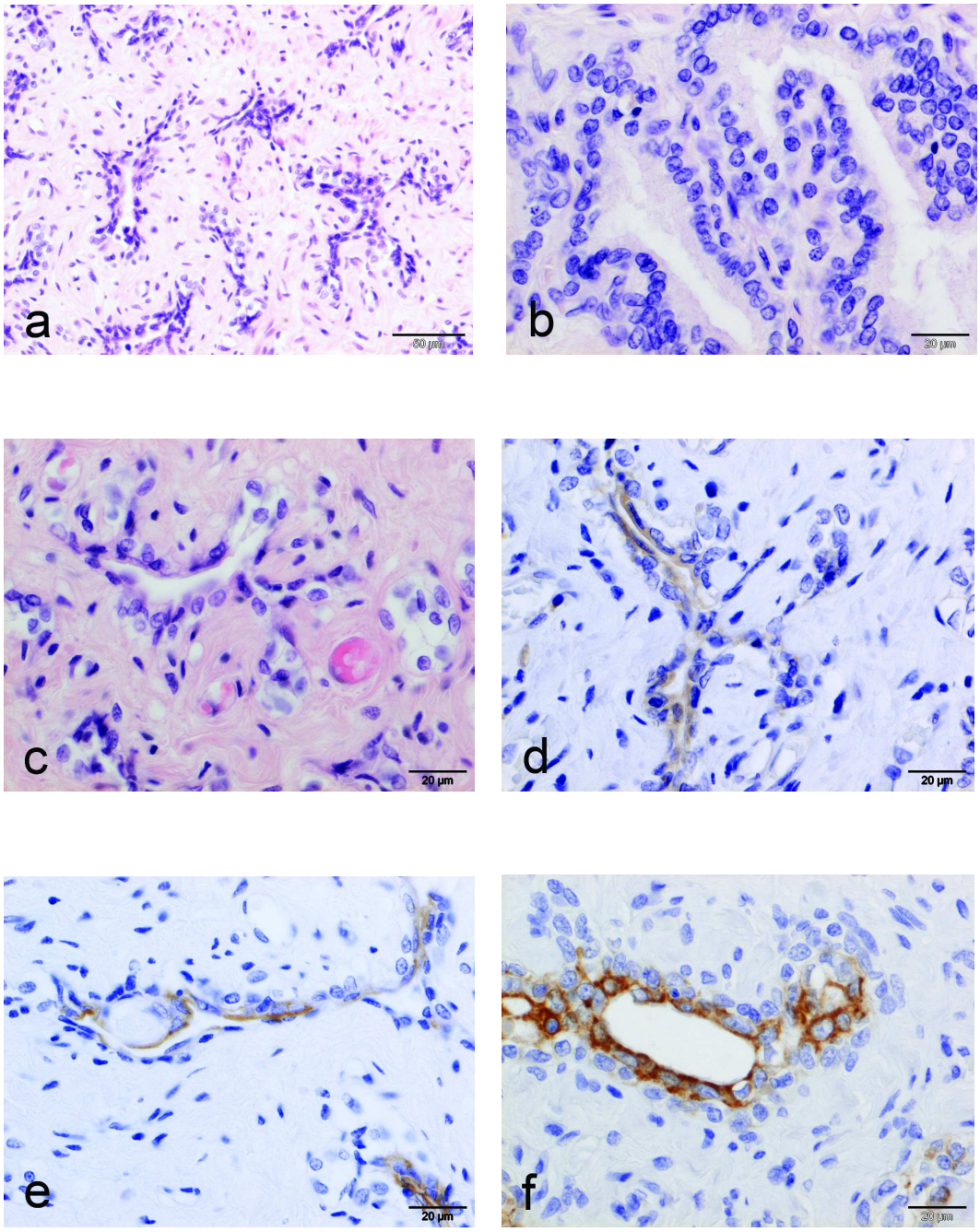
The prostate markers PSA and PSMA could be observed in the luminal cells of the urethra in 6 and 4 out of the 8 prostates of intact dogs respectively (Figure 1E and 1F). The urethral cells of all PSMA positive specimens were also positive for PSA. UPIII and CK7 staining was seen in the urethral luminal cells of 8 and 6 out of 8 prostates respectively (Figure 1G and 1H). Whereas CK7 staining was observed equally throughout these luminal cells, UPIII positivity was mainly restricted to the apical surface of the urethral luminal cells (Figure 1G and 1H). CK14 expression was scarce and could only be detected in the basal cells of the urethra of one prostate.

Positive staining for HMWCK and CK5 was present in the urethra of 6 and 8 animals, respectively.
Both markers intensively stained a continuous layer of basal cells. This intensity decreased towards the luminal cells of the urethra, and HMWCK was only expressed in the luminal cells of 3 of the 8 prostates. Expression of CK18 was generally seen in the luminal urethral cells of 7 prostates. The expression of the described markers in the normal prostate of intact dogs is summarized in Table II.

Castrated dogs
After castration, the prostate gland shows atrophy characterized primarily by atrophy of the acini. In advanced atrophy only tubular structures with a single lining of epithelial cells remain in the prostate. Distinction between ductal structures and atrophic acini by light microscopy and morphological criteria of HE stained sections alone is then hardly possible (Figure 2A). We will therefore further refer to these structures as 'tubules'. The three prostates of castrated animals used in this study show an increasing degree of atrophy; whereas in the least atrophic prostate several acinar structures with multiple layers of cells could still be detected (Figure 2B), only tubules with a single lining of epithelial cells remained in the 2 most atrophic prostates (Figure 2C). However, the lobular structure of the prostates could still be recognized by the interstitial stroma. Unfortunately, one of the prostate samples from the castrated animals did not contain a urethra.

Staining for PSA was observed in the tubules of all three prostates, i.e. in the single lining of tubular cells in the two most atrophic prostates and in the luminal lining of tubular cells in the least atrophic prostate. PSMA staining was present in the tubular cells of the 2 most atrophic prostates (Figure 2D). UPIII staining was only found in the single lining of tubular cells in the peri-urethral area of two prostates. In the least atrophic prostate, CK7 positive staining was only observed in the tubules at the peri-urethral area, whereas in the two most atrophic prostates, CK7 staining was present in the majority of the tubular structures across the whole section (Figure 2E). CK14 staining was found only in the scattered cells of the tubules at the periphery of the least atrophic prostate. HMWCK staining was seen in the tubules in the peri-urethral area in the least atrophic prostate, as well as in an estimated 20% and all tubules across the whole section of the two advanced atrophic prostates. CK5 positive staining was found scarcely in the basal lining of tubular cells in the least atrophic prostate and abundantly in the single lining of tubular cells in the other two prostates (Figure 2F). In addition, the absolute number of HMWCK and CK5 positive cells per cross section in the atrophied prostates appeared to be increased compared to their number in the intact dogs (Figure 2G and 2H). CK18 was expressed in the single or luminal lining of tubular cells in all three prostates of the castrated dogs.

No morphological differences could be detected in the urethra of the castrated dogs when compared to those of the intact animals. Likewise, immunostainings of the urethra were generally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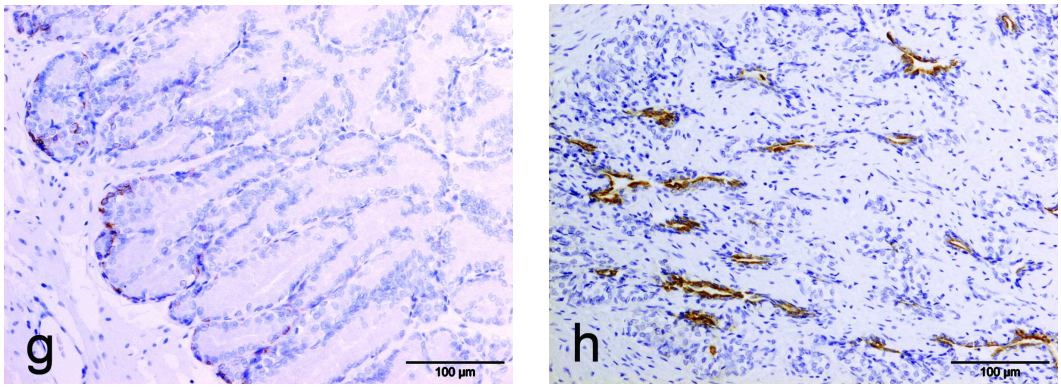

Figure 2
2A, histology of the prostate of a castrated dog (original objective: 10X, HE); 2B, the least atrophic prostate kept multiple layers of epithelia (original objective: 40X, HE); 2C, the most atrophic prostate showed only single lining of the tubular structure (original objective: 40X, HE); 2D PSMA stained the single lining of the tubule (original objective: 40X); 2E CK7 stained the single lining of the tubule (original objective: 40X); 2F, CK5 stained the single lining of the tubule (original objective: 40X); 2G, CK5 stained scattered basal cells of the acini at the periphery of the gland from an intact dog (original objective: 10X); 2H, increasing number of the CK5 positive stained cells in the prostate from a castrated dog (original objective: 10X).

similar to those observed in the intact animals. Staining of PSA, PSMA, UPIII and CK7 appeared in the luminal cells of the urethra of the two dogs. CK14 expression could not be detected, whereas HMWCK was present in the basal layer of the urethra in one prostate and CK5 in the basal and luminal cells of the urethra in two prostates. CK18 expression was noticed in the luminal cells of both urethras.

Discussion

Human and dogs share several similarities in their prostate disorders. The dog has therefore generally been regarded as a suitable animal model for the study of human prostate diseases. However, to better understand the significance of the dog as a spontaneous animal model for prostate disease, detailed comparative characterization of the normal canine prostate in terms of histomorphology and immunohistochemistry is a prerequisite. Since androgen depletion, or castration, affects the initiation of prostate disorders of both men and dogs (23,24), additional characterization of the canine prostate after castration may further improve our understanding of the initiation of prostate diseases.

The human prostate is characterized by the presence of different zones (the peripheral zone, the transition zone and the central zone), each of which has a characteristic normal histology and predisposition to developing a certain disease (25,26). Glands of the peripheral zone have a simple, rounded shape, with gentle ripples of the luminal borders. Of the carcinomas, 70-75% arise in this zone. Glands of the transition zone are similar to that of peripheral zone, and about 15-20% of prostate carcinomas arise in this area, while it is the main source of benign prostatic hyperplasia. Glands of the central zone are larger and are often arranged in lobules with luminal ridges and papillary foldings. The epithelium of the central zone usually has a granular cytoplasm. Only about 10% of carcinomas arise in the central zone (27).

The dog prostate lacks such zonal differences based on epithelial cell morphology, but rather displays a uniform morphology along the longitudinal axis. The canine prostate is organized with end ductal structures that surround the urethra from the peri-urethral area to the periphery of the gland, ending into secretory acini. The acinic structures in the dog are much more prominent in comparison to the human prostate, while there is less stromal tissue. As in humans, most dogs develop a certain degree of BPH with increasing age (28). In contrast to man however, where BPH develops in a nodular fashion in the transition zone of the prostate, the condition in dogs affects the gland diffusely (29). In addition, no preferential site of initiation of prostate cancer in the canine prostate has been mentioned in the literature.

The human prostate epithelium is morphologically composed of two cell layers. The basal layer consists of flat to tri-angular cells that are regarded as progenitors of the luminal epithelium. Luminal cells are cuboidal to columnar and secrete proteins such as PSA into the glandular lumina (30). Basal cells are characterized by the expression of CK5 and 14, while luminal epithelium highly expresses CK8 and 18 (11,12). Based on the expression pattern of these CKs, at least two intermediate cell populations have been identified. The first population is localized in the basal compartment and identified by the expression of CK5 in the absence of CK14. The second population is part of the luminal cell layer and expresses CK5 as well as CK8 and CK18 (12). It is postulated that these gradual shifts in cytokeratin expression reflect physiological differentiation from basal cells (CK5/14) to terminally differentiated luminal cells (CK8/18) (9).

In the vast majority of canine prostates, we did not find any CK14 expression by the basal cells of either the ductal or the acinic structures of the canine prostate. Only one prostate, the least atrophic prostate from the group of castrated dogs, had scattered expression of CK14. Thus, although CK14 expressing cells do exist in the canine prostate, they are generally very scarce. This corresponds to the results of LeRoy et al. (17), but is in contrast to what is seen in the human prostate, where CK14 expressing cells are more prominent (11). HMWCK and CK5 are, compared to CK14, somewhat more abundantly expressed in the canine prostate. Typically, we found expression of CK5 in scattered cells at the periphery of the acini. Leav et al. (1) investigated the role of the basal cells in the developing and the sexually mature dog. They found cell aggregations at the tips of canalizing ducts, radiating from the prostatic urethra in the developing prostate, that were

destined to form the peripheral acini. In the mature prostate, they also reported the scattered basal cells in the acini at the periphery of the mature prostate that expressed HMWCK and the proliferation marker, KI-67. From this, they suggested that the HMWCK-stained basal cells constituted the major proliferative component of the canine prostate epithelium throughout life. Although the number of HMWCK positive cells in the human prostate is generally higher compared to that in the canine prostate, the proliferative characteristic of the intermediate/amplifying/HMWCK (8,9) positive cells seems similar. In general, the normal canine prostate has a more differentiated character compared to the human prostate, with less of the 'immature' epithelial cells that finally differentiate to the CK8/CK18 expressing cells. The general scheme of prostate epithelial cell differentiation proposed by Isaacs and Coffey (31), however, seems also applicable to the canine prostate epithelium. The differentiated character of the epithelium, the dominance of acini and a relative lack of stromal tissue in the canine prostate compared to the human prostate account to the vast changes in the general morphology of the canine prostate seen after castration.

Several markers have been applied to differentiate the cellular origin of various tumors appearing in the prostate, such as PSA and PSMA to indicate prostate carcinomas (14,16), and UPIII or CK7 to identify transitional cell carcinomas (15, 16). Although PSMA is known as a prostate cancer marker in humans, its usefulness in canine specimens has never been examined. Immunoreactivity for human PSA has been reported in several studies in both normal prostate and prostate cancer in the dog (32,33), but so far no genes have been detected in the dog that share a high homology with human PSA (34). However, a gene encoding the prostatic arginine esterase has been identified as an ortholog to the progenitor of the PSA and hK2 genes, carrying the same conserved androgen responsive elements directing prostate transcription as these genes (35). Positive immunostaining with human PSA antibodies may be attributed to conserved epitopes in both proteins. With regard to PSMA, immunoreactivity to PSMA has been reported before for a canine prostate carcinoma cell line (DPC-1) (36) and MDCK cells (37), although the gene for PSMA in the dog has not yet been published. In a recent study it was stated that dogs do not express PSMA at all (38). However, in contrast to human PSA, a canine ortholog for human PSMA is clearly present in the canine genome. In a recent study using RT-PCR, we found clear expression of PSMA transcripts in the canine prostate, that was enhanced in carcinomas by a factor 5 (39). In addition, PSMA expression has also been shown by quantitative RT-PCR and western blot in several canine prostate cell lines (40).

As in humans (14,41), prostatic cells are positive to PSA, but negative to PSMA. In our experiments, however, both markers are moderately expressed in the urethral cells of the dog, disqualifying these two markers for the identification of a prostatic origin of tumors within the canine prostate. Although originally believed to be restricted to prostate, recent studies in humans have also demonstrated moderate PSMA expression in normal urothelium and endothelial cells of tumor-associated neovasculature (42) The expression of the urothelial markers UPIII and CK7 was generally restricted to the urethra and the ducts in the periurethral area. Thus, UPIII and CK7 are,

alone or in combination, good candidates to indicate a urethral or a ductal origin of canine prostate pathologies.

Castration status affects both the histomorphology and the expression of the investigated markers of the canine prostate. Prostate epithelia regress and the shape of acini changes to tubular formations with a single lining of epithelial cells, which makes it hardly possible to identify these tubules either to be regressed acini or pre-existing ducts. Androgen depletion causes mainly the depletion of CK18 expressing secretory cells from the acini (43). This is also seen in our castrated animals. Apart of this, there was an increase in the number of HMWCK+ and CK5+ cells in the atrophic areas of the prostates of the castrated dogs when compared to their number in the intact dogs. This finding may either indicate a simple accumulation of intermediate/amplifying cells that are not capable of differentiating further to secretory cells, or an active regeneration of the epithelium, trying to restore its original number of secretory cells. Similar mechanisms of epithelial regeneration have been described for the dermis and the testis (44,45). However, additional experiments will have to be performed to indicate such mechanisms in the canine atrophic prostate.

In addition to the increased number of HMWCK/CK5 expressing cells, the remaining tubules in the prostate of the castrated animals also showed the expression of CK7 across the whole prostate and UPIII in the tubules in the peri-urethral area. This indicates that these remaining atrophic tubules are not so much repopulated by cells from the pre-existing acini, but by cells with a ductal phenotype. The remaining tubules in the peri-urethral area are in turn populated by cells with a urethral phenotype. Whether these repopulation mechanisms involve active proliferation and/or migration of the ductal and urethral cells needs further investigation. The finding that the number of cells with a ductal phenotype appear to be increased in the prostate of castrated animals may connect the finding that castrated dogs are at greater risk of developing prostate cancer (23) and the postulation that canine prostate cancer originates from cells with a ductal phenotype (1).

Conclusions

In conclusion, the canine and human prostate differ to some extend in their histomorphology and the expression of several marker proteins. The canine prostate epithelium displays a much stronger differentiated phenotype compared to that in humans. The general scheme of prostate epithelial cell differentiation as proposed by Isaacs and Coffey (31) however, is most likely also applicable to the differentiation of the canine prostate epithelium. Finally, the finding that the remaining epithelial cells in the prostate of castrated animals display a ductal phenotype may have important implications to better understand the origin of canine prostate diseases.

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CHAPTER 3 *The Prostate, 68, 477-488, 2008*

Histopathological and immunohistochemical characterization of canine prostate cancer

Chen-Li Lai[1,2], René van den Ham[1], Geert van Leenders[3], Jaco van der Lugt[2], Jan A. Mol, and Erik Teske[1]

[1]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.154, 3508 TD Utrecht, The Netherlands
[2]Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[3]Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands

Abstract

BACKGROUND. In this study we try to identify the origin of canine prostate cancer (cPC) by classifying the tumors histological subtypes and relate these subtypes to their combined expressional characteristics of several tissue specific and differentiation markers.
METHODS. cPCs were examined histomorphologically and by immunohistochemical detection of the cytokeratin markers CK14, HMWCK, CK5, CK18, and CK7, and of the markers UPIII, PSA and PSMA.
RESULTS. Histopathologically, six growth patterns could be differentiated. The most frequent patterns were solid, cribriform and micro-papillary growth patterns, while sarcomatoid, small acinar/ductal, and tubulo-papillary growth patterns were less frequent present. Solid growth patterns were significantly (P=0.027) more often seen in castrated dogs. Immunohistochemically, about half of the cPC cases showed expression of PSA (8/20) and PSMA (10/20); 85% and 60% of the cPC expressed UPIII (17/20) and CK7 (12/20), while 13 and 12 cPC expressed CK5 and CK14, respectively; all cPC expressed CK18. CK14 was significantly more often and UPIII less frequent expressed in the solid growth patterns than in the micropapillary and cribriform patterns, respectively.
CONCLUSIONS. Canine prostate cancer appear to be more aggressive and of a less differentiated type than most common human prostate cancers. Comparing the expression patterns of the markers in cPC to those in normal canine prostate tissue, cPC most likely originates from the collecting ducts rather than from the peripheral acini. Given also the fact that canine prostate cancer is unresponsive to androgen withdrawal therapy, canine prostate cancer mostly resembles human, androgen refractory, poorly differentiated prostate cancer.

KEYWORDS: PSMA; growth pattern; animal model

Introduction

Apart from man the dog is the only species known to develop prostate cancer spontaneously. Interestingly, canine prostate cancer (cPC) shares several similarities with human prostate cancer (hPC). Both are most commonly found in the elderly patient (1), tumor growth outside the prostate is common and the distribution of distant metastases (bone and lung) is similar to that seen in humans (2,3). In this respect, cPC may serve as a precious model for human prostate cancer, filling a gap between the rodent model studies and human clinical research. Characterization of cPC in relation to its human counterpart is therefore important, both in terms of morphologic features and in the identification of the cell of origin.

Canine PC has been reported to show heterogeneity in its histopathology. According to the classification of tumors of domestic animals made by the World Health Organization, two major types of cPC can be discerned, adenocarcinoma and poorly differentiated carcinoma (4). Within the group of adenocarcinomas, intra-alveolar and acinar subtypes are recognized. However, several independent studies reported the occurrence of additional histopathologic types of cPC including glandular, urothelial, squamoid, sarcomatoid, and discrete epithelial types (2,3,5). A mixed morphology was often noticed, concurrently showing two or more different patterns (2,5). None of these authors however, reported the actual distributions of these patterns and any possible relationships between them. Nor were these patterns compared to those commonly observed in hPC.

Another matter of debate is which type of cell is the origin of prostate cancer in the dog. In hPC studies cytokeratins (CK) have been used to characterize prostate epithelial cells as they express different CKs at different stages of epithelial development and differentiation (6-9). Basal cells can be identified by the immunostaining with antibodies recognizing high molecular weight cytokeratins (HMWCK) including CK5 and 14, while luminal/secretory cells are stained by the antibodies to CK8 and 18. Both cell types have a low capacity of proliferation. A third group of cells, which co-express CK5 and 18, are regarded as intermediate cells. These intermediate cells have a short lifespan and a high proliferation rate (10-12).

In order to understand the pathogenesis of cPC it would be necessary to identify the cell of origin in this species. Mahapokai et al. investigated the three different developmental stages of the prostate epithelium in the dog (13). In addition, in this study HMWCK positive basal cells were recognized as the major proliferative cell type in the neonatal and adult canine prostate. The acinar basal cells are highly proliferative, and disappear after castration, while the majority of ductal basal cells are seldom Ki67 positive, but remain present after castration (14). As cPC is seen more often in castrated animals (15) these ductal HMWCK positive cells may be involved in the carcinogenesis of cPC (14). Further attempts have been made to identify the cellular origin of cPC with the help of PSA and CK7, however without definite proof (16,17).

In the present study we try to identify the cellular origin of cPC by classifying the tumors by their histological subtypes and relating these subtypes to the expression patterns of different cytokeratins (HMWCK, CK14, CK5, CK18, CK7) and Uroplakin III, PSA and prostate specific membrane antigen (PSMA).

Material and methods

Tissue specimens

Formalin-fixed paraffin-embedded specimens from 20 dogs with spontaneous prostate tumors were collected from the archives of the Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University. These comprised tumors collected at necropsy from 11 castrated and 9 non-castrated dogs. The basis for inclusion of cases in the study were: (1) a histological diagnosis of prostate carcinoma in biopsy material or in prostatectomy material, and (2) availability of formalin-fixed, paraffin-embedded tissue blocks. Four μm sections were cut and stained with haematoxylin and eosin for histological examination. From each tissue block at least 10 consecutive sections of 3 μm were cut for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using an indirect avidin-biotin-peroxidase staining procedure. The antibodies used are described in Table I. All incubations were performed at room temperature unless indicated otherwise. Following deparaffinization and rehydration of the sections, several antigen retrieval methods were used. For the antibody 34βE12, sections were incubated with 0.1% pronase (w/v in distilled water) (Roche Diagnostics, Almere, The Netherlands; catalog # 11459643001) at 37°C for 10 min. For the antibodies recognizing PSMA, CK14, CK5 and CK18, antigen retrieval was achieved by submerging the sections in pre-heated 0.1M sodium citrate (pH 6) and subsequent further heating in a microwave oven (700W, near boiling) for 10 min and cooling for 20 min. For the antibodies UPIII and CK7, sections were incubated with ready-to-use proteinase K (DAKO Corporation, Carpinteria, USA; catalog # S3020) at room temperature for 10 and 15 min, respectively. For the polyclonal antibody recognizing PSA, no antigen retrieval was necessary.

Then, endogenous peroxidase was neutralized by submersion of the slides in 0.3% H_2O_2 in 40% methanol-PBS for 30 min. After a short rinse with PBS the sections were pre-incubated with 10% normal goat serum (for anti-PSA) or normal horse serum (all other antibodies) for 15 min. Sections were incubated with the primary antibodies at 4°C overnight, using the antibody concentrations as indicated (Table I) in PBS or PBS with 10% normal goat serum (PSA staining). After washing the slides three times for 5 min in PBS/0.05% Tween, sections were incubated with biotinylated secondary antibody in PBS (for PSA: goat-anti-rabbit diluted 1:250, E0432, DAKO

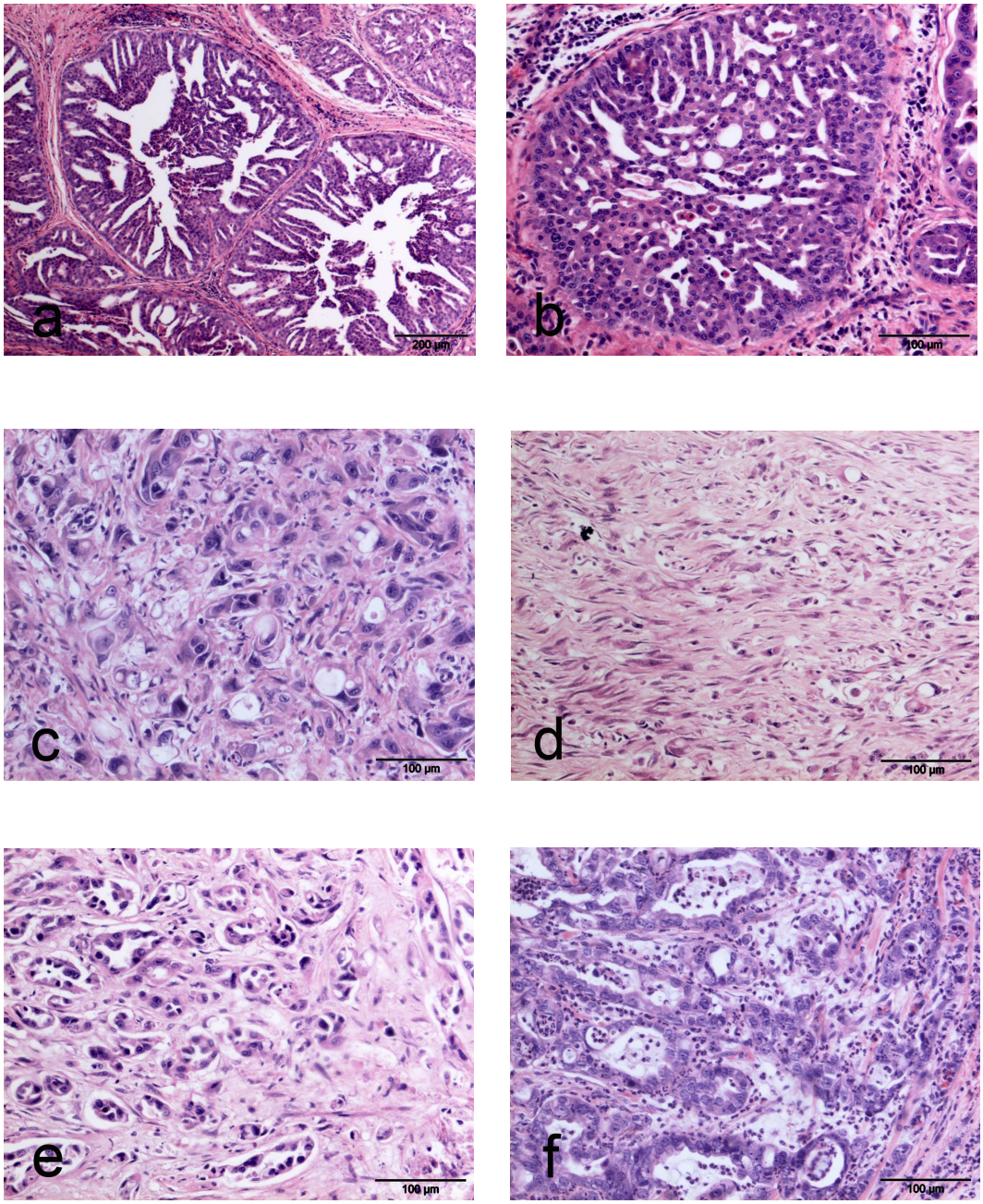
Table I. Monoclonal antibodies used in this study

Antigens	Clone	Dilution	Source
Cytokeratins 1,5,10,14	34βE12	1:50	DAKO, Carpinteria, USA
Cytokeratin 5	RCK103	1:5	Monosan, Uden, Netherlands
Cytokeratin 7	OV-TL12/30	1:40	BioGenex, San Ramon, USA
Cytokeratin 14	LL002	1:50	BioGenex, San Ramon, USA
Cytokeratin 18	DC-04	1:200	Abcam, Cambridge, UK
Uroplakin III	AU-1	1:10	Progen, Heidelberg, Germany
PSA	polyclonal	1:150	DAKO, Carpinteria, USA
PSMA	Y/PSMA1	1:40	Biodesign, Saco, USA

corporation; for all other antibodies: horse-anti-mouse diluted 1:125, BA-200, Vector Laboratories, Inc., Burlingame, USA) for 30 min. Slides were washed three times for 5 min in PBS/0.05% Tween and incubated with peroxidase coupled AB complex (ABC Kit, Vector Laboratories) for 30 min as indicated by the manufacturer, and washed three times for 5 min in PBS. Peroxidase activity was then visualized by incubation with 3,3'diaminobenzidine (0.5mg/ml in 0.05M Tris (pH 7.6)/0.3% H_2O_2, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) for 10 min in the dark. Slides were then washed two times for 5 min in MilliQ, counterstained with haematoxylin, dehydrated, and mounted. As a negative control, primary antibodies were substituted with PBS. To evaluate the specificity of the antibodies, known positive tissues were used as controls. Canine skin was used as a positive control for the antibodies recognizing HMWCK, CK5 and CK14, while canine intestine was used as a positive control for CK18. For CK7 and UPIII, reactivity of normal urinary bladder epithelium was assessed, whereas human prostate tissues were used as positive controls for PSA and PSMA.

PSMA expression

Isolation of total RNA and cDNA synthesis As the expression of PSMA in the canine prostate has been questioned recently additional expression studies were undertaken (18). Prostate tissue was collected from 10 control dogs, which had no disease related to the prostate, and from 11 dogs with prostate cancer. Tissues were snap frozen in liquid nitrogen and stored at –70 °C until further use. Total RNA was isolated using the RNeasy kit (Qiagen, Leusden, The Netherlands) according to the manufacturers protocol. cDNA was synthesized from 0.5 µg of total RNA using the iSscript™ cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) according to the manufacturer's protocol.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c
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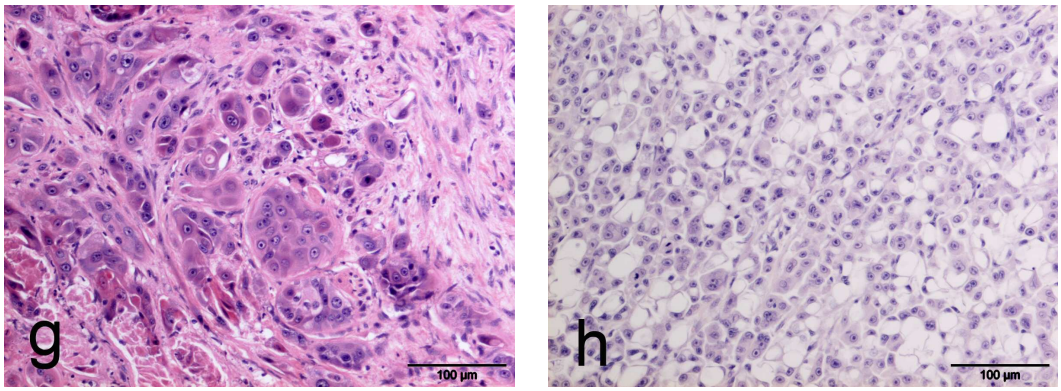


Figure 1
Different histologic growth patterns found in canine prostate carcinoma. 1A, Micropapillary pattern, in which papillary projections are formed in duct-like structures (original objective: 4x, HE); 1B, Cribriform pattern, the tumor cells form regular fenestrae (original objective: 4x, HE); 1C, Solid pattern, pleomorphic tumor cells situated as solid nests or individual cells within the stroma (original objective: 10x, HE); 1D, Sarcomatoid pattern, tumor cells with a spindle shape like morphology (original objective: 10x, HE); 1E, Small acinar/ductal pattern, several micro-acini are arranged in fibromuscular stroma (original objective: 10x, HE); 1F, Tubulo-papillary pattern, some dilated ducts with single layer columnar cells, irregularly formed are present. In addition, an inflammatory reaction is present (original objective: 10x, HE); 1G, Squamous differentiation of tumor cells (original objective: 10x, HE); 1H, Signet cells with cytoplasmic vacuoles, suggesting urothelial differentiation (original objective: 10x, HE)

Quantitative measurement of the PSMA mRNA levels Dog-specific primers for PSMA were designed using the sequence of the canine folate hydrolase 1 isoform-1 (sFOLH1/PSMA; GeneID: LOC476775), which is 89% homologous to human PSMA transcript. The sequences of the forward and reverse primers were 5'-GTGTTTGGTGGCATTGACC and 5'-TTCTGCATCCCAGCTTGC, respectively.

QPCR reactions were performed in a total volume of 25 µl containing 12.5 µl 2x SYBR green super mix (BioRad), 1 µl of each primer at 400 nM, 1 µl cDNA and 9.5 µl RNase and DNase free water. The PCR was performed using a BioRad MyiQ detection system with SYBR green fluorophore according to a scheme of a primary denaturation (95°C, 2 min), followed by 40 cycles of amplification (95°C for 20 sec), annealing (58°C for 20 sec) and elongation (72°C for 20 sec). Specificity of the QPCR reactions was validated by performing a melting curve procedure. Canine HPRT and RPS5 were used as reference genes for normalization.

Statistics
Differences among groups were assessed by the chi-square or Fisher exact test. Differences in

relative expression of PSMA mRNA in tumor and normal tissue were assessed using a Pair-wise fixed reallocation randomization test using the REST-XL software (19). Differences were considered to be significant at $P<0.05$.

Results

Histopathology

Tumor growth throughout the whole prostate, extending from periurethral to (sub)capsular areas was seen in almost all cases. Capsular penetration was found in 8 of the 18 cases in which a complete prostate could be evaluated. In five of these cases tumor growth in tissues surrounding the prostate was found, including the tunica muscularis of the rectum. Vessel invasion by tumor cells was present in 8 cases.

We observed a remarkable variation in tumor growth patterns, both between and within individual cases of prostate cancer. In total, we were able to differentiate six different growth patterns:

(i) micropapillary, in which delicate papillary projections of neoplastic cells were formed within an extended duct (Fig 1A)

(ii) cribriform, showing a duct completely extended by tumor cells with the formation of regular fenestrae (Fig 1B), often accompanied with central necrosis (‘comedonecrosis’)

(iii) solid, a highly infiltrating, anaplastic undifferentiated carcinoma characterized by pleomorphic tumor cells arranged as solid nests or sometimes apparently individual cells within the stroma (Fig 1C)

(iv) sarcomatoid, in which the tumor cells have a spindle like morphology with an irregular or fascicular orientation (FIG 1D)

(v) small acinar/ductal, with variously sized micro-acini, arranged within a scirrhous looking fibromuscular stroma (Fig 1E)

(vi) tubulo-papillary, dilated ducts comprising irregular, single to stratified layers of tall, columnar cells (Fig 1F).

The distribution of the cPC patterns among the individual dogs is described in Table II. In short, the most common morphological patterns were solid, cribriform and micropapillary, which were observed in 13, 11 and 10 cases respectively. In addition, the mixed morphologies of cPC were mostly composed of these three patterns. In six prostates, solid, cribriform, and micropapillary patterns were concurrently present, another three cases showed mixed micropapillary and cribriform patterns and one case showed mixed micropapillary and solid patterns. Sarcomatoid, small acinar/ductal and tubular papillary patterns were seen less often, sometimes in combinations but often in combination with a solid growth pattern. In total, 15 out of

TABLE II. Details of Antigen Expression in Each Growth Pattern of Each Prostate Tumor

	Micropapillary	Cribriform	Solid	Sarcomatoid	Acinar/ductal	Tubular papillary
A (intact dogs)						
1	H, CK7, CK18, UPIII, PSMA	CK7, CK18, UPIII, PSMA	H, CK5, CK7, CK14, CK18, PSMA			
2					H, CK5, CK7, CK18	
3			CK7, CK14, CK18, PSMA	All negative	CK18, PSMA	
4	H, CK5, CK7, CK14, CK18, UPIII, PSMA	H, CK7, CK18, UPIII, PSMA				
5					CK5, CK7, CK14, CK18, PSA, PSMA	
6						CK7, CK18, (PSA*), (PSMA*)
7	H, CK5, CK7, CK18, UPIII	H, CK7, CK18, UPIII				
8	H, CK7, CK18, UPIII, PSA, PSMA	H, CK7, CK18, UPIII, PSA, PSMA	H, CK5, CK14, CK18			
9	CK5, CK7, CK18, UPIII, PSA, PSMA	CK7, CK18, UPIII, PSA, PSMA				
B (castrated dogs)						
1	CK5, CK7, CK14, CK18, UPIII, PSA	CK7, CK18, UPIII, PSA	H, CK5, CK7, CK14, CK18			
2	H, CK7, CK18, UPIII	CK7, CK18		All negative		
3	H, CK5, CK7, CK14, CK18, UPIII, PSA, PSMA	H, CK5, CK7, CK18, UPIII, PSA, PSMA	H, CK7, CK18, UPIII, PSA, PSMA			
4	CK5, CK7, CK18, UPIII, PSA		H, CK5, CK7, CK18, UPIII, PSMA			
5	CK5, CK7, CK14, CK18, UPIII	CK5, CK7, CK14, CK18, UPIII	CK5, CK7, CK18, UPIII			
6	CK7, CK18, UPIII	CK7, CK18, UPIII	H, CK5, CK7, CK14, CK18, PSA			
7			CK5, CK7, CK18, PSA, PSMA		CK7, CK18, PSA	CK14, CK18, PSA, PSMA
8			(CK14*), CK18			
9			(CK14*), CK18		CK18	
10			CK7, CK14, CK18, UPIII, PSMA		CK7, CK14, CK18, PSMA	
11			H, CK14, CK18			

H, HMWCK; (monoclonal*): missing value of that monoclonal.

Table III. Frequency of expression of the antigens in each tumor and each growth pattern

	whole tumor (n=20)	micro-papillar (n=11)	cribriform (n=10)	solid (n=13)	sarcomatoid (n=2)	acinar/ ductal (n=6)	tubular papillary (n=2)
HMWCK	11	6	4	7	0	1	0
CK5	13	7	2	7	0	2	0
CK14	12	4	1	7[2]	0	2	1
CK18	20	11	10	13	0	6	2
PSA	8	5	4	3	0	2	1[1]
PSMA	10	5	5	6	0	3	1[1]
CK7	17	11	10	9	0	4	1
UPIII	12	11	9	4	0	0	0

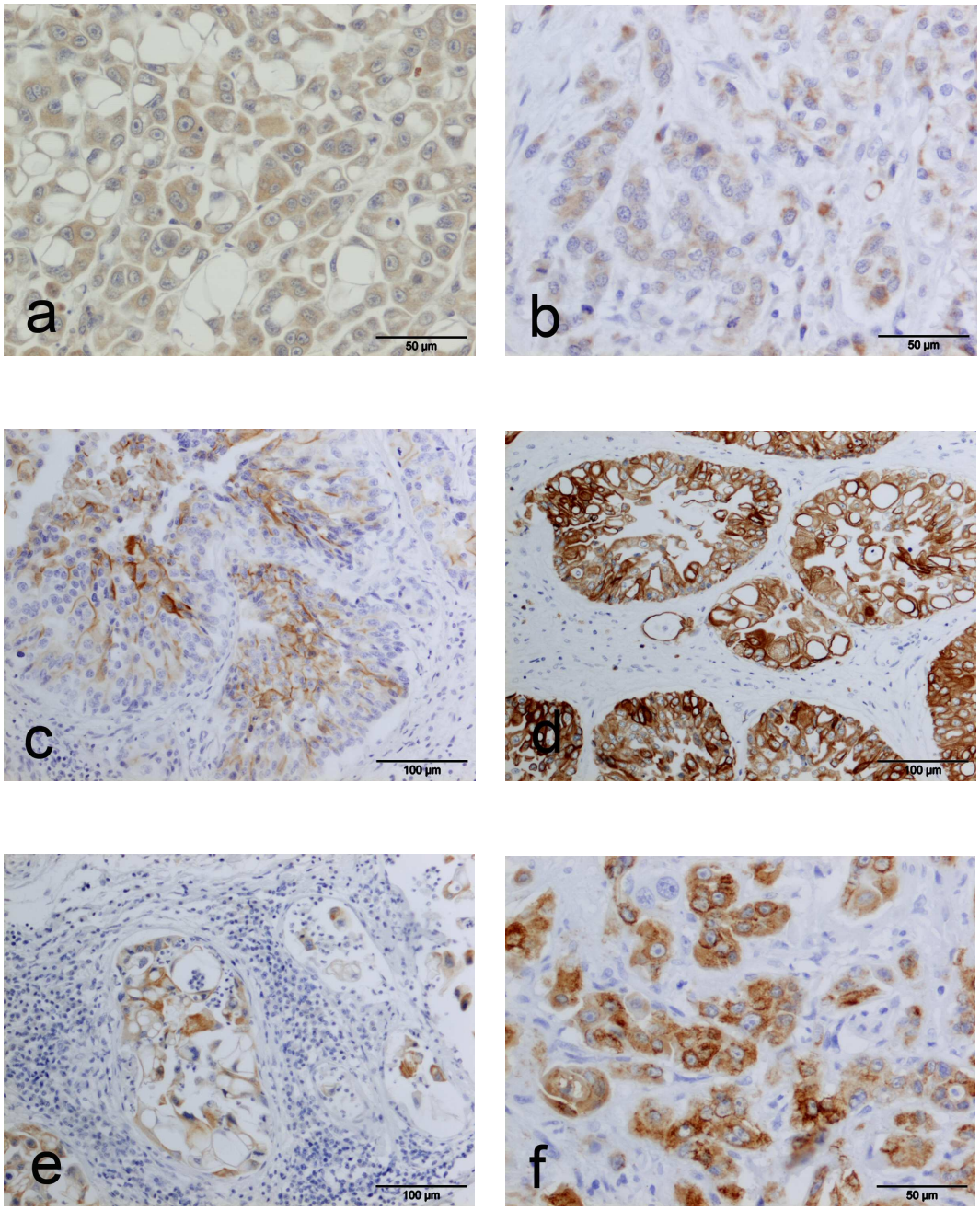
[1] one sample missing
[2] two samples missing

the 20 cases (75%) of cPC showed mixed histological growth patterns of the tumor within the same cross section. Solid growth patterns were significantly (P=0.027) more often observed in castrated dogs than in non-castrated dogs. No significant differences existed between the other histological types and castration status. Apart from histologic growth patterns there was also a heterogeneity of cell types within these growth patterns. Mixtures of squamoid cells (Fig 1G), mesenchymal cells and signet cells (Fig 1H) could be found in all growth patterns.

Immunohistochemistry

A detailed description of the immunostaining results is presented in Tables II and III. In general, about half of the cPC cases showed expression of PSA (Fig 2A) and PSMA (Fig 2B), whereas 12 and 17 of the 20 cases showed expression of UPIII (Fig 2C) and CK7 (Fig 2D), respectively. Thirteen and 12 cases of cPC expressed CK5 (Fig 2E) and CK14 (Fig 2F) respectively, whereas all 20 cases of cPC expressed CK18 (Fig 2G).

To better characterize cPC, we also analyzed the expression of the different markers per growth pattern. Starting with the most abundant growth pattern, three and six out of the 13 solid patterns expressed PSA and PSMA, respectively. All solid patterns expressed CK18, whereas seven out of the 13 solid patterns expressed CK14, CK5, or HMWCK (comprising of CK1, CK5, CK10, CK14) (Fig 2H). Nine and four of the 13 solid patterns expressed CK7 and UPIII respectively. A comparable expression pattern was seen in the micropapillary and cribriform patterns, with the exception that there was a significant higher (P=0.03) expression frequency of CK14 (7/11) in the solid pattern than in the cribriform pattern and a significant lower (P=0.007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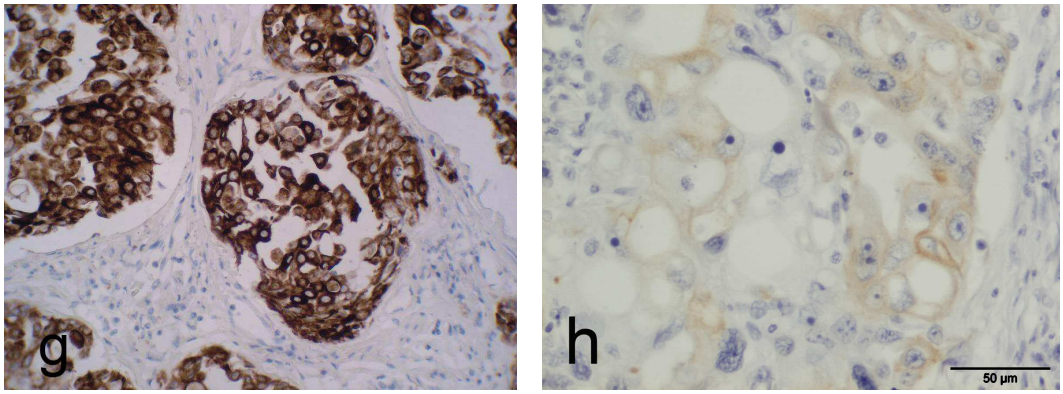

Figure 2
Immunohistochemistry in canine prostate carcinoma. 2A, Clear PSA positivity in solid pattern of carcinoma with several signet cells (original objective: 20x); 2B, PSMA expression in small acinar/ductal pattern (original objective: 20x); 2C, UPIII expression in micropapillary tumor pattern (original objective: 10x); 2D, Strong CK7 expression in micropapillary growth pattern (original objective: 10x); 2E, CK5 expressed by tumor cells of small acinar/ductal pattern (original objective: 10x); 2F, The majority of the solid pattern tumor cells has a strong CK14 expression (original objective: 20x); 2G, Micropapillary tumor cells expressing CK18 (original objective: 10x); 2H, Irregular faint staining with HMWCK by tumor cells of solid pattern (original objective: 20x).

and P=0.01) expression frequency of UPIII in the solid pattern than in the micropapillary and cribriform patterns, respectively. The other growth patterns were less abundantly present in our tissue sections, and no significant differences in expression of the markers could be detected. Remarkably, none of the sarcomatoid patterns expressed any of the investigated markers. The expression of the markers in the small acinar/ductal, and tubular papillary growth patterns grossly resembled those observed in the solid, micropapillary and cribriform patterns: PSA and PSMA were expressed in about half of the cases, CK18 expression was detected in all, and CK5, HMWCK and CK14 in decreasing numbers of the individual growth patterns. In addition, most small acinar/ductal, and tubular papillary growth patterns expressed CK7. None of the small acinar/ductal or tubular papillary growth patterns investigated expressed UPIII.

PSMA expression
As the presence of PSMA in canine prostate tissues has been questioned recently (18), the PSMA expression was further investigated using quantitative RT-PCR. Expression analysis in a panel of 11 frozen prostate carcinomas and 10 control prostate samples revealed the presence of PSMA mRNA in all tissues with a mean threshold cycle (C_t) value of 28.84 for control tissues and 26.75 for carcinomas. After normalization to the house-keeping genes HPRT and RPS5 the expression in carcinomas was 5.2 ± 2.8 times higher in comparison to control tissue.

Discussion

Canine cPC may serve as a precious spontaneous animal model for human prostate cancer. In order to understand the use and limitations of such a model however, it should first be thoroughly characterized and compared to human prostate cancer. In this study, we characterized 20 individual cPC cases in terms of histological growth patterns and their expression of several marker proteins. Canine prostate tumors are very aggressive: the cancer lesion usually occupies the whole prostate, the tumor cells are often not confined within the natural boundary of the basal membrane, and we observed cancer cells invading the prostate capsule and surrounding tissues in a significant number of cPC cases. Although there is currently no Gleason like grading system to score the aggressiveness of canine prostate cancer, the majority of clinically diagnosed cPC cases would probably be graded highly malignant.

Previously, other authors have attempted to classify prostate cancer (2,4,5,20) . In the WHO classification, a distinction between adenocarcinoma and poorly differentiated tumors is made. In this classification, the adenocarcinomas are further subclassified as intra-alveolar, which is the most common form, and acinar. Bell et al. (5) classified cPC in five groups, namely, intra-alveolar, small acinar, syncitial, discrete epithelial and poorly differentiated. In his study, the small acinar type was the most frequent type in intact dogs, but in castrated dogs the only subtypes seen were intra-alveolar and poorly differentiated. In the classification of Cornell et al. (2) tumors were classified as either adenocarcinoma, urethelial carcinoma, squamous-cell carcinoma or mixed morphology (including two or more types of differentiation: glandular, urothelial, squamoid, or sarcomatoid). In their study adenocarcinoma was the most common subtype (36%), although more than half of the cPC exhibited intratumoral heterogeneity. In many cases, primary tumors showed a mixed morphology, characterized by two or more growth patterns.

Our classification agrees with several of the findings of the above-mentioned authors in that we also observed alveolar and small acinar growth patterns. In our study however, the group of alveolar growth types is further refined and classified as a micropapillary, a cribriform and a tubular papillary growth pattern (21), based on differences in the histologic patterns and confirmed by differences in cytokeratin expression patterns. The previous studies in general simply categorized the rest of the morphological growth patterns as “poorly differentiated” or “undifferentiated”, which is insufficient to describe the distinct details and differences between these different growth patterns we observed. In addition, the classifications of the above mentioned authors all contain both descriptions of histological growth patterns and of cell morphologies. The presence of certain cell types, however, is not specific for the histological growth patterns. Mixtures of squamoid, mesenchymoid and signet cells were found within the solid growth patterns, but e.g. signet cells could sometimes also be observed in the other growth patterns. In short, our study (and that of Cornell et al. (2)) shows that canine prostate cancer (cPC) displays a high degree of morphologic heterogeneity, in terms of the number and combinations of growth patterns per

tumor as well as of cellular morphology. Finally, castration leads to an increased appearance of less differentiated growth patterns in canine prostate cancer. In our study, solid growth patterns were significantly (P=0.027) more often seen in castrated dogs than in non-castrated dogs, similar to Cornell et al. (2), who observed less adenocarcinomas in castrated dogs.

In humans, the majority (up to 95%) of prostate cancer is adenocarcinoma, mainly characterized by an acinar differentiation. Five to ten percent of the histologic variants of prostate adenocarcinoma have distinctive features, however (22). Among these variants, Randolph et al. (22) differentiated sarcomatoid carcinoma, adenosquamous carcinoma, ductal carcinoma, and transitional cell carcinoma. Next to the adenocarcinomas, poorly differentiated carcinomas were recognized, of which the tumor cells are not confined to the acini. Differences in pathomorphological appearance of androgen-sensitive and androgen-refractory prostate cancer have also been reported in human prostate cancer. On basis of the WHO classification, Kondo et al. (23) classified the glandular (including large and/or small simple glands), micro-glandular, and cribriform growth patterns as androgen sensitive, while fused glands, medullary/solid and columnar/trabecular patterns were classified as androgen refractory components. Moreover, they reported that metastases tend to have androgen refractory components. In addition, Shah et al.(24) studied the characteristics of metastatic, hormone-refractory prostate cancer in terms of morphology, immunophenotype, and genotype. They concluded that androgen-independent prostate cancer is a heterogeneous group of diseases. The majority of cases showed a mixture of Gleason grades 4 and 5, including growth in solid sheets and nests with or without comedonecrosis, and confluent cribriform glandular patterns. This mixture of growth patterns seen in human androgen refractory prostate cancer remarkably resembles the mixture of growth pattern we have seen in canine prostate cancer.

About half of the investigated tumors expressed a positive reaction to PSA, although expression was only weak to mild. This is substantially more than what is found by others. McEntee et al. (25) reported a positive PSA reaction in 2 out of 31 canine prostate tumors, whereas Sorenmo et al. (17) found only 1 out of 58 cPCs to be positive for PSA expression. In humans, PSA has been used routinely in serum screening and immunohistochemical diagnosis of prostate disorders. In general, patients with prostate cancer show a higher PSA concentration in the serum, while PSA immunostaining intensity decreases in tumor cells with higher Gleason's grade (26-28). Although several studies report immunoreactivity for human PSA in both the normal prostate and prostate cancer in the dog (17,25,29-31), so far no PSA could be found in the plasma of dogs with prostate cancer nor have PSA (hKLK3) orthologous genes been detected in the dog (31,32). However, a gene encoding the prostatic arginine esterase has been identified as a canine ortholog of the related hKLK2 gene, and it carries the same conserved Androgen Responsive Elements directing prostate transcription as these genes (33,34). Although the amino acid sequence homology between the canine arginine esterase and the human PSA is not very high (<60%), positive immunostaining with polyclonal human PSA antibodies may be attributed to conserved epitopes in both proteins (35). In a previous study we found that in normal canine prostate strong

positivity to human PSA is observed both in the acinar cells and in the ductal cells, even after castration, as well as a moderate expression in the urethral cells (36).

Besides PSA, PSMA is expressed at low levels in normal human prostatic epithelium but markedly increased in prostate cancer, and maintained in poorly differentiated tumors and prostate cancer metastases (37). PSMA has therefore been used to identify cells of prostatic origin (38). Although originally believed to be restricted to the prostate, recent studies have also demonstrated moderate PSMA expression in normal human urothelium and endothelial cells of tumor-associated neovasculature in several solid cancers (39).

In a comparative study of PSMA expression (18) it was stated that mice, monkeys and dogs do not express PSMA at all, making PSMA unique for humans, and these animals, including the dog, not suitable for investigating PSMA-activated intraprostatic prodrug therapies. However, in contrast to human PSA, a canine ortholog for human PSMA is clearly present in the canine genome. In our study, we found clear expression of PSMA transcripts in the canine prostate, that was enhanced in carcinomas by a factor 5. In addition, PSMA expression has also been shown by quantitative RT-PCR and western blot in several canine prostate cell lines (40).

The absence of a positive RT-PCR for PSMA in canine prostate tissue in the study of Aggarwal et al. (18) is most probably the result of the use of a reverse primer with a very low predicted melting temperature. They also only investigated normal canine prostate tissue in which the expression, as found by quantitative RT-PCR is some 5-fold lower than in carcinomas.. Immunohistochemically, PSMA could not be detected in the prostate of intact animals, and only weakly in the ductal and urothelial cells of castrated animals (36). Half of the investigated cPC expressed detectable levels of PSMA protein, although this could not be associated to a histomorphological subtype or to the castration status of the dogs.

Seventeen (85%) and twelve (60%) out of the 20 prostate tumors expressed UPIII or CK7, respectively. A high percentage (79.3%) of CK7 expression in cPC has also been reported by Sorenmo et al (17), but they didn't associate the expression of CK7 with the histopathological growth patterns in the tumors. In our study we found a significant lower (P=0.007 and P=0.01) expression frequency of UPIII in the solid patterns compared to the micropapillary and cribriform patterns, respectively. In addition, we also observed some reduction of the CK7 staining frequency in these solid patterns. This observation corresponds to previous studies concerning human urothelial carcinoma (41) and canine transitional cell carcinoma (42), in which it was mentioned that tumor cells of increasing invasiveness lose their expression of CK7 and UPIII. In human urogenital cancers, CK7 and UPIII have been used to identify the ductal and/or urothelial origin of tumor cells (43,44). Mhawech et al. (44), showed that 27.5% of the human prostate tumors (and 86.6% of the urothelial carcinomas) shows CK7 positivity, while none of them expresses UPIII. In normal prostates of dogs, we (36) found that UPIII staining is restricted to the urethra, while CK7 is also expressed by the periurethral ductal cells. In normal prostates of castrated animals however, the vast amount of acinar cells disappear and the basal membranes of the remaining tubules are occupied by cells expressing CK7 and, to a lesser extend UPIII. Notably, castration increases the

incidence of diagnosed prostate cancer (15). Thus, our current and previous results taken together point to a ductal origin of canine prostate cancer, and underline an earlier suggestion by Leav et al. (14).

All canine prostate tumors, and more precisely, all growth patterns of cPC express CK18. This appears to indicate a predominance of differentiated cell types in the canine prostate tumors and parallels with observations in human prostate carcinoma (45). CK5 and CK14 however, indicators for prostatic intermediate and basal cell types respectively, are also quite abundant in our canine tumors (65% and 60% of the tumors, respectively), and more often in the tumors of castrated animals. Since CK14 is also more abundantly expressed in solid growth patterns (compared to cribriform and micropapillary patterns) and solid growth patterns are seen more often in castrated animals compared to intact dogs, the conclusion may simply be that the less-differentiated growth patterns also contain more less-differentiated tumor cells. Markedly, in normal dog prostates, no CK14 expression was seen in intact dogs, while it could be observed in castrated animals (36). This is in line with the notion that androgens are the major driver of differentiation of the prostatic epithelium (46). Taken together, castration of dogs leads to a less differentiated prostatic epithelium (36), an increased risk of prostate cancer development (15), and an increase in less differentiated tumor types (this study), resulting in a higher content of stem cells and/or transiently amplifying cells. The induction of CK14 positive cells in the dog prostate may reflect a reduced drive towards differentiation of the epithelium.

Hormone refractory prostate cancer is the second leading cause of death due to cancer in western men. Most of these deaths are caused by hormone refractory prostate cancer for which an efficient treatment is urgently needed. A suitable animal model is important to develop an effective therapy. The aim of our study was to explore to what extend spontaneous prostate cancer of the dog provides such a model. In conclusion, we claim that our histomorphological classification is the most consistent and accurate presentation of the different subtypes of cPC to date, and that it provides an excellent basis for future cPC studies. In addition, canine prostate tumors appear to be more aggressive and of a less differentiated type than the most common, androgen withdrawal responsive, human prostate cancers. Canine prostate cancer is less differentiated in terms of histomorphology (solid, infiltrating growth patterns versus acinar type adenocarcinomas in humans) and cellular development (high percentage of CK14 and CK5 positive cells compared to hPC), and seems to originate from collecting ducts area rather than from peripheral acini. Given also the fact that canine prostate cancer is androgen withdrawal unresponsive (5), canine prostate cancer mostly resembles human, androgen refractory, poorly differentiated prostate cancer. Further studies may address genome wide expression changes involved in cPC in comparison to human (poorly differentiated) prostate cancer.

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CHAPTER 4 *The Veterinary Journal, accepted for publication*

Immunostaining of the androgen receptor and sequence analysis of its DNA-binding domain in canine prostate cancer

Chen-Li Lai[a,b], René van den Ham[a], Jan Mol[a], and Erik Teske[a,*]

[a]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.154, 3508 TD Utrecht, The Netherlands
[b]Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Abstract

Prostate cancer in the dog (cPC) has many features in common with hormone refractory human prostate cancer. As PC is seen more often in castrated dogs, the contribution of the androgen receptor (AR) to the development of prostate cancer remains questionable. The aim of the present study was to evaluate the presence of the AR by immunohistochemistry in cPC. A predominant, although low cytoplasmic AR staining was found in prostate cancer of both castrated and non-castrated dogs. The cytoplasmic localization was not related to mutations in exon 3 of the DNA-binding domain of the AR, as shown by sequence analysis of microdissected AR positive tumor cells. Other mechanisms that lead to an impaired androgen-AR signaling or a basal/stem cell like origin may explain the low cytoplasmic AR staining in cPC.

KEYWORDS: Dog, prostate cancer, androgen receptor, sequence analysis, immunostaining.

Introduction

Other than humans the dog is the only species that regularly develops prostate cancer (PC) spontaneously. Despite the much lower incidence in the dog (0.2-0.6 %) (Bell et al., 1991) compared to humans, canine prostate cancer (cPC) has characteristics similar to its human counterpart in that it occurs in aged patients and has the propensity to metastasize to sites such as lung, regional lymph nodes and bone (Rosol et al., 2004). Human PC is initially androgen sensitive and relapses after androgen deprivation as a hormone refractory tumor. The pathomorphology at this phase tends to be more undifferentiated and more heterogeneous compared to the more often seen adenocarcinoma in primary, androgen responsive PC (Shah et al., 2004).

Canine PC occurs in intact as well as castrated animals (Rosol et al., 2004), with a remarkably higher incidence in castrated dogs (Teske et al., 2002). It is not responsive to androgen deprivation therapy and has an undifferentiated morphology, which resembles the refractory phase of human PC (Lai et al., 2008). Originally, androgen resistance in human PC was thought to be the result of the loss of androgen receptor (AR) expression in the tumor cells, as AR expression was reported to be down-regulated during PC progression (Quarmby et al., 1990; Segawa et al., 2002). However, a growing body of evidence exists that an increased expression of AR occurs with development of hormone refractory tumors (Linja et al., 2001; Trapman and Cleutjens, 1997). In the dog, immunoreactive AR has been shown to be uniformly present in the nuclei of all prostate epithelial cells and many stromal cells of the developing prostate, as well as in the acinar and ductal epithelium of prostates of sexually mature dogs. However, after castration and in PC, this expression was lost in all but one out of the 19 cases studied (Leav et al., 2001).

In a pilot experiment, we found perinuclear and cytoplasmic AR staining in cPC (data not shown). This was the impetus to start a re-evaluation of immunohistochemical AR staining in cPC. As such perinuclear or cytoplasmic staining may be due to disruptive mutations in the DNA binding domain of the AR, we sequenced exon 3 of the AR in DNA extracted from immunopositive AR cells obtained by microdissection.

Materials and methods

Tissue specimens

Formalin-fixed and paraffin-embedded specimens from 20 dogs with spontaneous prostate tumors were collected from the archives of the Department of Pathology, Faculty of Veterinary Medicine, Utrecht University. These comprised tumors from 11 castrated (mean age: 10 years, range between 6.8 years to 12.8 years) and nine non-castrated (mean age: 9.4 years, range between 9 years to 11.6

years) dogs. Four normal prostates from two intact dogs and two castrated dogs served as control. Sections for histological examination were cut at 4 µm and stained with haematoxylin and eosin (HE).

Immunohistochemistry

Immunohistochemistry was performed using an indirect avidin-biotin-peroxidase staining procedure. Following deparaffinization and rehydration of the sections, antigen retrieval was achieved by submerging the sections in pre-heated 0.1M sodium citrate, pH 6 and by subsequent further heating in a microwave oven (700W, near boiling) for 10 min and cooling for 20 min. Endogenous peroxidase was neutralized by submersion of the slides in 0.3% H_2O_2 in 40% methanol-PBS for 30 min. After a short rinse with PBS, the sections were pre-incubated with 10% normal goat serum for 15 min. Sections were incubated with the primary antibody against the AR (dilution 1:150; N-20, Santa Cruz) overnight at 4°C. After washing the slides three times for five min in PBS/0.05% Tween, sections were incubated with biotinylated secondary antibody in PBS (goat-anti-rabbit diluted 1:250, E0432, DAKO Corporation) for 30 min. Slides were washed three times for five min in PBS/0.05% Tween and incubated with peroxidase coupled avidin-biotin complex (ABC Kit, Vector Laboratories) for 30 min, and washed three times for five min in PBS. Peroxidase activity was visualized by incubation with 3,3'diaminobenzidine (0.5mg/mL in 0.05M Tris, pH 7.6 containing 0.3% H_2O_2, Sigma-Aldrich) for 10 min in the dark. Slides were then washed twice for five min in water, counterstained with haematoxylin, dehydrated, and mounted. As a negative control, primary antibodies were substituted with PBS.

Laser microdissection and DNA extraction

Formalin-fixed and paraffin-embedded samples were cut in 5 µm thick sections, mounted on membrane slides (Molecular Machines and Industries), and stained with anti-AR as described above. Immunopositive cells, irrespective of the localization of the staining, cytoplasmic or nuclear, were dissected by laser microdissection using a MMI CellCut® (Molecular Machines and Industries) mounted on a Nikon TE 300 microscope and equipped with MMI Celltools® software. Approximately 1000 cells were cut using the following settings: Speed 9, Focus 45, Energy 95 and 10x objective magnification. The cells were then isolated by lifting to the cap of specific Isolation Cap Vials® (Molecular Machines and Industries). After microdissection, the genomic DNA of collected samples was extracted by incubation with 10 µL of PicoPure DNA extraction solution (Arcturus) for 48 h. At the start and at 24 h of incubation, 0.2 µL proteinase K (20 mg/mL) was added to the solution.

PCR amplification

Exon 3 of the canine androgen receptor was amplified by polymerase chain reaction (PCR; Peltier thermal cycler 9700; MJ Research) in a total volume of 50 µL containing 1 µL of extracted DNA,

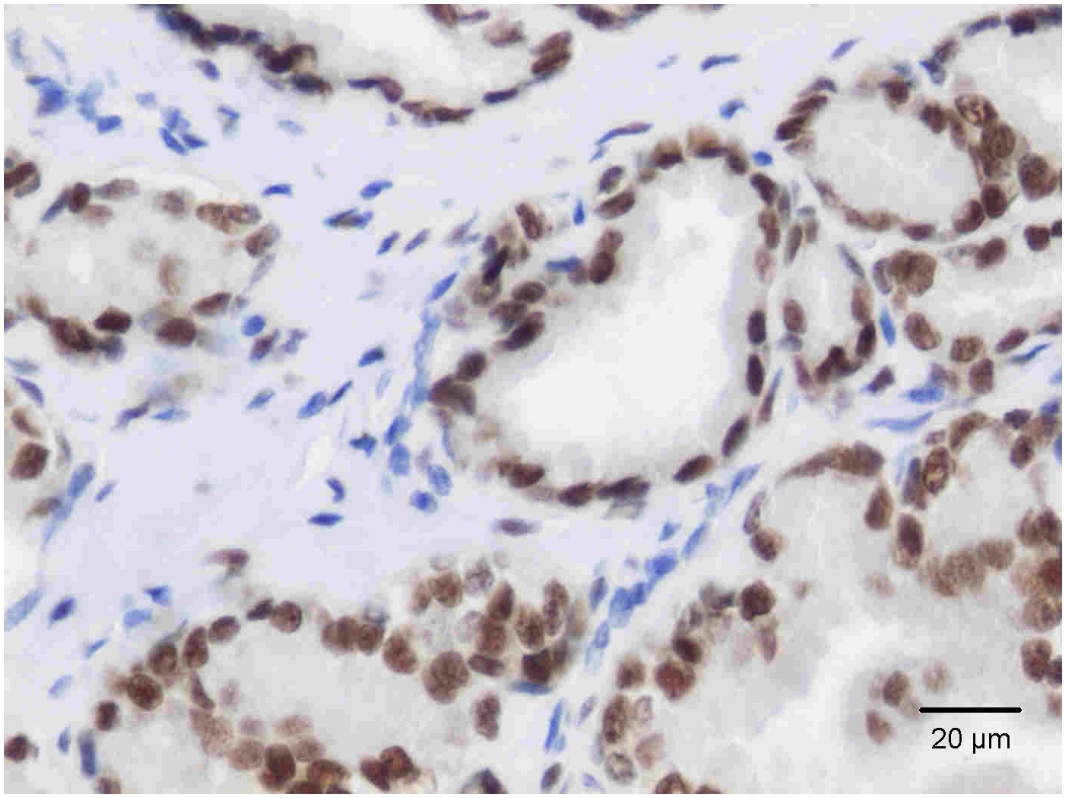

Figure 1
Photomicrograph illustrating AR expression in the nuclei of prostate epithelial cells and of some stromal cells in a normal intact dog. AR immunohistochemistry with hematoxylin counterstain.

0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 U Platinum® Taq DNA Polymerase in 1X PCR buffer (Invitrogen) for 30 cycles. This was followed by a second round of PCR using a similar mixture as the first round of PCR but containing 1 µL of 100 times diluted product from the first PCR as a template. PCR amplification was carried out under the following conditions: denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 61.5 °C for 30 sec; extension at 72 °C for 30 sec; and a final extension at 72 °C for 10 min. The lengths of the PCR products were checked after electrophoresis in a 1.2% agarose gel under UV transillumination. The primers flanking the canine exon 3 were:
Forward: 5'-ATGGCCATTCTCTGTTCACTTCT-3';
Reverse: 5'-ATCCCTGCTTCACACCTTCTG-3'

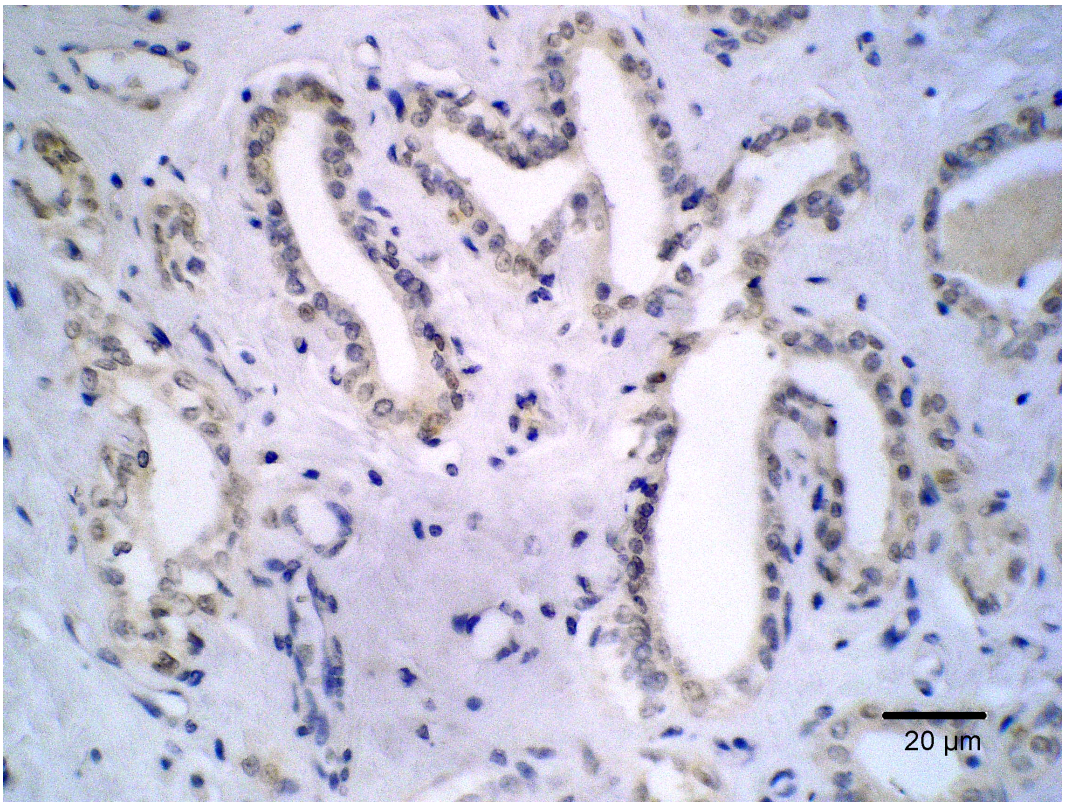

Figure 2
Photomicrograph illustrating the decreased nuclear expression and low cytoplasmic expression of the AR in prostate epithelial cells in a normal castrated dog. AR immunohistochemistry with hematoxylin counterstain.

Sequencing
Tercycle reactions were performed in a 10 µL volume containing 2 µL of ten times diluted PCR product, 3.2 pmol primer and 1 µL Big Dye Terminator v 3.0 Ready Reaction Mix (Perkin Elmer Applied Biosystems) in 1x sequence buffer (400 mM Tris, 10 mM MgCl2, pH 9.0; final concentrations). The tercycle reactions consisted of 25 cycles of denaturation at 96 ˚C for 30 sec, annealing at 50 ˚C for 15 sec and extension at 60 ˚C for 2 min. Tercycle products were then purified using a multiscreen 96-well filtration plate filled with Sephadex G-50 superfine (Amersham Biosciences) and sequenced in 20 µL distilled water using an automatic sequencer (ABI 3100 Genetic Analyzer; Perkin Elmer Applied Biosystems). Sequences were aligned to the published canine AR sequence (Lu et al., 2001) using the DNAstar software package (DNAstar Inc.).

Results

Immunohistochemistry

In the normal prostate of the intact dogs, AR staining was seen in the nuclei of all the epithelial cells and of about 10-15% of the stromal cells (Fig. 1). In the prostates of the two castrated dogs, nuclear AR staining was absent in most epithelial cells or decreased in intensity in the others (Fig 2.). In some epithelial cells a vague cytoplasmic staining was noticed. No staining was seen in the stromal compartment after castration.

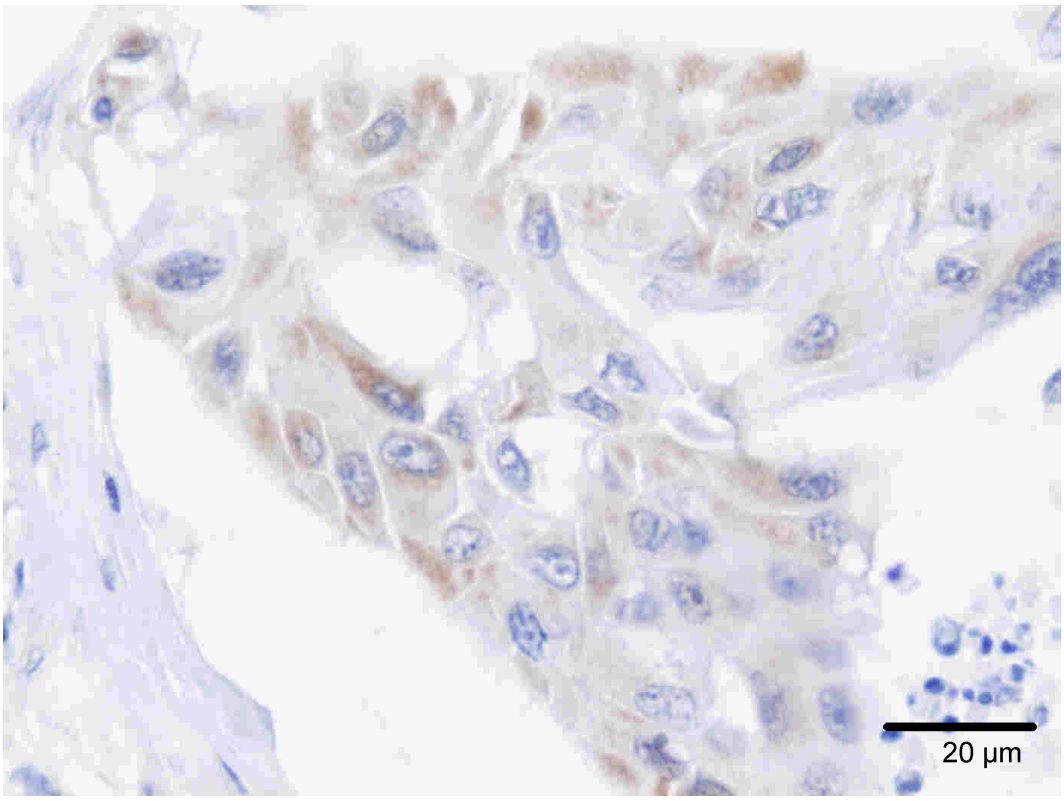

Figure 3

Photomicrograph illustrating AR expression in the cytoplasm of prostate cancer cells. Note the lower staining intensity and lack of nuclear expression when compared to normal intact dogs.

Table I. Immunostaining of androgen receptor in canine prostate cancer samples

	Positive (n)	Tumor cells		Stroma*	
Intact		Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
(n= 9)	8	6	8	3	0
Castrated		Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
(n= 11)	8	3	8	2	0

* AR staining was seen only in few, scattered cells

In the 20 dogs with PC, AR staining was observed in 8 of the 9 tumors of the intact dogs and 8 of the 11 tumors of the castrated dogs, although the proportion of positive cells and the staining intensity were much lower than in the prostate tissue of healthy, non-castrated animals. Most of the positive staining was seen in the cytoplasm rather than in the nuclei of the tumor cells (Fig. 3, Table I). Of those 20 dogs with PC, 15 provided enough material for laser-microdissection and subsequent sequence analysis.

PCR and sequence analysis

A clear PCR product was seen on gel electrophoresis after PCR amplification. After purification and sequencing of the PCR product, the results were aligned with the sequence acquired from the NCBI database (gi:6578766), which confirmed that the PCR product was the canine AR exon 3. The alignments showed no mutation or polymorphism in exon 3 of the AR in any of the cPCs investigated.

Discussion

The transition of prostate cancer to a hormone refractory phase in humans was originally attributed to a loss of AR expression (Quarmby et al., 1990; Segawa et al., 2002). However, the expression of the AR appeared to be rather heterogeneous in human prostatic carcinomas, regardless of tumor differentiation and progression (Ruizeveld de Winter et al., 1994), and AR gene amplification and protein expression was found in hormone refractory PC (Edwards et al., 2003).

In our control prostate tissue of non-castrated dogs, we confirmed the widespread AR immunoreactivity in the nuclei of prostate cells of the dog (Leav et al., 2001). In contrast, we observed a partial loss of nuclear AR expression and appearance of cytoplasmic expression in prostate epithelial cells after castration. In the cPC samples, AR immunoreactivity was detected in 16/20 cases, although at a lower intensity in comparison to the normal prostate tissue present

adjacent to the cancer tissue. In both intact and castrated animals a shift from nuclear to cytoplasmic localization was observed in tumor tissue. The presence of androgens is necessary for stabilizing the AR dimer and its translocation to the nucleus. The absence of androgens in the castrated animals may lead to cytoplasmic staining due to diminished receptor activation, comparable to what is seen in androgen sensitive LNCap cells in vitro in the absence of androgen (Gregory et al., 2001).

However, we also observed a predominantly cytoplasmic staining in the tumors of intact animals in spite of the presence of androgens. This may point to a disruption of the androgen-AR signaling pathway. Mutations in the AR may render the AR unable to enter the nucleus. Another explanation may be that cPC is derived from the basal/stem cell like cells that do not or hardly express AR. A cytoplasmic AR expression has also been found in human PCs and was found to be an independent predictor of biochemical recurrence after androgen depletion (Diallo et al, 2008). In the latter study cytoplasmic AR expression was seen more often in androgen-independent prostate cancer than in hormone-sensitive prostate cancer.

We hypothesized that the cytoplasmic localization of the AR in prostate tumors of intact animals might have been due to mutations in exon 3 of the DNA binding domain. Many AR mutations have been documented in the AR in human prostate cancer tissue, including in the N-terminal domain, the DNA-binding domain (DBD), the hinge region, and in the ligand-binding domain (LBD) (Gottlieb et al., 2004). In order to examine the possible presence of such mutations, we isolated DNA only from AR-expressing tumor cells using laser microdissection. We then amplified exon 3 by PCR and analyzed the nucleotide sequence. No mutations were found in exon 3 of the DNA binding domain in any of the samples, leading to a rejection of the above hypothesis. However, other regions are also involved in nuclear translocation. For instance, mutations in the hinge region may greatly inhibit nuclear translocation and DNA binding, although a strong androgen response can still be seen using several reporters (Haelens et al., 2007).

There remains a lack of understanding of the role of the AR in human and canine PC. The androgen insensitivity in hormone resistant/refractory PC is likely to be caused by clonal selection, the selection of cells that are adapted to an environment without androgens. Several adaptation mechanisms have been reported for this, including AR amplification, AR mutation, altered expression of AR coactivator and corepressor proteins, and activation of other pathways that can enhance AR function (Balk, 2002).

In humans, AR amplification at the DNA and RNA level has been reported in about 20-30% of hormone-refractory PC (Edwards et al., 2003; Feldman and Feldman, 2001; Fenton et al., 1997), whereas mutations in the AR gene have been reported in about 10-20% of PC specimens, with higher percentages in hormone refractory and metastatic tumors (Gaddipati et al., 1994; Suzuki et al., 1996; Suzuki et al., 1993; Taplin et al., 1999; Taplin et al., 1995). AR mutation can occur in response to the strong selective pressure exerted by androgen antagonists like flutamide or low levels of androgen concentration after castration, thus increasing receptor sensitivity to its ligands (Suzuki et al., 2003). These mutations might contribute to cancer progression after androgen

ablation therapy. Most significantly, the selection for rare tumor cells with flutamide-stimulated mutant ARs indicates that ongoing AR activity is critical for tumor cell survival after androgen ablation and that mutation of the AR is an important mechanism for achieving this activity in flutamide-treated patients (Taplin et al, 1999).

In the human prostate, four subsets of prostate cells have been identified by a variety of cytokeratin markers: basal cells, intermediate cells, secretory cells, and finally neuroendocrine cells. In the subset of basal cells a small proportion are thought to be the stem cells of prostate epithelium. AR expression is mainly seen in secretory cells whereas basal cells are thought to be negative for AR expression. However, in a subset of basal cells, low AR mRNA expression has been found, and shown to be increased in medium containing fibroblast growth factor (FGF)-7 (Planz et al., 2001). In addition, stromal cells induced expression of the androgen reporter prostate specific antigen (PSA) gene in CD57- positive basal cells (Liu et al., 1997). It is most likely that the androgen-AR axis does not affect secretory cell proliferation directly but rather recruits stem cell-like epithelial cells to proliferate and differentiate into secretory cells after interacting with stromal cells. These cells subsequently express AR, which may mediate androgen activation of secretory target genes. Similar subsets of prostate cells in the dog have been observed in our previous studies (unpublished data), and the expression in the canine prostate follows the distribution of the AR in the human prostate. Therefore, the regulation of the cellular response to androgen in the canine prostate may be similar to that in the human prostate. Our results are in line with the proposed basal/ductal origin of tumor cells–postulated by others (Leav et al, 2001; Sorenmo et al, 2003).

Conclusion

A lack of nuclear AR expression and the presence of cytoplasmic AR expression were shown by immunohistochemistry in the majority of cPC cells. This altered pattern of AR expression as compared to that in normal prostate cells is not caused by mutations in exon 3 of the DNA-binding domain of the AR. However, other disruptive androgen-AR signaling mechanisms such as mutations in other regions cannot be excluded. Alternatively, cPC may be derived from the basal/stem cell like epithelial cells that express low levels of AR. These data indicate that the function of the AR in cPC development is not as clear as initially thought.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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CHAPTER 5 *J Vet Intern Med 2008;22:1380–1384*

Androgen receptor CAG repeat polymorphisms in canine prostate cancer

Chen-Li Lai, Henry L'Eplattenier, Rene van den Ham, Femke Verseijden, Astrid Jagtenberg, Jan A. Mol and Erik Teske*

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University

Abstract

BACKGROUND: Relatively shorter lengths of the polymorphic polyglutamine repeat-1 of the androgen receptor (AR) have been associated with an increased risk of prostate cancer (PC) in humans. In the dog, there are 2 polymorphic CAG repeat regions.
OBJECTIVE: To investigate the relationship of CAG-repeat length of the canine AR-gene and the development of PC.
ANIMALS: Thirty-two dogs with PC and 172 control dogs were used.
METHODS: DNA was extracted from blood. Both CAG repeats were amplified by PCR and PCR products were sequenced.
RESULTS: In dogs with PC, CAG-1 repeat length was shorter (P=0.001) by an increased proportion of 10 repeats (P=0.011) and no 12 repeats (P=0.0017) than in the control dogs. No significant changes were found in CAG-3 length distribution. CAG-1 and CAG-3 polymorphisms proved not to be in linkage disequilibrium.
Breed difference in allelic distribution was found in the control group. Of the prostate-disease sensitive breeds a high percentage (64.5%) of the shortest haplotype 10/11 was found in the Doberman, whereas Beagles and German Pointers had higher haplotype 12/11 (47.1% and 50%). Bernese Mountain dogs and Bouvier dogs both shared a high percentage of 11 CAG-1 repeats and 13 CAG-3 repeats. Differences in (combined) allelic distributions among breeds were not significant.
CONCLUSIONS AND CLINICAL IMPORTANCE: In this preliminary study, short CAG-1 repeats in the AR-gene were associated with an increased risk of developing canine PC. Although breed specific differences in allelic distribution of CAG-1 and CAG-3 repeats were found, these could not be related to PC risk.

KEY WORDS:
Progression, breed specificity, transactivation domain, haplotype, androgen resistance

Introduction

The prostate is an androgen dependent organ.[1] Androgens mediate their effect through activation of the androgen receptor (AR). Upon binding of the biologically most active testosterone metabolite dihydrotestosterone (DHT), proliferation, survival, and expression of differential markers of prostate epithelial cells are stimulated.[2]

In humans, activation of the androgen-AR axis is believed to play an important role in the development and progression of prostate cancer (PC). Epidemiologic studies demonstrated that high plasma androgen concentrations are associated with a high incidence of PC.[3,4] Continuous administration of testosterone pellets to Nb rats promotes development of prostate adenocarcinoma.[5] Androgen deprivation initially blocks tumor growth in humans. However, an androgen independent tumor eventually re-occurs in these patients and leads to a uniformly lethal drug-resistant stage.[6] In this stage, there is still expression and activity of the AR in the recurrent PC cells,[7-9] which demonstrates the importance of the AR in oncogenesis and progression of PC.

The human AR gene is located on the X chromosome and is composed of 8 exons. The first exon encodes the N-terminal domain which possesses transcriptional activity. This N-terminal transactivation domain of the AR gene contains 3 polyglutamine repeats and one polyglycine repeat. One of the polyglutamine (CAG) repeats is polymorphic and consists of 11 to 31 repeats in the germline DNA of the normal population.[10] The length of this repeat has been reported to correlate with the risk of PC. Humans with shorter CAG repeat (CAGr) length are at higher risk of developing PC compared to those with longer variants.[11-13]

The dog is the only non-primate species that develops PC spontaneously. Canine PC shares several similarities with human PC. Both tumors are commonly found in older individuals,[14] tumor growth beyond the prostate is common and the distribution of distant metastases (bone and lung) is similar to that seen in humans.[15,16]

The role of androgens and their receptor in canine PC is not yet clear. Most canine tumors do not respond to androgen depletion.[17] Furthermore, an increased risk of PC is found in castrated male dogs and in the Bouvier des Flandres breed.[18] These findings may point to no inductive, or even a protective role of androgens in canine PC development. The fact that prostate tumors in non-castrated dogs generally are of a more differentiated type compared to tumors in castrated dogs may further support this hypothesis.[19]

Similar to the human, rat and mouse AR, the canine AR sequence contains 3 polyglutamine repeats and 1 polyglycine repeat.[20] Two of these $(CAG)_n$ microsatellites in the canine AR are reported to be polymorphic.[21] The first polymorphic CAGr (CAG-1) was found at a similar position as the human polymorphic CAG-I repeat, whereas the second polymorphic CAGr (CAG-3) was located at a position corresponding to that of the CAG-3 region in the rat.[20]

The aim of this study was to investigate whether polymorphisms in the CAGr of the

canine AR gene can be related to the development of PC in dogs.

Material and methods

Subjects

For analysis of the allelic distribution of the CAG repeat, blood samples were obtained from 32 dogs with PC and 172 male control dogs that were admitted to the Utrecht University Clinic for Companion Animals during 2002-2005. The group of dogs with PC came from 17 different breeds, including 3 Bouviers des Flandres. All were older dogs with a mean age of 10.7 yrs (range, 6.7-13.5). Control dogs were selected from those referred to the hospital for reasons unrelated to the prostate. The control group consisted of 17 Beagles, 45 Bernese Mountain dogs, 30 Bouviers des Flandres, 31 Dobermans, 36 German Pointers, 6 Scottish Terriers, and 7 dogs of other breeds. DNA extraction was performed using a salt extraction method as previously reported.[22]

Table 1 Primers used in this study

Primer	Sequence	Annealing Temperature (°C)
CAG-1 Forward	GTTTTCCCAGTCACGACGAACCTGTTCCAGAGTGTGC*	57
CAG-1 Reverse	TCCTCATCCAGAGCCAGGTA	
CAG-3 Forward	CCAGCACCACCGGACGAGAATGA	
CAG-3 Reverse	GTTTTCCCAGTCACGACCGGCGGCCTCCCTTGCTCTC*	64

* The underlined sequence denotes the M13-tail

Primer development

The complete coding sequence of the canine AR was obtained from Genbank (gi:6578766; http://www.ncbi.nlm.nih.gov). Two primer pairs were used to amplify the 2 polymorphic repeats within exon 1 of the canine AR gene. One of each primer pair was attached to a M13 tail, which was used to introduce a phosphoramidite (FAM) dye attached to a M13 primer in order to label the PCR product (Table I). The PCR product size for the first CAGr was 238 base pairs, and for the second CAGr was 270 base pairs, both calculated if the CAGr was 11 repeats long.

Polymerase chain reaction (PCR)

The polymorphic CAG repeats of the canine AR were amplified by PCR, using 25 ng of genomic DNA in a final volume of 15 µl PCR mixture, containing 1x PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.03 µM primer with M13 tail, 0.3 µM FAM-labeled M13 primer, 0.3 µM complementary primer and 0.3 units AmpliTaq Gold. The cycle conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 10 cycles of denaturation at 95°C for 30 sec, annealing at specific temperatures (Table 1) for 15 sec, and extension at 72°C for 30 sec. This was followed by an additional 25 cycles using a denaturation temperature of 92°C. Finally, an extension step for 10 min at 72°C was performed.

Estimation of repeat length

Two mircoliters of a 10-fold diluted PCR-product was mixed with 10 µl of formamide and 0.2 µl of a carboxytetramethylrhodamine (TAMRA) labeled 500 base pair size standard[a] in a 96-optical well plate. Products were denatured at 95ºC for 3 minutes and immediately placed on ice for 5 minutes. The length of the PCR product was determined on an ABI 3100 Genetic Analyzer[b] and evaluated using the GeneScan Analysis program.

Sequencing analysis

Sequence analysis for the 2 different PCR products was performed at least twice, using independently amplified PCR products. Tercycle reactions were performed in a 10 µl volume and contained 2 µl of 10-fold diluted PCR product, 3.2 pmol primer and 1 µl Big Dye Terminator v 3.0 Ready Reaction Mix[b] in 1x sequence buffer (400 mM Tris, 10 mM MgCl2, pH 9.0). The tercycle consisted of 25 cycles of denaturation at 96ºC for 30 seconds, annealing at 53ºC for 15 seconds and extension at 60ºC for 2 minutes. Tercycle products then were purified using multiscreen 96-well filtration plates filled with Sephadex-gel G-50 superfine[c] and sequenced in 20 µl distilled water using the ABI 3100 Genetic Analyzer. The length of the AR-CAG repeats in each individual dog was analyzed by counting the number of CAG trinucleotides using the DNAstar software package[d].

Statistics

Analysis of data was performed using the statistical software SPSS (version 12.0). CAGr length distributions between the canine PC and the control group were compared by Fisher's exact test and Mann-Whitney test with 95% confidence interval. In order to compensate for multiple testing, statistical significance was accepted for P-values ≤ 0.01. The linkage disequilibrium measure r^2 was calculated using previously described formulae[e] .[23]

Table 2 Allelic distribution in dogs with PCA and in normal control dogs

	CAG-1					CAG-3			
Length	**n**	**PCA**	**n**	**Normal**	**Length**	**n**	**PCA**	**n**	**Normal**
10	17	54.8 %	51	29.7 %	11	19	61.3 %	130	75.6 %
11	14	45.2 %	84	48.8 %	12	4	12.9 %	13	7.6 %
12	0	0.0 %	37	21.5 %	13	8	25.8 %	29	16.9 %
Total	31*	100.0 %	172	100.0 %	Total	31*	100.0 %	172	100.0 %

* In both CAG-1 and CAG-3 one sample for repeat length measurement was inadequate

Results

Allelic distribution in the population

From the length of the PCR products obtained by GeneScan analysis, the length of the CAG-1 and CAG-3 repeats was calculated. This calculated number of trinucleotide repeats was confirmed by sequence analysis, using 2 independent PCR products for both repeats. For each repeat PCR, the estimation of 1 sample was inadequate for the assessment of the CAG length in tumor DNA and therefore excluded. The number of CAG repeats in CAG-1 varied between 10 and 12 in our study group, whereas CAG-3 contained 11 to 13 repeats. Next, the allelic distribution for each repeat in both the PC and the control group was calculated (Table 2). A significant difference (P= 0.001) in

Table 3 Haplotype distribution in dogs with PCA and in normal control dogs

		PCA dogs		Normal dogs	
		Frequency	**Percentage**	**Frequency**	**Percentage**
haplotype	10/11	8	26.7	35	20.3
	10/12	1	3.3	2	1.2
	10/13	8	26.7	14	8.1
	11/11	10	33.3	60	34.9
	11/12	3	10	9	5.2
	11/13	0	0	15	8.7
	12/11	0	0	35	20.3
	12/12	0	0	2	1.2
	12/13	0	0	0	0
Total		30*	100	172	100

* In two dogs one CAG repeat length could not be established

Table 4 Differences in allelic distribution between breeds in control dogs

Breed	**CAG-1 (%)**			**CAG-2 (%)**		
Length	10	11	12	11	12	13
Beagle (n=17)	5.9	47.1	47.1	82.4	17.6	0.0
Bernese Mountain dog.(n=45)	24.4	64.4	11.1	55.6	0.0	44.4
Bouvier des Flandres (n=30)	30.0	56.7	13.3	60.0	16.7	23.3
Doberman (n=31)	67.7	32.3	0.0	93.5	3.2	3.2
German Pointer (n=36)	8.3	41.7	50.0	97.2	0.0	2.8
Scottish Terrier (n=6)	66.7	33.3	0.0	83.3	16.7	0.0
Other breeds (n=7)	28.6	42.9	28.6	57.1	42.9	0.0

the CAG-1 repeat length was found between the PC dogs and the normal dogs. The proportion of 10 repeats was significantly increased (P=0.011) and no 12 repeats were present in dogs with PC. No significant changes were found in the distribution of the CAG-3 length between control dogs and dogs with PC. Haplotype analysis revealed that the CAG-1 and CAG-3 polymorphisms are not in linkage disequilibrium ($r^2 = 0.112$).

From the 6 different alleles, 9 potentially different haplotypes can be constructed, denoted as CAG-1/CAG-3 repeats by numbers (Table 3). Haplotype 12/13 was not found in any of the samples investigated in the combined group. Calculation of the sum of repeat lengths resulted in a variation of 21-24 repeats. However, no significant difference of the total repeat length was found between the PC group and the normal group (P = 0.243).

Comparison between breeds

Among the control dogs, the difference of allelic distribution among breeds was examined (Table 4). For the polymorphic CAG-1 region, the Doberman and Scottish Terrier showed a relatively larger proportion of the shortest repeat length (allele 10), compared to other breeds, and lacked the longest allele 12. In contrast, German Pointers and Beagles had the largest proportion of allele 12 and relatively less allele 10. With regard to CAG-3 repeats, the majority of the dogs in each breed had mostly the shortest allele 11. The Beagle, Scottish Terrier and the group of “other breeds” didn’t have the longest allele 13, whereas the Bernese Mountain dog and German Pointer didn’t have allele 12. The Bernese Mountain dog had the largest proportion of the longest allele 13. No significant differences in the (combined) allelic distributions among breeds could be detected (Table 5).

Table 5 Differences in haplotype between breeds in control dogs.

Breed	Haplotype							
	10/11	10/12	10/13	11/11	11/12	11/13	12/11	12/12
Beagle (n=17)	1	0	0	5	3	0	8	0
Bernese Mountain dog (n=45)	5	0	6	15	0	14	5	0
Bouvier des Flandres (n=30)	2	0	7	13	4	0	3	1
Doberman (n=31)	20	1	0	9	0	1	0	0
German Pointer (n=36)	2	0	1	15	0	0	18	0
Scottish Terrier (n=6)	4	0	0	1	1	0	0	0
Other breeds (n=7)	1	1	0	2	1	0	1	1

Discussion

In order to determine whether polymorphisms in the CAGr length of the AR gene can be related to the development and progression of PC in the dog as in humans,[11-13] we analyzed the length of AR-CAGr in genomic DNA of 32 dogs with PC and 172 male control dogs. Our results demonstrated that the CAG-1 region of the AR gene in the canine population contained 10-12 CAGr, whereas the CAG-3 region contained 11-13 CAGr. The range of the CAG-3 repeats in this study was somewhat narrower than reported by Shibuya et al (1993), in which the CAG-1 also contained 10-12 repeats but the CAG-3 contained 10-13 repeats.[21] Differences in study populations may explain this variability in CAGr range. In the study of Shibuya et al.,[21] a larger number of breeds was included and 27 of the 80 dogs were female.

The allelic distribution of CAG-1 appeared to be significantly different between the dogs with PC and the normal dog population. Similar to humans, [11,24-26] shorter CAG-1 repeats were found more often in the canine PC group, with an overall lack of the longest length CAG-1. In humans, shorter CAGr in exon-1 of the AR gene has been suggested to play a causal role in PC development[11], because it encodes an AR with higher transcriptional activity.[27] However, all canine AR CAG-1 repeat lengths are short when compared to the human length of 11 - 31 repeats in this allele.[10] Increased transcriptional activity for the short canine CAG-1 repeat remains to be investigated.

Reports about the inverse association between CAGr length of the AR gene and PC risk in human are contradictory. Some studies found statistical significance,[11,24-26] whereas others failed to find significance.[28-31] Giovannucci[32] summarized this discordance and proposed a “two pool” model, one of relatively aggressive, early onset “androgen-driven” PC cancers and the other

occurring at older ages which may be less driven by androgens and more related to pathologic processes such as oxidative insults. A recent study from the same group found that patients with higher plasma total or free testosterone concentrations had lower-grade cancer whereas those with lower plasma total or free testosterone concentrations had higher-grade cancer.[28] This fact implies a more protective role of androgenic stimulation in PC cells or even prevention of PC [6] and was in agreement with our previous studies in which we observed that non-castrated dogs have a lower risk of PC and that PC in castrated dogs more often had poorly differentiated histology.[18,19] The features of canine PC, low incidence and lack of prostate marker screening, is supportive of an inverse association between CAGr length and PC in humans and is concordant with Giovannucci's suggestion. It is possible that those AR encoded by genes with shorter CAG-1 have higher transactivation function which accelerates the transit of nascent cancer from the latent phase to a symptomatic phase.

The fact that the differences in the dog may be less than 2 repeats is in concordance with a recent meta-analysis of 23 epidemiologic studies on the association between AR receptor CAG and GGN polymorphisms and PC in humans.[33] In this study the presence of shorter repeats was found to be modestly associated with PC risk, with the absolute difference in number repeats between cases and controls less than 1 repeat. By far the main contribution in this meta-analysis is from Caucasian men. In a recent study by Lange et al, [34] the authors failed to find such an association in African-American men. However, African-American men already have shorter CAGr compared to Western white and Asian men and have a higher incidence of PC.[35] In the European canine population, the Bouvier has been reported to have a higher risk of PC.[18] However, the effect of a shorter CAGr was not reflected in the allelic distribution of the 2 repeats that were investigated. Nevertheless, a breed difference in allelic distribution was found. Doberman and German Pointer dogs have quite different allelic distribution despite the fact that both breeds are predisposed to benign prostate hyperplasia and PC.[18,36] Bernese Mountain dogs and Bouvier dogs both shared a high percentage of 11 CAG-1 repeats and 13 CAG-3 repeats, whereas of the 2 breeds only the Bouvier is reported to have higher PC risk.[18] The relatively low numbers of other breeds represented in this study cannot exclude other breed differences.

In summary, our study showed an overall lack of 12 repeats and an increased short CAG-1 repeat within exon 1 of the AR gene in the canine PC group, which was significantly different from the control group. No significant differences were found in the third CAG polymorphic region or in the combined CAGr length between cases and controls. CAG-1 and CAG-3 polymorphisms were found not to be in linkage disequilibrium. Although breed specific differences in allelic distribution of CAG-1 and CAG-3 repeats were found, these could not be related to PC risk. More studies are needed to further elucidate the role of this finding in the development of PC.

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Footnotes:
[a] LIZ™, Applied Biosystems Part No. 4322682
[b] Perkin Elmer Applied Biosystems, Foster City, CA
[c] Amersham Bioscience, Roosendaal, The Netherlands
[d] DNAstar Inc, Madison, WI
[e] Excel®, Microsoft Corporation, Redmond, WA

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CHAPTER 6 *J Vet Intern Med 2007;21:776-782*

Regulation of COX-2 expression in canine prostate carcinoma: increased COX-2 expression is not related to inflammation

Henry F. L'Eplattenier[a], Chen Li Lai[a], René van den Ham[a], PhD, Jan Mol[a], PhD, Freek van Sluys[a], PhD, Erik Teske[a], PhD

[a] Department of Clinical Sciences of Companion Animals,University of Utrecht, PO Box 80154, 3508 TD Utrecht, Netherlands

Abstract

BACKGROUND: Cyclooxygenase-2 (COX-2) expression has been documented in human and canine prostate carcinoma (PCA). Canine PCA is a histologically heterogeneous tumor, sometimes including inflammatory infiltrations. However, it is unknown whether COX-2 expression in canine PCA is related to the histological type of the tumor, to the presence of inflammation, or to both. Moreover, little is known about the mechanisms regulating COX-2 expression in neoplastic tissue.
HYPOTHESIS: COX-2 expression is related to the presence of inflammation in canine PCA and correlates with the degree of tumor differentiation.
METHODS: The expression of COX-2 was examined in 28 cases of canine PCA by immunohistochemistry. In addition, a neoplastic and a nonneoplastic canine prostatic cell line were used to investigate the effects of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), phorbol 12-myristate 13-acetate (PMA), epithelial growth factor (EGF) and specific signal transduction pathway inhibitors on COX-2 expression.
RESULTS: Twenty-four of the 28 prostate tumors showed COX-2 expression. The presence of inflammatory infiltrates in tumor tissue was associated with lower COX-2 expression scores. In vitro, TNF-α, IL-6 and EGF increased COX-2 expression in nonneoplastic cells but not in PCA cells, where baseline expression was high. COX-2 expression in PCA cells could be suppressed by means of specific phosphatidyl inositol-3 kinase (PI3K), protein kinase c (PKC), or inhibitor of extracellular signal-related kinase (ERK/MAPK) inhibitors.
CONCLUSIONS AND CLINICAL IMPORTANCE: COX-2 is expressed in canine PCA; however, this is not related to the presence of inflammatory infiltrates. This conclusion is further supported by the finding that the cytokines TNF-α and IL-6 and their involved signaling pathways do not stimulate COX-2 expression in malignant canine prostate cells.

KEY WORDS: Cell lines; Epithelial growth factor; Immunohistochemistry; Interleukin-6; Phorbol 12-myrisate 13-acetate; Tumor necrosis factor alpha.

Introduction

The enzyme prostaglandin-endoperoxide synthase, also referred to as cyclooxygenase (COX), exists in two main isoforms. COX-1 is constitutively expressed in many cell types[1], whereas COX-2 is the inducible form of the enzyme and is only expressed in cells involved in various inflammatory and neoplastic diseases.[1-3] The COX-2 enzyme itself and its RNA expression have been detected in several forms of human and canine cancer.[3] In the dog, these include oral squamous cell carcinoma, mammary tumors, transitional cell carcinoma of the urinary bladder and prostatic carcinoma.[4-9] There is a growing body of evidence showing that COX-2 and its product prostaglandin E_2 (PGE_2) promote tumor development by a variety of mechanisms, such as an increase in proliferation, a decrease in apoptosis and an induction of angiogenesis, possibly by generating free radicals and carcinogens.[1,10]

COX-2 is expressed in both human and canine prostate carcinoma (PCA). In two immunohistochemistry studies of canine PCA, COX-2 expression was found in 75% and 88.2% of the tumors, respectively [4,9], whereas it was not expressed in normal prostatic tissue. These findings suggest that COX-2 may play a role in the pathogenesis of PCA in dogs. In human PCA, COX-2 expression has been reported to be significantly higher in poorly differentiated compared to well-differentiated tumours[11], and to correlate with local chronic inflammation.[12] Canine PCA is histologically a heterogeneous tumor that may present several patterns of histological differentiation[13-15] and a possible correlation between COX-2 expression and histological classification of the tumors or presence of inflammation has not yet been examined.

Little is known about the mechanisms regulating the expression of COX-2 in tumor cells. However, it has previously been shown that the expression of COX-2 can be up-regulated by oncogenes, growth factors, cytokines, endotoxins and phorbol esters.[16-19]

The objectives of this study were to examine whether there is a relationship between the expression of COX-2 and either the histological morphology of the tumors or the presence of inflammation in canine PCA, as well as to gain insight into the mechanisms responsible for COX-2 expression in canine PCA. For this, COX-2 expression in naturally occurring canine PCA was examined by immunohistochemistry. Furthermore, the ability of the tumor promoter phorbol 12-myristate 13-acetate (PMA), the cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) as well as epithelial growth factor (EGF) to induce up-regulation of COX-2 expression was investigated in a normal and a neoplastic canine prostatic cell line. In order to understand which signaling pathways are used for this up-regulation, the cells were also incubated with three inhibitors of the various signaling pathways: PD98059, inhibitor of Extracellular signal Related Kinase (ERK/MAPK)[20,21], GF109203X, inhibitor of Protein Kinase C (PKC)[22], and LY294002, inhibitor of Phosphatidyl Inositol-3 Kinase (PI3K).

Materials and methods

Tissue samples

Prostate tissue from 28 dogs with prostatic carcinoma was used for immunohistochemistry staining of COX-2. The dogs included 21 castrated and 7 intact males with prostatic carcinoma. Tissue was obtained either during surgery or during necropsy. Tissue samples were fixed in formalin and embedded in paraffin using standard protocols.

Immunohistochemistry staining

Five micrometer paraffin-embedded tissue sections were cut and mounted on Superfrost slides.[a] Subsequently, the slides were dewaxed in xylene[a] and rehydrated in descending concentrations of alcohol[b] (100%, 96%, 70%). After rinsing the slides in distilled water, the tissue was pre-treated by boiling the slides in 0.01 M citrate[b] buffer, pH 6, for 10 minutes in a microwave oven. The slides were left to cool for 30 minutes and rinsed in phosphate buffered saline (PBS) before the endogenous peroxidases were inhibited by immersing the tissue in 0.3% hydrogen peroxide[b] in methanol[b] for 30 minutes at room temperature. The slides were again rinsed in PBS, and non-specific antibody binding was blocked by incubating the sections with normal horse serum (NHS) diluted 1:10 in PBS for 30 minutes at room temperature. Next, the tissue sections were incubated overnight at 4°C with the primary antibody (monoclonal mouse anti-COX-2[c]) diluted 1:50 in PBS. After rinsing with PBS, the sections were treated for 45 minutes at room temperature with the secondary antibody (horse-anti-mouse biotinylated IgG[d]), at a concentration of 1:125 in PBS. Visualization of the immune reaction was then performed using an ABC/peroxidase kit[d], and diaminobenzidine[e] as a substrate (0.05% solution in 0.05 M Tris-HCl, pH 7.8, with 0.003% H_2O_2). After a final rinse in distilled water, the sections were briefly counterstained (15 seconds in hematoxylin) and dehydrated (successive concentrations of 70%, 96%, 100% ethanol and finally xylene). The specificity of the primary antibody was tested by performing a Western blot on canine PCA cells and obtaining a single stained band just lower than the 75 kDa marker, which corresponds with the molecular weight of COX-2 (72 kDa). Sections of normal canine bladder were used as negative control and sections of canine transitional carcinoma of the bladder were used as negative and positive control, respectively.

Classification of histological subtypes

The tumors were classified according to criteria described by Leav and Ling.[13] The different histological subtypes described and observed were ductal, micropapillary, sarcomatoid, small acinar and solid.

Immunohistochemistry scoring

The slides were evaluated with regard to the number of stained cells, the intensity of the staining and the presence of inflammation. The number of stained tumor cells was estimated and scored as 0 (0% positive cells), 1 (>0-20% positive cells), 2 (>20-40% positive cells), 3 (>40-60% positive cells), 4 (>60-80% positive cells) and 5 (>80-100% positive cells). Intensity was scored as 0 (no staining), 1 (mild staining), 2 (intermediate staining) and 3 (strong staining). Inflammation was scored as 0 (absent), 1 (mild), 2 (intermediate) and 3 (marked). In addition, a staining index was calculated by multiplying the score for the number of positive cells (0 to 5) by the score for staining intensity (0 to 3), as previously described [4,23]. Different areas of the same slide were scored separately if they differed in histological subtype or degree of inflammation.

Cell culture

The non-malignant canine prostate cell line CAPE[24,25] was a generous gift of Dr. Tom Rosol (Colombus, OH) and the malignant cell line ACE was obtained from Dr. Helen Jones (Cardiff, UK). Both cell lines were maintained at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium[e] (DMEM) with the addition of 5% fetal calf serum[f] (FCS).

Twenty-four hours before the experiments, the culture medium was replaced with DMEM without FCS. Then the cells were incubated for 24 hours with putative activators of COX-2 (PMA[g], 10 ng/ml, IL-6 [g], 10 ng/ml, EGF[g], 10 ng/ml and TNFα[g], 50 ng/ml), either alone or in combination with several specific signal transduction pathway inhibitors (PD98059[g], GF109203X[h] and LY294002[h], at a concentration of 10 μM). After 24hours of incubation, the cells were washed with Hanks' balanced salt solution[e] (HBSS) and harvested by scraping them from the culture flask and suspending them in approximately 0.5 ml HBSS. The cells were spun down and stored without buffer at –80 °C until further processing. All experiments were performed in duplicate.

Measurement of protein content

Cells were lysed with lysis buffer containing 1% igepal[e], 0.5% sodium deoxycholate[e] and 0.1% sodium dodecyl sulfate[b] (SDS) in phosphate buffered saline (PBS). Samples were incubated for 30 minutes on ice, and centrifuged at 4 °C for 3 minutes at 1000 rpm. The supernatant was carefully removed and stored at –20 °C until further use. The protein content of the samples was determined using a protein assay kit[k] and serial dilutions of bovine serum albumin[e] (BSA) as a standard.

Western blot

Five μg protein from each sample was suspended in a buffer containing 20% glycerol[b], 2.5% SDS and 0.5% brome phenol blue[b] in 0.125 M Tris-HCl[b] buffer at pH 6.8, and separated by electrophoresis on ready-made 10% Tris-HCl gels[k]. Then, the gels were equilibrated for 30 minutes in transfer buffer (25 mM Tris, 192 mM glycine[e], 20% methanol[l]), before the protein was transferred from the gels to nitrocellulose membranes[m]. These blots were then washed in Tris-

Table 1. Distribution of histological types among castrated and intact males

	Castrated	Intact	Total
No. of dogs	21	7	28
No. of dogs with 1 histological type	7	2	9
No. of dogs with several histological types	14	5	19
Total no. of histologically distinct areas:	38	12	50
Subtypes:			
Solid	21	3	24
Micropapillar	13	7	20
Ductal	2	1	3
Small acinar	1	1	2
Sarcomatoid	1	0	1

buffered saline (TBS) and blocked for 1 hour in 3% non-fat dry milk[b] in TBS at room temperature. They were then incubated for 1 hour at room temperature with the primary antibody (Mouse anti-COX-2) at a concentration of 1:1000 in TBS containing 0.1% Tween-20[n] (TBST). The blots were washed in TBST and incubated for 1 hour at room temperature with the secondary horse radish peroxidase (HRP) linked anti-mouse antibody[o] at a concentration of 1:20,000. The blots were washed in TBST and TBS, and stained using a chemiluminescent substrate kit[p] before being exposed to an x-ray film for 30 seconds.

Statistical methods

Where appropriate, differences in immunohistochemical scoring were analyzed using the non-parametric Mann-Whitney U test. Statistical significance was accepted for $P < 0.05$.

Results

COX-2 expression in prostate carcinoma tissue

Nineteen of the 28 dogs examined (68%) had more than one histological subtype identified in the sections investigated. A total of 50 distinct tumor areas were identified in the 28 prostates examined (i.e. areas with different histological subtypes or different degrees of inflammation). The distribution of histological types between intact and castrated dogs is summarized in Table 1. In both castrated and intact dogs, the micropapillary and the solid tumor subtypes were the most frequently seen. Both subtypes together represented 88% (44/50) of the different tumor areas (micropapillary 40% [20/50] and solid 48% [24/50]). The ductal, small acinar and sarcomatoid

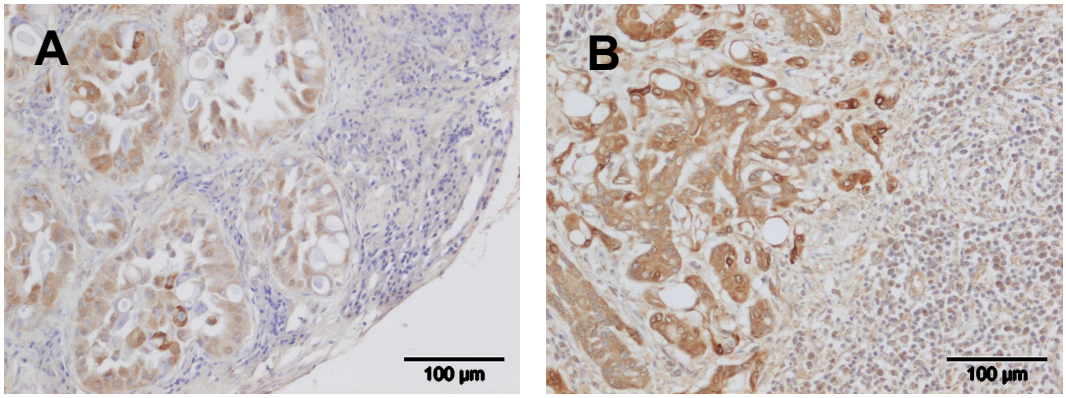

Figure 1
Prostate carcinoma: Immunoperoxidase-DAB for COX-2. Hematoxylin counterstain. (A) Micropapillary subtype with a moderate degree of inflammation. (B) Solid subtype with strong COX-2 staining.

subtypes were seen in 6% (3/50), 4% (2/50) and 2% (1/50) of the cases, respectively. The micropapillary type was seen in 34% (13/38) of the cases in castrated dogs and in 58% (7/12) of the cases in intact dogs. The solid type was seen in 55% (21/38) of the cases in castrated males and in 25% (3/12) of the cases in intact males. These differences between castrated and intact males were not statistically significant.

The immunohistochemistry scoring is summarized in Table 2. There was no statistically significant difference in the mean degree of inflammation between the micropapillary and solid tumor subtypes. Inflammation was present as frequently in tumors of castrated dogs as in those of intact dogs, however the degree of inflammation in intact dogs was significantly lower than in castrated dogs (P=.021).

Positive COX-2 staining (Fig 1) was typically seen as granules in the cytoplasm, often with an increased density around the nucleus of the cells. There was no staining of stromal tissue. Three of the 28 tumors (10.7%) stained negative for COX-2. Three of the 4 negative tumors were from castrated dogs and 1 from an intact male. Two were ductal, 1 micropapillary, and 1 solid. There was no significant difference in staining index among histological subtypes (P=.767). The median staining index was 7 for the micropapillary subtype, and 5.5 for the solid subtype. Although there was no significant difference in the number of positively stained cells or the intensity of staining between castrated and intact dogs, there was a lower but nonsignificant staining index in castrated dogs compared with intact dogs (P=.07), with a median staining index of 5 for castrated dogs and 8 for intact dogs.

The relationship between degree of inflammation and staining index is shown in Figure 2. The presence of inflammation (score 0 versus score 1, 2, or 3) was significantly associated with a lower staining index for COX-2 (P=.001) in both the micropapillary and solid histological subtypes. This was mainly due to a significantly lower number of positive cells (P=.04) rather than

Table 2. Summary of the immunohistochemistry scoring (mean ± SD)

	No. of Positive Cells	Intensity of Staining	Staining Index	Degree of Inflammation
Castrated males	2.82 ± 1.75	2.21 ± 0.95	6.12 ± 4.11	1.73 ± 1.11
Intact males	3.60 ± 1.71	2.50 ± 0.71	9.20 ± 4.94	0.80 ± 0.79
(P value)[1]	(P=0.17)	(P=0.43)	(P=0.07)	(P=0.02)
Solid type	3.00 ± 1.83	2.25 ± 0.91	7.15 ± 4.93	1.65 ± 1.18
Micropapillary type	3.00 ± 1.72	2.29 ± 0.91	6.54 ± 4.08	1.42 ± 1.06
(P value)[a]	(P=0.92)	(P=0.85)	(P=0.77)	(P=0.46)
Ductal type[b]	N/A	N/A	N/A	N/A
Small acinar type[b]				
Sarcomatoid type[b]				
No inflammation	4.36 ± 0.81	2.45 ± 0.69	10.82 ± 4.02	
Inflammation score 1, 2 or 3	2.55 ± 1.75	2.21 ± 0.96	5.48 ± 3.77	
(P value)[a]	(P=0.004)	(P=0.58)	(P=0.001)	

[a]Using the non-parametric Mann-Whitney test (statistical significance accepted for P≤.05)
[b]Not included in the statistical evaluation because of the small number of cases

to a lower intensity of staining (P=.630). However, the degree of inflammation (scores 1 to 3) was not significantly associated with different staining indices. In general, when present, inflammatory infiltrates were homogeneously distributed throughout the entire tissue section on the slides.

COX-2 expression in cultured cells

Results are shown in Figures 3 and 4. Baseline expression of COX-2 was barely detectable in the nonneoplastic CAPE cells, but was marked in the neoplastic ACE cells. This baseline COX-2 expression could be completely inhibited by blocking PI3K, PKC or ERK/MAPK in the ACE cells. In the CAPE cells, inhibition of ERK/MAPK pathway by PD98059 induced COX-2 expression to clearly higher levels than baseline.

TNF-α and IL-6 had similar effects. Both cytokines induced higher COX-2 expression in the CAPE cells, compared with baseline. This induction was blocked by inhibition of the PI3K and PKC pathways. Blockage of the ERK/MAPK pathway did not decrease but rather enhanced the inductive effect of TNF-α and IL-6 in CAPE cells. Conversely, in the ACE cells, TNF-α and IL-6 both caused a slight reduction in COX-2 expression. COX-2 expression was further reduced by blocking the PI3K pathway. Blocking the PKC and the ERK/MAPK left COX-2 expression

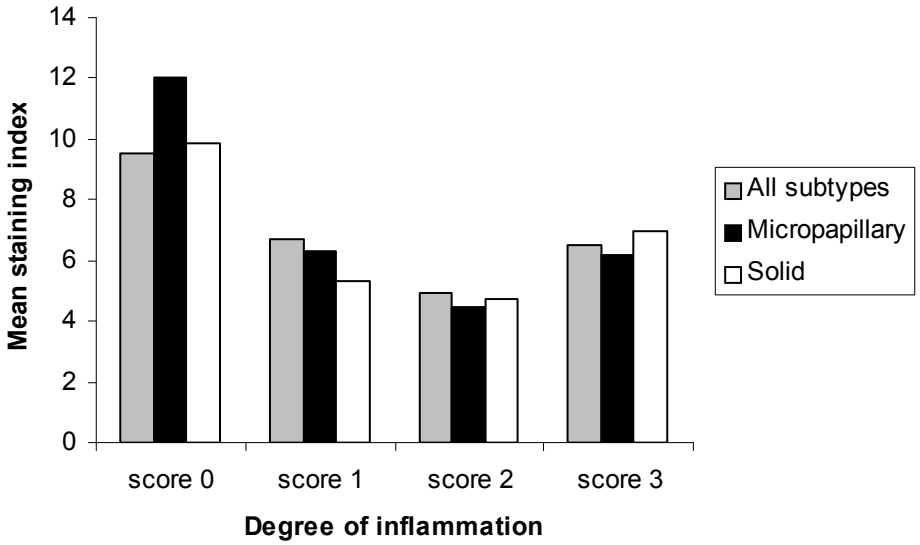

Figure 2.
Relationship between degree of inflammation and staining index for all PCA samples and for the 2 most common histologic subtypes (micropapillary and solid).

unchanged compared with incubation with TNF-α or IL-6 alone.

Incubation of cell cultures with PMA increased COX-2 expression in both the nonneoplastic CAPE cell line and the neoplastic ACE cell line. In the CAPE cell line, this inductive effect of PMA could not be blocked by inhibition of any of the pathways. In the neoplastic ACE cells, blockage of the PKC pathway caused almost complete inhibition of COX-2 expression. Blocking other pathways did not alter induction of COX-2 expression by PMA.

EGF caused an induction of COX-2 expression in CAPE cells similar to that caused by TNF-α and IL-6. This induction was only partially reduced by blocking the PI3K and PKC pathways. In the ACE cells, EGF caused no induction of COX-2 expression compared with control cells but partially reversed inhibition of COX-2 expression by inhibitors of the PI3K and ERK/MAPK pathways and completely reversed blockage by inhibitors of the PKC pathway.

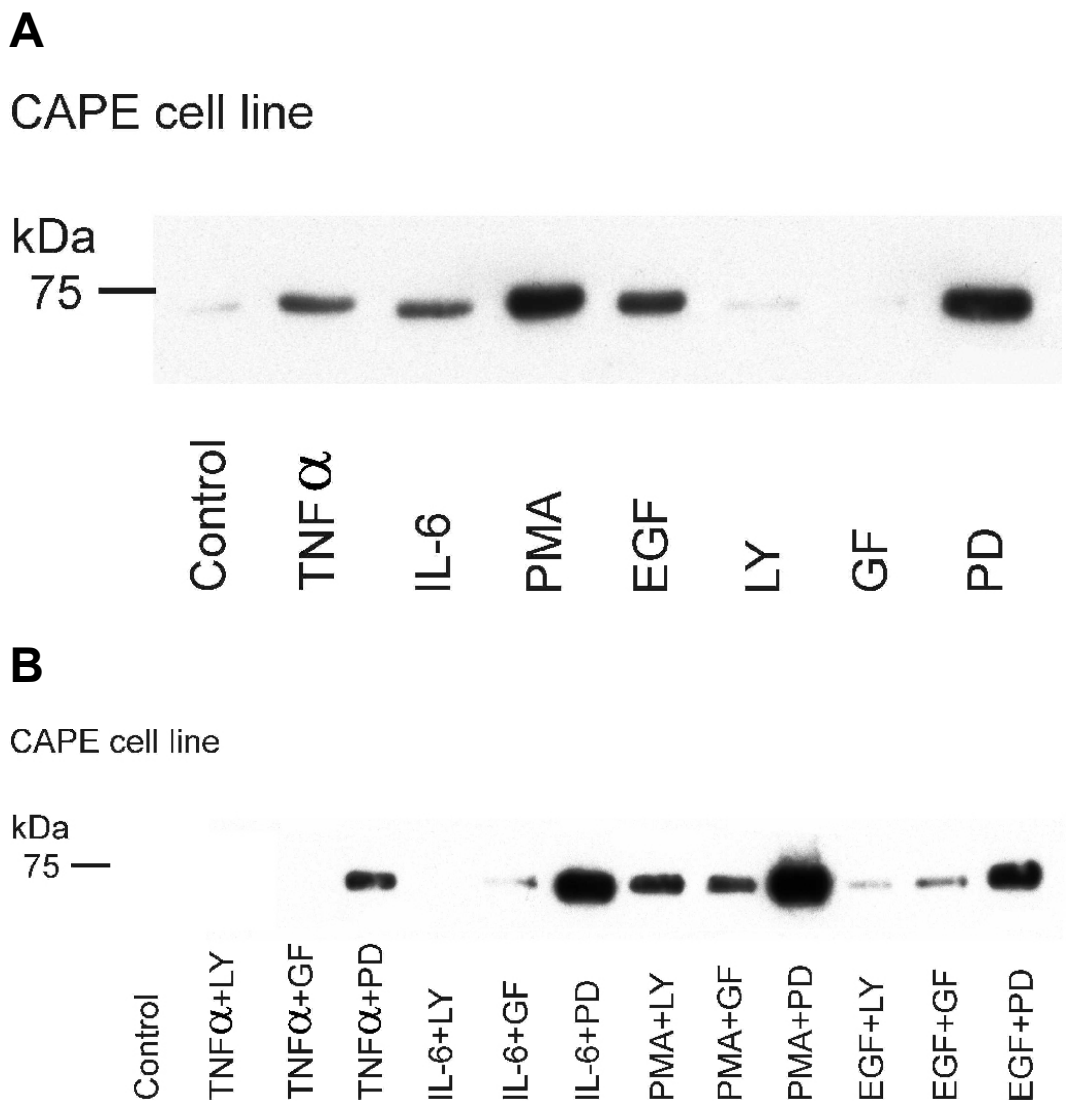

Figure 3.
Western blot showing COX-2 expression in the nonneoplastic CAPE cell line after incubation with various combinations of stimulators and cell signaling pathway inhibitors. Stimulators: TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; PMA, tumor promoter phorbol 12-myristate 13-acetate; EGF, epithelial growth factor. Inhibitors: PD98059, inhibitor of extracellular signal-related kinase (ERK/MAPK); GF109203X, inhibitor of protein kinase C (PKC); LY294002, inhibitor of phosphatidyl inositol-3 kinase (PI3K).

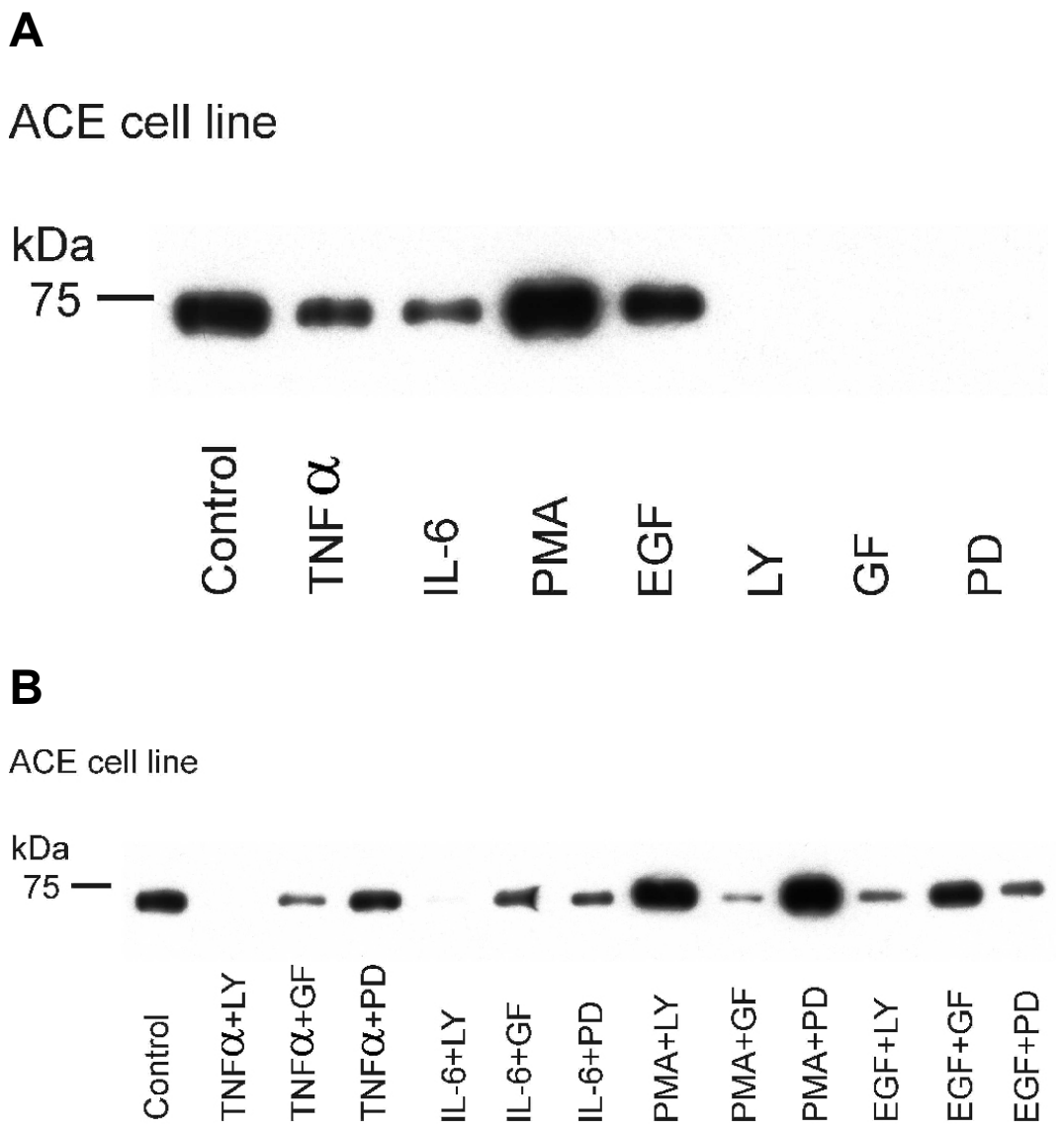

Figure 4.
Western blot showing COX-2 expression in the neoplastic ACE cell line after incubation with various combinations of stimulators and cell signaling pathway inhibitors. Stimulators: TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; PMA, tumor promoter phorbol 12-myristate 13-acetate; EGF, epithelial growth factor. Inhibitors: PD98059, inhibitor of extracellular signal-related kinase (ERK/MAPK); GF109203X, inhibitor of protein kinase C (PKC); LY294002, inhibitor of phosphatidyl inositol-3 kinase (PI3K).

Discussion

The proportion of dogs with positive staining for COX-2 (89.3%) is consistent with findings in other studies in dogs [4,9] and in humans [5,6,11], where 80-90% of prostatic tumors showed COX-2 expression. The classification of canine PCA into different histological subtypes and the finding in this study that many tumors consist of more than 1 morphological tumor subtype, confirm the earlier described heterogeneous character of PCA in dogs.[15,26] Two subtypes (micropapillary and solid) clearly dominated, whereas the other subtypes occurred far less frequently.

The micropapillary subtype is characterized by tumor cells within the original glandular acini and an intact basement membrane, whereas the solid tumor type is characterized by tumor invasion and breach of the basement membrane and normal acinar structure, and therefore can be considered less well differentiated. There was a tendency in this study towards a higher proportion of less-differentiated tumor subtypes (solid type) in castrated dogs, compared with intact males. This finding is consistent with observations in humans, where androgen ablation often leads to the development of less well-differentiated PCA (higher Gleason grades).[27] Also consistent with findings in humans was the tendency towards lower COX-2 expression in PCA in castrated dogs compared with intact males. A similar infiltration of inflammatory cells has also been observed in humans as a consequence of androgen ablation therapy.[28] However, the COX-2 staining indices found in this study did not correspond to what would be expected based on the reported COX-2 expression in human PCA. The COX-2 expression found in the canine prostatic tumors examined in this study did not vary with the degree of differentiation of the tumors; whereas in humans, intensity of COX-2 staining has been found to be higher in less-differentiated tumors than in well-differentiated tumors.[11] Furthermore, canine tumors without inflammation had a significantly higher staining index than did tumors with inflammation, although no relationship between the degree of inflammation in these tumors and COX-2 staining could be detected. In humans, COX-2 expression correlates with the presence of local chronic inflammation[12], and it is even hypothesized that inflammation may initiate and promote prostate cancer.[29] Findings in this study suggest, therefore, that factors other than inflammation are responsible for the induction in COX-2 expression in canine prostate cancer.

In vitro, a clear difference was found between neoplastic and nonneoplastic prostate cells. Evidence of a much higher COX-2 expression in neoplastic ACE cells than in nonneoplastic CAPE cells reflect the in vivo situation, where COX-2 expression is seen in neoplastic prostatic tissue but not in normal prostate tissue.[4,9,30]

Cytokines TNF-α and IL-6 and the growth factor EGF all caused induction of COX-2 expression in the nonneoplastic CAPE cells, confirming earlier findings by others that COX-2 may be induced by a variety of cytokines and growth factors.[31-33] PD98059, an inhibitor of the ERK/MAPK pathway, also induced COX-2 expression in the CAPE cells. This finding suggests that the ERK/MAPK pathway may be important in suppressing COX-2 expression in prostate cells

of nonneoplastic origin. Blockage of the PI3K and PKC pathways completely suppressed induction of COX-2 expression caused by the cytokines TNF-α and IL-6 and greatly reduced the induction caused by EGF, indicating that both pathways are necessary for induction of COX-2 by these cytokines and growth factors.

In the neoplastic ACE cells, TNFα and IL-6 slightly decreased COX-2 expression compared with baseline value. This finding correlates with the reduced COX-2 staining found in the presence of inflammation in the canine prostate carcinomas examined in this study, because TNF-α and IL-6 are important inflammatory cytokines. In addition, the findings reveal that endogenous COX-2 expression in the ACE cell line is dependent on all investigated pathways (PI3K, PKC and ERK/MAPK) functioning, because blockage of either one of these signal transduction molecules caused a significant inhibition of COX-2 expression to non-detectable concentrations.

In the presence of exogenous TNF-α and IL-6, however, COX-2 expression in the ACE cell line is maintained despite blockage of the PKC and ERK/MAPK pathways. The PKC pathway therefore seems to be involved in the regulation of COX-2 by TNF-α, IL-6 and EGF in normal prostate cells but not in neoplastic cells.

COX-2 expression stimulation of the ACE cells by EGF is partially blocked by inhibition of either PI3K or ERK/MAPK. Thus, while in the nonneoplastic cell line, COX-2 expression is increased by blocking ERK/MAPK, COX-2 expression in the neoplastic cell line is increased by stimulating this kinase.

The phorbol ester PMA was a strong inducer of COX-2 expression in this study. This confirms findings by others; PMA is known to activate the PKC signaling pathway.[17]

The results of the present study. Taken together, reveal that COX-2 is expressed in canine prostate carcinoma and is inversely correlated to the presence of inflammation in these tumors. This finding is confirmed in the in vitro study. Although COX-2 expression in nonneoplastic cells may be induced by cytokines and growth factors, inflammation (in vivo) and cytokines (in vitro) tend to decrease the already high baseline COX-2 expression found in neoplastic PCA cells. Although stimulation of PI3K and PKC lead to COX-2 in nonneoplastic prostate cells, only PI3K preserved this function in neoplastic cells. Moreover, while inhibition of ERK/MAPK in nonneoplastic cells induces COX-2 expression, inhibition of the same protein in neoplastic cells exerts a similar effect. These results point to changed signal transduction pathways in PCA leading to increased COX-2 expression. Additional studies are required to fully understand the mechanisms regulating COX-2 expression in canine prostate carcinoma.

Footnotes

[a] Superfrost slides, Menzel Glaser, Braunsweig, Germany

[b] xylene, alcohol, citrate buffer, hydrogen peroxide, methanol, SDS, glycerol, bromo phenol blue, Tris-HCL buffer, nonfat dry milk; Merck, Darmstadt, Germany

[c] Monoclonal mouse anti-COX-2, Zymed Laboratories Inc, San Francisco, CA

[d] Horse anti-mouse biotinylated IgG and ABC/peroxidase kit, Vector Laboratories, Burlingame, CA
[e] Diaminobenzidine, igepal, deoxycholate, BSA, glycine; Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[f] DMEM, FCS, HBSS; Gibco Invirogen, Paisley, Scotland, UK
[g] Putative activators of COX-2 (PMA, EGF, TNFα); Sigma Chemical Company, St Louis MO
[h] PD98059, LY294002; Calbiochem, La Jolla, CA
[i] GF109203X, Biomol, Plymouth Meeting, PA
[j] DC Protein Assay, BioRad Laboratories, Hercules, CA
[k] Tris-HCL gels, BioRad Laboratories, Hercules, CA
[l] Methanol, Labscan Ltd, Dublin, Ireland
[m] Nitrocellulose membranes, Hybond C, Amersham Biosciences, Amersham, UK
[n] TBST, Boom, Meppel, The Netherlands
[o] Anti-mouse IgG, R&D Systems, Minneapolis, MN
[p] Immune-star HRP kit, Biorad Laboratories, Hercules, CA

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CHAPTER 7

Summarizing Discussion

Human and dogs share several similarities in their prostate disorders. The dog has therefore generally been proposed as a suitable animal model for the study of human prostate diseases. The aim of this thesis was to evaluate the dog as a suitable animal model for human prostate cancer by obtaining basic information of spontaneous canine prostate cancers, validating the cellular origin of canine prostate cancer and comparing the results with the current knowledge of human prostate cancer development.

In humans, the intermediate/transiently proliferating type of cells of the prostatic acini have been postulated to constitute the cellular origin of prostate cancer[65, 66]. In dogs however, the prostatic ductal cells have been hypothesized as the cellular origin of prostate cancer[37]. To clarify this issue, we first investigated and described the canine hierarchical system of cell differentiation of the normal prostate epithelium (Chapter 2). We then classified the different growth patterns of canine prostate cancer (cPC) and characterized the differentiation status of the cPC cells with various differentiation markers by immunohistochemistry (Chapter 3).

Another important aspect of human prostate cancer is that about 70% of the patients respond to androgen withdrawal therapy favorably[48], only to become therapy unresponsive some time after the androgen withdrawal. Thus, about thirty percent of the human patients do not respond to this therapy, and strikingly, none of the canine patients benefits from castration. In humans, DNA sequence mutations, expression and regulation changes of the androgen receptor (AR) are among the causes of androgen unresponsiveness[4, 15, 18]. In addition, men with a decreased size of a CAG repeat within the AR gene are more prone to developing prostate cancer than others[24, 61, 68]. We therefore investigated the contribution of the androgen receptor to the development of canine prostate cancer by evaluating AR expression and AR sequence analysis in canine prostate cancer specimens (Chapter 4). In order to further address the contribution of the AR to the development of canine prostate cancer, we investigated the influence of a similar canine AR CAG repeat on the incidence of canine prostate cancer (Chapter 5).

Many studies report the importance of chronic inflammation as the cause of prostate cancer initiation and progression and the expression of the inflammatory mediator COX-2 has been documented in both human and canine prostate cancer [7, 28, 32, 40, 45, 50, 56, 60, 63]. We evaluated whether COX-2 expression in cPC is related to the tumour histology and to the presence of inflammation (Chapter 6).

Normal organization of the prostatic acinar-, ductal- and urothelial epithelium

In intact dogs, PSA expression was seen in the luminal cells of the prostatic acini, the periurethral ducts, and the prostatic urethra (Chapter 2). PSMA expression was not found in prostatic cells, but we found a clear positive PSMA reaction in discrete epithelial cells of the prostatic urethra in the

normal intact dog, despite earlier reports that denied the existence of PSMA in the dog[1]. UPIII expression was only observed in the apical surface of the prostatic urethra. CK7 expression was largely comparable to this UPIII expression but in some dogs it could also be demonstrated in luminal acinar cells. CK14 expression was found scarcely, only in the basal cells of the prostatic urethra in the intact dog, whereas a positive staining of HMWCK and CK5 was observed discontinuously in the basal cells of few peripheral acini, and continuously in the periurethral ducts and the prostatic urethra. A strong CK18 expression was seen in all luminal cells of the whole gland.

The observed expression patterns of the various cell markers in the canine prostate are only slightly different from what is seen in humans. In human prostate, PSA staining is specific to the luminal cells of prostatic acini[43, 47]. A positive PSMA reaction is, similar to our results in the dog prostate, also not found in normal human prostatic cells[46, 59], whereas recent studies have demonstrated a moderate PSMA expression in the normal human urothelium as well[12]. The UPIII and CK7 staining patterns shown in our studies were generally similar to those seen in the normal human prostate [43, 46]. However, different from the expression in the human prostate[69, 70], CK14 expression was much less abundant in the basal layer of the dog prostate. The expression patterns of the other cell differentiation markers (CK5, CK18, HMWCK) in the dog prostate were similar to what is seen in the human prostate, except that HMWCK and CK5 expression in basal cells is discontinuous in the dog while continuous in the human prostate[69, 70].

A stem cell driven hierarchical arrangement of the epithelium has been postulated in the human prostate[26]. In this hypothesis of human stem cell driven prostate development, the basal cells of the prostatic acini represent the progenitor cells of the fully differentiated secretory cells. A similar scheme of cellular differentiation of the prostate epithelium may be applied to the dog prostate. Like the human prostate, the canine prostate epithelium is morphologically composed of two cell layers: a basal and a luminal layer. The luminal layer is dominated by fully differentiated secretory cells while basal cells occupy the major part of the basal layer. Immunohistologically, we discerned a similar type of intermediate/transiently proliferating cells as found in the human prostate, that co-express both basal and luminal cell markers (HMWCK/CK5 and CK18).
Taken together, the canine and human prostate epithelium appear to be similarly organized, with the canine prostatic epithelium displaying a more differentiated character; the less differentiated cells are localized more towards the ductal/urothelial sites and less migrated towards the acini, compared to their human counterpart.

After castration, a positive staining for PSA was still observed in the remaining tubular structures, while PSMA positive cells were present in both the urethra and the tubules of atrophic prostates. UPIII staining was also not only found in the urethra but in the single lining of tubular cells in the peri-urethral area as well. Besides in the urethra, CK7 positive staining was seen in the tubules at the peri-urethral area and the peripheral acini. CK14 staining was found in the scattered cells of the tubules at the periphery of one atrophic prostate but not in the urethra. The HMWCK and CK5 staining patterns were similar to the CK7 staining patterns. Furthermore, the absolute number of HMWCK and CK5 positive cells per cross section in the atrophied prostates appeared

to be increased compared to their number in the intact dogs. CK18 was seen less obvious than in the un-castrated dogs and was only expressed in the single or luminal lining of tubular cells in the prostates of the castrated dogs.

The canine prostate has a rather differentiated character with a dominance of prostatic acini and a lack of stromal tissue. Castration causes a great change in this morphology with an obvious relative increase of stroma. The increasing number of HMWCK and CK5 positive cells in the atrophied prostates of castrated dogs may either indicate an accumulation of intermediate/transiently proliferating cells that are not capable of differentiating further to secretory cells, or an active regeneration of the epithelium, trying to restore its original number of secretory cells. The increase of basal and intermediate type cells in the prostate of castrated dog suggests that castration leads to an increase of the intermediate cell type and a less differentiated composition of the prostatic epithelium. Furthermore, our results imply that the remaining atrophic tubules in the prostate of castrated dogs are not so much repopulated by cells from the pre-existing acini, but by cells originating from the ducts. The remaining tubules in the peri-urethral area are in turn populated by cells with a urethral phenotype.

The origin of canine prostate cancer

A ductal origin of canine prostate cancer?

Prostate cancer is sometimes difficult to discriminate from transitional cell carcinoma growing into the prostate[14, 38, 41, 42]. For this, cell type specific expression markers are used to discriminate both types of cancer. PSA is the most common used marker in humans to identify prostate cancer [43, 59]. Despite a moderate PSMA expression in normal human urothelium and in endothelial cells of tumour-associated neovasculature in several solid cancers[12], PSMA has been used to identify cells of prostatic origin[27, 30, 54]. Both UPIII and CK7 are used to identify transitional cell carcinomas and cells of urothelial origin in the human prostate[43, 46].

We applied the same panel of markers to study canine prostate cancer. About half of the investigated cPC only weakly expressed PSA in our study (Chapter 3). In addition, only half of the canine prostate tumours were positive for PSMA. Although we found a significant lower number of cells expressing UPIII and CK7 in the solid patterns compared to the micropapillary and cribriform patterns (Chapter 3), the vast majority of tumours in the dog express UPIII and CK7.

In the normal prostate, ductal cells express CK7 (Chapter 2). In normal prostates of castrated animals, the vast amount of acinar cells disappears, and the basal membranes of the remaining tubules are occupied by cells expressing CK7 and, to a lesser extend UPIII. Interestingly, the incidence of diagnosed prostate cancer is increased in castrated animals. Taken these characteristics together, a ductal origin of canine prostate cancer is likely, which underlines an earlier suggestion by Leav and coworkers[37]. However, based on our results, an urothelial origin of canine prostate cancer can not be excluded.

Are intermediate/transiently proliferating cells the origin of canine prostate cancer?

Our study on the pathomorphological and immunohistochemical characterization of canine prostate cancer revealed that all growth patterns of cPC express CK18 (Chapter 3). This indicates a predominance of differentiated cell types and parallels the observations in human prostate carcinoma[49]. Different from human prostate cancer we frequently found the expression of HMWCK, CK5 and CK14, indicators of prostatic intermediate/transiently proliferating and basal cell types respectively, in cPC, especially in the tumours of castrated animals (Chapter 3).

In humans, van Leenders et al [64] was never able to find expression of CK14 in prostate cancer specimens; not in early prostate cancer, advanced prostate cancer or prostate cancer cell lines. The least differentiated cell type they were able to detect were sparse CK5 expressing cells. Since, in their line of thought, the least differentiated cell type are the cells of origin of prostate cancer, they postulated the intermediate/transiently proliferating cells the cells of origin of human prostate cancer. Following a similar reasoning as van Leenders et al, we hypothesize that early basal cells are the cells of origin of canine prostate cancer; the least differentiated cells found in our canine prostate cancer specimens are CK14 expressing cells. Since CK14 expressing basal cells are only found in the urothelium of the canine prostate, we further hypothesize that basal cells of the prostatic urethra/ducts are at the origin of canine prostate cancer.

Prostate cancer growth patterns in humans and dogs.

Canine prostate cancer is a very aggressive tumour. The cancer lesion usually occupies the whole prostate, the epithelial tumour cells are often not confined within the natural boundary of the basal membrane, and the cancer cells often invade the prostate capsule and surrounding tissues. Although there is currently no Gleason like grading system to score the aggressiveness of canine prostate cancer, the majority of clinically diagnosed cPC cases would certainly be graded as highly malignant when compared to human PC.

In our study, we observed a remarkable variation in the growth patterns of canine prostate cancer, both between and within the individual cases. Six growth patterns were distinguished: micropapillary, cribriform, solid, sarcomatoid, small acinar/ductal, and tubulo-papillary. A heterogeneous morphology was often seen even in individual specimens, both in terms of the number of combinations of growth patterns per tumour and in terms of cellular morphology. Castration leads to an increased appearance of less differentiated growth patterns in canine prostate cancer. In our study, solid growth patterns were significantly ($P=0.027$) more often seen in castrated dogs than in intact dogs. This resembles the observation of Cornell et al, that well differentiated adenocarcinoma is seen more often in un-castrated dogs than in castrated dogs[6].

In humans, the majority (up to 95%) of prostate cancer is of the adenocarcinoma type, mainly characterized by an acinar differentiation. However, variants like sarcomatoid carcinoma,

adenosquamous carcinoma, ductal carcinoma, and transitional cell carcinoma are also observed[53]. Next to these well differentiated carcinomas, poorly differentiated carcinomas are recognized. Differences in pathomorphological appearance of androgen-sensitive and androgen-refractory prostate cancer have also been reported in human prostate cancer[22, 23, 34]. Kondo et al[34] classified the glandular (including large and/or small simple glands), micro-glandular, and cribriform growth patterns as androgen sensitive, while medullary-solid and columnar-trabecular patterns, occasionally with fused cells, were classified as androgen refractory. They also reported that metastases tend to have an androgen refractory appearance. In addition, Shah and co-workers[57] concluded that androgen-independent prostate cancer is a heterogeneous group of diseases. The majority shows a mixture of Gleason grades 4 and 5, including growth in solid sheets and nests with or without comedonecrosis, and confluent cribriform glandular patterns. This mixture of growth patterns seen in human androgen refractory prostate cancer does resemble the mixture of growth patterns we have observed in prostate cancer in the dog.

As mentioned, the pathological morphology of cPC shows more often mixed patterns, resembling human androgen refractory PC. The mixed patterns were not only seen in castrated dogs but also in intact dogs although cPC in intact dogs appeared relatively more often differentiated (Chapter 3). The serum PSA screening for hPC increases the chance to discover the potential patient in an early stage, in which the prostate cancer may show a less aggressive and more differentiated morphology. This is a distinct difference with the dog where PC is mostly diagnosed in a late stage. Furthermore, the different cells of origin, which might be at a different stage of the hierarchical system, may explain the morphological difference between human and dog. And thirdly, the differences of the microenvironment within the human and canine prostate might also contribute to this difference in tumour differentiation. Histologically, the ratio between stroma and parenchyma differs between man and dog, indicating differences in matrix components. A difference of any molecule involved in the pathways of proliferation and differentiation can contribute to the difference between the two species. So, a different expression of growth factors, cytokines, or enzymes involved in the epithelial-stromal interaction, and often originating from the stroma, might influence the process of tumour growth.

The role of AR in the development of canine prostate cancer

Androgens play a critical role in regulating the growth, differentiation and survival of epithelial cells in the normal prostate[25, 52, 55]. Castration leads to a massive loss of secretory cells but does not affect the basal cells[5, 11, 35], which suggests that the survival of secretory cells is dependent on androgens, either directly or indirectly through the interaction of androgens with the surrounding environment. In humans, not only normal prostate cells depend on androgen for survival, but also cancer cells may behave like this[2, 16]. Patients with hPC often respond favourably to androgen ab-

lation in the early stage. However, subsequent to a successful androgen ablation treatment prostate cancer often relapses and progresses to a more aggressive, hormone-refractory type of tumour [48]. The mechanisms involved in the development of resistance of the tumour cells to hormonal ablation therapy are poorly understood.

Canine prostate cancer is not characterized by an early phase of androgen withdrawal responsiveness: it is well known that castration does not lead to better outcomes of canine patients with prostate cancer. This does however, not mean that androgens and the androgen receptor do not play a role in canine prostate cancer development. Non-castrated dogs for example have a lower risk of developing PC than castrated dogs[36, 62]. In addition, we found that PC in castrated dogs more often has a poorly differentiated histology. Finally, in previous studies we observed that although the AR was expressed in 80% of the cPC cases, a shift from a nuclear to a cytoplasmic localization was observed in tumour tissues. Since the presence of androgens is necessary for both stabilizing the AR dimer and its translocation to the nucleus, the absence of androgens in castrated animals may lead to such a cytoplasmic staining. However, we also observed a predominantly cytoplasmic staining in the tumours of intact animals, in spite of the presence of androgens. This may point to a disruption of the androgen-AR signalling pathway. Mutations in the AR gene may render the AR unable to enter the nucleus. We therefore examined whether mutations in the genomic sequence of the AR DNA-binding region caused the expression of the AR to switch from the nucleus to the cytoplasm in the prostate cancer cell of the dogs. Our sequencing results showed no mutation in this DNA-binding region (Chapter 4), but other regions of the AR gene such as the hinge region are also involved in its nuclear translocation. Mutations in the hinge region have been reported that may greatly inhibit nuclear translocation and DNA binding[18].

We also examined a possible association between prostate cancer risk and CAG repeat length of a short tandem repeat in the transcriptional region of the AR. The allelic distribution of CAG-1 appeared to be significantly different between dogs with PC and the normal dog population (Chapter 5). Similar to humans[3, 24, 44, 58], shorter CAG-1 repeats were found more often in the canine PC group, with an overall lack of the longest length CAG-1. In humans, shorter CAG repeats in exon-1 of the AR gene have been suggested to play a causal role in PC development[24] because they encode an AR with a higher transcriptional activity[13]. However, all canine AR CAG-1 repeat lengths are short when compared to the human length of 11 - 31 repeats in this allele[10]; a possible increased transcriptional activity for the short canine CAG-1 repeat remains to be investigated.

The role of inflammation in prostate carcinogenesis

An elevated expression of the enzyme prostaglandin-endoperoxide synthase, or cyclooxygenase-2 (COX-2), has been detected in several forms of human and canine cancer[9, 31, 32, 60, 63]. In addition,

inhibition of COX-2 suppresses tumour growth in several animal models of carcinogenesis[8, 17, 67]. There is a growing body of studies that show that COX-2 and its product prostaglandin E2 (PGE2) promote tumour development by a variety of mechanisms, such as an increase in proliferation, a decrease in apoptosis and an induction of angiogenesis, possible by generating free radicals and carcinogens[29, 32]. The induction of COX-2 expression in malignant cells is mostly due to activation of oncogenes, such as p53, and inactivation of tumour suppressor genes, such as HER-2/neu. Subsequent activation of signal transducers, such as MAPKs and pAkt, and dysregulation of mRNA stability factors may also contribute to this induction.

We investigated the expression and regulation of COX-2 in canine prostate cancer (Chapter 6). In this study, we found that COX-2 is not expressed in the normal prostate tissue. However, COX-2 expression is induced in canine prostate cancer. To shed light on the mechanisms leading to this COX-2 expression, we investigated the involvement of inflammatory pathways. The inflammatory cytokines TNF-α and IL-6 and the growth factor EGF all indeed caused induction of COX-2 expression in the non-neoplastic CAPE cells, confirming earlier findings by others that COX-2 may be induced by a variety of cytokines and growth factors[19, 20, 21]. However, the presence of inflammatory infiltrates in canine tumour tissue was rather associated with lower COX-2 expression scores, which indicates that COX-2 expression in cPC is not related to the presence of inflammatory infiltrates. This was confirmed by in vitro experiments in which the earlier mentioned cytokines did not cause induction of COX-2 expression in neoplastic ACE and CPA cell lines, where COX-2 baseline expression was high. By using inhibitors which specifically blocked the PI3K, the PKC or the ERK/MAPK pathway, we found that the ERK/MAPK pathway is important in suppressing COX-2 expression in prostate cells of non-neoplastic origin, and the PI3K and PKC pathways are necessary for induction of COX-2 by the cytokines TNF-α and IL-6 and EGF. Moreover, while in the non-neoplastic cell line, COX-2 expression is increased by blocking ERK/MAPK, COX-2 expression in the neoplastic cell line is increased by stimulating this kinase.

Suggestions for further research

AR expression is mainly seen in the secretory cells of human prostates whereas basal cells are generally considered to be devoid of AR expression. However, our AR immunohistochemistry staining results showed scattered AR positive basal cells in the normal prostate (data not shown). Previous studies also reported that a low AR mRNA expression was found in a subset of human basal cells and that the AR mRNA expression increased in medium containing fibroblast growth factor (FGF)-7 [51]. In addition, stromal cells induced the expression of the androgen receptor and PSA gene in CD57 positive basal cells[39]. As suggested earlier, cell differentiation of prostatic cells may be indirectly promoted by stromal cells. It is not clear yet whether these AR expressing basal cells are the same subset as the proliferative basal cells suggested by Leav et al [37] . To further elu-

cidate the role of the AR in the normal prostate and in PC, it will be worthwhile to elucidate the role of AR expressing basal cells in the homeostasis and tumorgenesis of the canine prostate. Do they proliferate in response to androgen stimulation? Are they progenitors of the rapidly growing intermediate/transiently proliferating cells? Do they play any role in the initiation and development of an androgen irresponsive clone in case of hormone refractory prostate cancer? More in general, the role of the epithelial-stromal interaction in the process of oncogenesis has traditionally been underrepresented in prostate carcinogenesis, and will be an interesting angle to disclose the mystery of prostate cancer oncogenesis.

To resolve the questions we just raised, we may first try to localize the basal cells co-expressing CK5 and AR with immunohistochemistry or in situ hybridization. By primary culture, the basal cells might be isolated and co-cultivated with matrix or prostatic stroma dissected from different conditions of prostate (young, old, intact, castrated, normal, cancer). By providing the growth factors, hormones, or antagonists which can block the signalling pathway, we might observe the impact of stroma on the epithelial proliferation and differentiation.

Furthermore, to elucidate the possible mechanisms of carcinogenesis of canine prostate cancer, we may look into the difference of epithelial-stromal interaction between young and old dogs, intact and castrated dogs, or normal and cancerous dogs. With the current powerful tools such as microdissection and gene expression microarrays, gene expression profiles of the epithelia and the stroma could be readily available. By comparing the difference of expression profiles between groups, an insight into the molecular change in the epithelia and stroma during the carcinogenesis might be obtained and a potential target for therapeutic intervention, or potential factors that can induce cell differentiation for the cells stuck in proliferation might be suggested.

Conclusions

The hierarchical system of prostatic epithelial cell differentiation is observed in dogs as well as in humans. The histopathological classification shows that canine prostate cancer appears to be more aggressive and of a less differentiated type than the most common human prostate cancer. From the immunohistochemical results we suggest that canine prostate cancer most likely originates from the collecting ducts. Our results also suggest that canine prostate cancer mostly resembles the androgen refractory, poorly differentiated prostate cancer of human. A predominant cytoplasmic AR staining was found in prostate cancer of both castrated and intact dogs. The cytoplasmic localization was not related to mutations in the DNA binding domain of the AR, which suggests that mechanisms that lead to an impaired androgen–AR signalling or a basal or stem cell like origin may explain the low cytoplasmic AR staining in cPC. Moreover, our study about the CAG repeats of AR gene suggest that short CAG-1 repeats are associated with an increased risk of developing prostate cancer in the dog. Although COX-2 expression was demonstrated in cPC, a potential role

for inflammation in the carcinogenesis of cPC could not be proven due to an inverse correlation between inflammation and COX-2 expression. This was further supported by the finding that the cytokines TNF-α and IL-6 and their involved signalling pathways do not stimulate COX-2 expression in malignant canine prostate cells.

In human patients with prostate cancer, most cancer deaths happen in the hormone refractory stage. Thus, an efficient treatment is urgently needed and for that purpose a suitable animal model would be very helpful. Apart from dogs, spontaneous benign prostate hyperplasia and prostate tumours are rare in animals. In rodents, prostate tumours are usually induced experimentally. Our results indicate that spontaneous canine prostate cancer resembles human late stage prostate cancer in several histological and molecular aspects. Hence, further research using spontaneous canine prostate cancer as a model for its human counterpart may generate beneficial results for both humans and dogs.

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CHAPTER 8

Samenvattende Bespreking

Mensen en honden hebben verscheidene overeenkomsten in de aandoeningen van hun prostaat. De hond is daarom vaak voorgesteld als een passend diermodel voor bestudering van menselijke prostaatziekten. Het doel van deze studie was te beoordelen of de hond een goed diermodel is voor het prostaatcarcinoom van de man (human prostate cancer, hPC) door basale informatie te vergaren over het prostaatcarcinoom van de hond (canine prostate cancer, cPC), door de cellulaire herkomst van het prostaatcarcinoom van de hond te bepalen en door de resultaten hiervan te vergelijken met de huidige kennis over de ontwikkeling van menselijke prostaatkanker.

Bij de mens is het intermediaire/tijdelijk vermenigvuldigende celtype uit de acini van de prostaat genoemd als de cel van herkomst van prostaatkanker, maar bij de hond worden de ductale cellen (cellen van de afvoerbuizen) verondersteld dat te zijn. Om meer helderheid op dit punt te krijgen onderzochten we het hiërarchische systeem van de cellulaire differentiatie van het normale prostaatepitheel (Hoofdstuk 2). Vervolgens klasseerden we de verschillende groeipatronen van prostaatkanker bij de hond en karakteriseerden we door middel van immunohistochemie de differentiatiestaat van de cPC cellen met verschillende differentiatie merkers (Hoofdstuk 3).

Ongeveer 70% van de patiënten met hPC reageren gunstig op het onttrekken of blokkeren van androgenen, maar responderen enige tijd na dat onttrekken niet meer op die therapie. Aanvankelijk reageert ongeveer 30% van de mannen dus niet op deze therapie, en merkwaardig genoeg, geen van de honden met cPC heeft voordeel van castratie. Oorzaken van het niet reageren van mannen op androgeen onttrekking zijn o.a. DNA mutaties van en veranderingen in expressie en regulering van de androgeen receptor (AR). Bovendien hebben mannen met een verkorte reeks van CAG herhalingen (een CAG repeat) in het AR gen meer kans hPC te ontwikkelen dan anderen. Daarom bestudeerden wij de bijdrage van de androgeen receptor aan de ontwikkeling van cPC door de expressie van AR te bepalen en de AR sequentie te analyseren in preparaten van cPC (hoofdstuk 4). Om de invloed van de AR op de ontwikkeling van cPC nog verder te bestuderen onderzochten we de invloed van een vergelijkbare CAG repeat op het voorkomen van cPC (Hoofdstuk 5).

In veel studies wordt het belang van chronische ontsteking als oorzaak van initiatie en progressie van prostaatkanker aangegeven en de expressie van de ontstekingsmediator COX-2 is beschreven in zowel hPC als cPC. Wij bepaalden of COX-2 expressie in cPC gerelateerd is aan de histologie van de tumor en aan de aanwezigheid van ontsteking (Hoofdstuk 6).

Normale organisatie van het acinaire, ductale en urotheliale epitheel van de prostaat

Bij intacte (niet gecastreerde) honden werd expressie van PSA (Prostate Specific Antigen) gezien in de luminale cellen van de acini, de ducten rondom de urethra en in de urethra in de prostaat

(Hoofdstuk 2). Expressie van PSMA (Prostate Specific Membrane Antigen) werd in prostaatcellen niet gevonden, maar we vonden een duidelijke PMSA reactie in verspreide epitheelcellen van de prostaaturethra van de normale intacte reu, ondanks eerdere publicaties die het voorkomen van PMSA bij honden ontkenden. UPIII (Uroplakin III) werd alleen waargenomen in het apicale oppervlak van de prostaaturethra. Expressie van CK7 (Cytokeratin7) was grotendeels vergelijkbaar met deze UPIII expressie, maar in sommige honden kon het ook worden aangetoond in de luminale acinaire cellen. CK14 (Cytokeratin14) expressie werd nauwelijks gevonden en dan nog alleen in de basale cellen van de prostaaturethra van de intacte reu. Aankleuring voor HMWCK (High Molecular Weight Cytokeratins (HMWCK: CK1, CK5, CK10, CK14) en CK5 (Cytokeratin5) was discontinu aanwezig in de basale cellen van enkele perifere acini en continu in de periurethrale ducten en de prostaaturethra. Een sterke CK18 (Cytokeratin18) expressie werd in alle luminale cellen van de hele klier gezien.

De waargenomen expressiepatronen van de verschillende celmerkers in de hondenprostaat verschillen slechts weinig van die van mannen. In de humane prostaat is PSA kleuring specifiek voor de luminale cellen van de acini. Een positieve PSMA reactie wordt, overeenkomend met onze resultaten bij de reu, niet gevonden in normale prostaat cellen van de man, terwijl recente studies ook een matige PMSA expressie hebben laten zien in het normale humane urotheel. De patronen van UPIII en CK7 kleuring in onze studies waren in het algemeen gelijk aan die in de normale humane prostaat. Maar de CK14 expressie in de basale laag van de prostaaturethra van de hond was, in tegenstelling tot de expressie in de humane prostaat, veel minder rijkelijk. De expressiepatronen van de andere celdifferentiatie-merkers (CK5, CK18, HMWCK) in de hondenprostaat waren gelijk aan wat gezien wordt in de humane prostaat, behalve dat de HMWCK en de CK5 expressie in de basale cellen discontinu is in de hondenprostaat en continu in de humane prostaat.

Er is gesteld dat de ordening van het epitheel in de humane prostaat is gestoeld op een hiërarchie die door de stamcellen wordt aangestuurd. In deze hypothese van een prostaatontwikkeling die door stamcellen wordt aangestuurd vertegenwoordigen de basale cellen van de prostaatacini de voorlopercellen van de volledig gedifferentieerde secretoire cellen. Een overeenkomstig differentiatieschema van de prostaatepitheelcellen kan worden toegepast op de hondenprostaat. Net als de humane prostaat is de hondenprostaat morfologisch opgebouwd uit twee cellagen: een basale en een luminale laag. De luminale laag wordt gedomineerd door volledig gedifferentieerde secretoire cellen terwijl de basale laag voor het grootste deel uit basale cellen bestaat. Immunohistochemisch konden we, net als in de humane prostaat, een type van intermediaire/tijdelijk prolifererende cellen onderkennen dat zowel basale als luminale celmerkers tot expressie brengt (HMWCK/CK5 en CK18).

Concluderend blijkt het prostaatepitheel van mens en hond op gelijke wijze te zijn georganiseerd, waarbij het prostaatepitheel van de hond een meer gedifferentieerd karakter vertoond; de minder gedifferentieerde cellen zijn meer richting de ducten en het urotheel te vinden en minder naar de acini getrokken, in vergelijking met de menselijke tegenhanger.

Na castratie werd nog steeds een kleuring voor PSA gezien in de overgebleven tubulaire

structuren, terwijl PSMA positieve cellen zowel in de urethra als in de tubuli van de atrofische prostaten aanwezig waren. UPIII werd ook niet alleen in de urethra gevonden maar ook in de enkelvoudige bekleding met tubulaire cellen in de periurethrale gebieden. CK7 positieve cellen werden gezien in de urethra, de tubuli in het periurethrale gebied en in de perifere acini. CK14 kleuring werd gevonden in verspreide cellen van de tubuli in de periferie van een atrofische prostaat maar niet in de urethra. De patronen van HMWCK en CK5 kleuring waren gelijk aan die van CK7. Verder bleek het absolute aantal HMWCK en CK5 positieve cellen per dwarsdoorsnede in de geatrofieerde prostaten toegenomen, vergeleken met de aantallen in intacte honden. CK18 was minder opvallend dan in de niet-gecastreerde honden en werd alleen tot expressie gebracht in de enkelvoudige of luminale laag van tubulaire cellen in de prostaten van de gecastreerde reuen.

De hondenprostaat heeft een nogal gedifferentieerd karakter met veel acini en weinig stromaal weefsel. Castratie veroorzaakt een grote verandering in deze morfologie met een opvallende relatieve toename van stroma. Het toenemende aantal HMWCK en CK5 positieve cellen in de geatrofieerde prostaten van gecastreerde honden kan óf op een ophoping van intermediaire/tijdelijk prolifererende cellen wijzen die niet verder kunnen differentiëren tot secretoire cellen, óf op een actieve regeneratie van het epitheel in een poging het oorspronkelijke aantal secretoire cellen te herstellen. De toename van basale en intermediair type cellen in de prostaat van gecastreerde honden suggereert dat castratie leidt tot een toename van het intermediaire/tijdelijk prolifererende celtype en een minder gedifferentieerde samenstelling van het prostaatepitheel. Verder blijkt uit onze resultaten dat de overgebleven atrofische tubuli in de prostaat van gecastreerde honden niet zo zeer opnieuw gekoloniseerd worden door cellen uit de pre-existente acini, maar door cellen die van de ducten komen. De overgebleven tubuli in het periurethrale gebied worden op hun beurt herbevolkt door cellen met een urethraal fenotype.

De oorsprong van prostaat kanker van de hond

Een ductale oorsprong van prostaatkanker van de hond?

Prostaatkanker is soms moeilijk te onderscheiden van transitioneel cel carcinoom dat de prostaat ingroeit. Hierom worden celtype-specifieke expressiemerkers gebruikt om de beide typen kanker te onderscheiden. Bij de mens is PSA de meest algemeen gebruikte merker om prostaatkanker te identificeren. Hoewel een matige PSMA expressie in normaal humaan urotheel en in endotheelcellen van tumorgeassocieerde nieuwe vaatgroei in verschillende solide kankers voorkomt, is PSMA gebruikt om cellen van prostaatoorsprong te identificeren. Zowel UPIII als CK7 worden gebruikt om transitioneel cel carcinomen en cellen van urotheliale herkomst in de humane prostaat te onderkennen. Wij pasten hetzelfde panel van merkers toe om prostaatkanker van de hond te bestuderen. In onze studie bracht ongeveer de helft van de bestudeerde cPC gevallen slechts zwak PSA tot expressie (Hoofdstuk 3). Bovendien was slechts de helft van de prostaattumoren van de hond posi-

tief voor PSMA. Hoewel wij in de solide partijen een significant lager aantal cellen vonden die UPIII en CK7 tot expressie brengen dan in de micropapillaire en cribriforme partijen (Hoofdstuk 3) bracht toch de overgrote meerderheid van de prostaattumoren UPIII en CK7 tot expressie.

In de normale prostaat brengen ductale cellen CK7 tot expressie (Hoofdstuk 2). In de normale prostaat van gecastreerde dieren verdwijnt het merendeel van de acinaire cellen en de basaalmembranen van de resterende tubuli worden bezet door cellen die CK7 en in mindere mate UPIII laten zien. Belangwekkend is dat het voorkomen van gediagnosticeerde prostaatkanker is toegenomen in gecastreerde dieren. Als deze kenmerken worden gecombineerd is een ductale oorsprong van prostaatkanker van de hond waarschijnlijk, wat de eerdere suggestie van Leav en medewerkers onderschrijft. Maar op basis van onze resultaten kan een urotheliale oorsprong van cPC niet worden uitgesloten.

Vormen intermediaire/tijdelijk prolifererende cellen de oorsprong van prostaatkanker van de hond?

Onze studie over de pathomorfologische en immunohistochemische karakterisering van prostaatkanker van de hond onthulde dat alle groeipatronen van cPC CK18 tot expressie brengen (Hoofdstuk 3). Dit wijst op een overheersen van gedifferentieerde celtypen en komt overeen met de waarnemingen in hPC. Verschillend van hPC was dat wij in cPC vaak expressie vonden van HMWCK, CK5 en CK14, indicatoren voor resp. intermediaire/tijdelijk prolifererende en basale celtypen, vooral in tumoren van gecastreerde honden (Hoofdstuk 3).

Bij de mens konden Leenders et al nooit expressie van CK14 vinden in prostaatkanker, niet in vroege prostaatkanker, noch in gevorderde prostaatkanker of in prostaatkanker cellijnen. Het minst gedifferentieerde celtype dat zij konden detecteren waren spaarzame CK5 positieve cellen. Aangezien in hun redenering het minst gedifferentieerde celtype de cellen van oorsprong van prostaatkanker vormen achtten zij de intermediaire/tijdelijk prolifererende cellen de cellen van oorsprong van hPC. Op basis van een gelijke redenering als van Leenders et al opperen wij de hypothese dat vroege basale cellen de cellen van oorsprong vormen van cPC; de minst gedifferentieerde cellen die wij in onze cPC gevallen vonden zijn CK14 positieve cellen. Aangezien CK14 positieve basale cellen alleen gevonden worden in het urotheel van de hondenprostaat, breiden wij onze hypothese uit door te stellen dat basale cellen van de prostaaturethra en/of ducten aan de oorsprong van cPC staan.

Groeipatronen van prostaatkanker bij mens en hond

Prostaatkanker van de hond is een erg agressieve tumor. De kanker laesie betreft in het algemeen de hele prostaat, de epitheliale tumorcellen blijven vaak niet besloten binnen de natuurlijke grens van de basaalmembraan, en de kankercellen groeien vaak in de prostaatkapsel en omgevende weefsels in. Hoewel er momenteel geen Gleason–achtig graderingsysteem is om de agressiviteit

van cPC te scoren zou het merendeel van de klinisch gediagnosticeerde cPC gevallen, vergeleken met hPC, zeker worden gegradeerd als hoogmaligne.

In onze studie zagen wij een opvallende variatie in de groeipatronen van cPC, zowel tussen als binnen de individuele gevallen. Zes groeipatronen werden onderscheiden: micropapillair, cribriform, solide, sarcomatoïde, kleinacinair/ductaal en tubulo-papillair. Een heterogene morfologie werd zelfs vaak gezien binnen individuele gevallen, zowel in termen van het aantal combinaties van groeipatronen per tumor als in termen van cellulaire morfologie. Castratie leidt tot een toename van minder gedifferentieerde groeipatronen in cPC. In onze studie werden solide groeipatronen significant (P=0.027) vaker gezien in gecastreerde honden dan in intacte honden. Dit komt overeen met de waarneming van Cornell et al dat goed gedifferentieerde adenocarcinomen vaker worden gezien in niet-gecastreerde reuen dan in gecastreerde.

In mannen is het merendeel (tot 95%) van de prostaatkankergevallen van het type adenocarcinoom, vooral gekenmerkt door een acinaire differentiatie. Er worden echter ook varianten zoals sarcomatoïde carcinoom, adenosquameus carcinoom, ductaal carcinoom en transitioneel cel carcinoom gezien. Naast deze goed gedifferentieerde carcinomen worden slecht gedifferentieerde carcinomen onderkend. Er zijn ook verschillen in de pathomorfologische verschijningsvormen gerapporteerd tussen androgeengevoelige en androgeenrefractaire prostaatkanker van de mens. Kondo et al classificeerden de glandulaire (waaronder groot- en enkelvoudig kleinglandulair), microglandulaire en cribriforme groeipatronen als androgeengevoelig, terwijl medullair-solide en columnair-trabeculaire patronen, soms met gefuseerde cellen, geclassificeerd werden als androgeenrefractair. Zij rapporteerden ook dat metastasen naar een androgeenrefractaire verschijningsvorm neigden. Shah en medewerkers concludeerden verder dat androgeenonafhankelijke prostaatkanker een heterogene groep van ziekten is. De meerderheid vertoont een mengeling van Gleason graad 4 en 5, hetgeen groei in solide velden en nesten inhoudt met of zonder comedonecrose, en confluerende cribriforme glandulaire patronen. Deze mengeling van groeipatronen in humane androgeenrefractaire prostaatkanker lijkt veel op de mengeling van groeipatronen die wij hebben waargenomen in prostaatkanker van de hond.

Zoals vermeld laat de pathomorfologie van cPC vaak gemengde patronen zien die lijken op androgeenrefractaire hPC. De gemengde patronen werden niet alleen gezien in gecastreerde honden maar ook in intacte reuen, hoewel cPC in intacte reuen relatief vaker gedifferentieerd bleek (Hoofdstuk 3). De screening op PSA in het serum vergroot de kans de mogelijke patiënt met hPC in een vroeg stadium te ontdekken, waarin de prostaatkanker meestal een minder agressieve en meer gedifferentieerde morfologie vertoont. Dit is een duidelijk verschil met de hond, waarbij PC meestal in een laat stadium wordt gediagnosticeerd. Verder kan het morfologische verschil tussen hPC en cPC mogelijk verklaard worden door de verschillende cellen van oorsprong, die in een verschillende differentiatiefase van het hiërarchische systeem zouden kunnen verkeren. En ten derde zouden de verschillen in het micromilieu binnen de mensen- of de hondenprostaat ook kunnen bijdragen aan de verschillen in tumordifferentiatie. Histologisch verschilt de ratio tussen stroma en parenchym tussen man en hond, wat wijst op verschillen in matrixcomponenten. Een ver-

schil in een of ander molecuul dat betrokken is in de proliferatie- en differentiatiepaden kan bijdragen aan het verschil tussen de twee species. Een verschillende expressie van groeifactoren, cytokines of enzymen die betrokken zijn bij de interactie tussen stroma en epitheel, en die vaak van het stroma afkomstig zijn, zou dus het proces van tumorgroei kunnen beïnvloeden.

De rol van de AR in de ontwikkeling van prostaatkanker van de hond

Androgenen spelen een kritische rol in het reguleren van de groei, differentiatie en overleving van epitheelcellen in de normale prostaat. Castratie leidt tot een enorm verlies van secretoire cellen maar tast de basale cellen niet aan. Dit wekt de suggestie dat de overleving van secretoire cellen afhankelijk is van androgenen, direct of indirect via de interactie van androgenen met het omgevende milieu. Bij de mens zijn niet alleen de normale prostaat cellen voor hun overleven afhankelijk van androgeen, maar kunnen ook kankercellen zich zo gedragen. Patiënten met hPC reageren in een vroeg stadium vaak gunstig op het wegnemen van androgeen. Na een dergelijke geslaagde androgeenablatie therapie komt de prostaatkanker echter vaak terug en ontwikkelt zich tot een agressiever en hormoonrefractair type tumor. De mechanismen die betrokken zijn bij het resistent worden van tumorcellen voor hormonale ablatietherapie zijn slecht begrepen.

Prostaatkanker van de hond wordt niet gekenmerkt door een vroege fase van gevoeligheid voor androgeenonttrekking: het is goed bekend dat castratie niet leidt tot betere resultaten voor de reuen met prostaatkanker. Dit betekent echter niet dat androgenen en de androgeenreceptor (AR) geen rol spelen in de ontwikkeling van cPC. Zo hebben intacte reuen een kleinere kans cPC te ontwikkelen dan castraten. Bovendien vonden wij dat cPC in gecastreerde reuen vaker een slecht gedifferentieerde histologie vertoonde. En ten slotte, in vroegere studies zagen wij de AR in 80% van de cPC gevallen tot expressie komt, maar met een verschuiving van de lokalisatie in het tumorweefsel van de kernen naar het cytoplasma. Aangezien de aanwezigheid van androgenen noodzakelijk is voor zowel het stabiliseren van de AR dimeer als voor de translocatie ervan naar de kern leidt de afwezigheid van androgenen in gecastreerde reuen mogelijk tot een dergelijke cytoplasmatische kleuring. We zagen echter ook een overwegend cytoplasmatische kleuring in cPC van intacte dieren, ondanks de aanwezigheid van androgenen. Dit wijst wellicht op een verstoring van het androgeen-AR signaalpad. Mutaties in het AR gen zouden de AR ongeschikt kunnen maken de kern binnen te gaan. Daarom onderzochten wij of mutaties in de gensequentie van het AR DNA-bindende gebied de oorzaak waren dat de expressie van de AR verschoof van de kern naar het cytoplasma van de prostaatkankercel van de reu. Onze sequentieresultaten vertoonden geen mutatie in dit DNA-bindende gebied (Hoofdstuk 4), maar andere gebieden van het AR gen, zoals het ‘scharnier gebied’ (hinge region), zijn ook betrokken in de translocatie naar de kern. Er zijn mutaties in het scharniergebied gerapporteerd die de translocatie naar de kern en de DNA binding sterk kunnen remmen.

Wij bestudeerden ook of er een mogelijk verband was tussen het risico van cPC en de lengte van een reeks CAG herhalingen (een CAG repeat) in een korte tandem repeat in het transcriptiegebied van de AR. De allelverdeling van CAG-1 bleek significant te verschillen tussen reuen met cPC en de normale hondenpopulatie (Hoofdstuk 5). Net als bij de man werden kortere CAG-1 repeats vaker gevonden in de cPC groep, met een totale afwezigheid van het allel met de langste CAG-1 repeat. Er is verondersteld dat bij de man de kortere CAG repeats in exon-1 van het AR gen een oorzakelijke rol zouden spelen in het ontwikkelen van hPC, omdat zij coderen voor een AR met een hogere transcriptieactiviteit. De lengtes van alle AR CAG-1 repeats van de reu zijn echter allemaal kort in vergelijking met de lengtes van 11-31 repeats in dit allel bij de mens; een eventueel toegenomen transcriptieactiviteit van de korte CAG-1 repeat van de reu moet nog worden onderzocht.

De rol van ontsteking bij de carcinogenese in de prostaat

Een verhoogde expressie van het enzym prostaglandin-endoperoxide synthase, of cyclooxygenase-2 (COX-2), is ontdekt bij diverse vormen van kanker bij mens en hond. Bovendien onderdrukt remming van COX-2 de tumorgroei in verschillende diermodellen voor carcinogenese. Er is een groeiende massa studies die tonen dat COX-2 en het product ervan, prostaglandin E2 (PGE2), tumorontwikkeling bevorderen door een veelheid van mechanismen, zoals een toename van proliferatie, een afname van apoptose en een inductie van angiogenese, mogelijk door het genereren van vrije radicalen en carcinogenen. De inductie van COX-2 expressie in maligne cellen komt meestal door het activeren van oncogenen, zoals p53, en het inactiveren van tumorsuppressorgenen, zoals HER-2/neu. Hierop volgende activering van signaaloverdragers als MAPKs en pAkt, en ontregeling van factoren die mRNA stabiliseren dragen wellicht ook bij aan deze inductie.

Wij bestudeerden de expressie en regulering van COX-2 in cPC (Hoofdstuk6). In deze studie vonden we dat COX-2 niet tot expressie komt in normaal prostaatweefsel, maar COX-2 expressie wordt wel geïnduceerd in cPC. Om enig licht te werpen op de mechanismen die tot deze COX-2 expressie leiden onderzochten wij de betrokkenheid van ontstekingspaden. De inflammatoire cytokines TNF-α en IL-6 en de groeifactor EGF veroorzaakten inderdaad allemaal inductie van COX-2 expressie in de niet-neoplastische CAPE cellen, wat vroegere bevindingen van anderen bevestigt dat COX-2 kan worden opgewekt door een veelheid van cytokines en groeifactoren. De aanwezigheid van ontstekingsinfiltraten in het prostaattumorweefsel van de hond was echter juist geassocieerd met een lagere COX-2 expressie, wat laat zien dat COX-2 expressie in cPC geen verband houdt met de aanwezigheid van ontstekingsinfiltraten. Dit werd bevestigd door in vitro experimenten. De eerdergenoemde cytokines wekten hierbij geen inductie van COX-2 expressie op in neoplastische ACE en CPA cellijnen waarin de basale COX-2 expressie al hoog was. Door remmers te gebruiken die specifiek de PI3K, the PKC of de ERK/MAPK paden blokkeren vonden we

dat het ERK/MAPK pad belangrijk is bij het onderdrukken van COX-2 expressie in prostaatcellen van niet-neoplastische herkomst en dat de PI3K en PKC paden noodzakelijk zijn voor de inductie van COX-2 door de cytokines TNF-α en IL-6 en EGF. Bovendien is de COX-2 expressie in de neoplastische cellijn toegenomen door ERK/MAPK te stimuleren, terwijl in de niet-neoplastische cellijn COX-2 expressie is toegenomen door ERK/MAPK te blokkeren.

Suggesties voor verder onderzoek

AR expressie wordt voornamelijk gezien in de secretoire cellen van de humane prostaat, terwijl de basale cellen gewoonlijk geacht worden AR expressie te missen. Onze immunohistochemische kleuringen lieten echter verspreide AR positieve basale cellen zien in de normale prostaat (niet vertoonde gegevens). Eerdere studies rapporteerden ook dat een lage AR mRNA expressie werd gevonden in een subset van humane basale cellen en dat de AR mRNA expressie toenam in een medium met fibroblasten groeifactor (FGF)-7. Bovendien induceerden stromale cellen de expressie van de AR en het PSA gen in CD57 positieve basale cellen. Zoals al eerder werd gesuggereerd wordt de differentiatie van prostaatcellen wellicht indirect bevorderd door stromale cellen. Het is nog onduidelijk of deze basale cellen met AR expressie dezelfde subset vormen als de door Leav gesuggereerde proliferatieve basale cellen. Om de rol van de AR in de normale prostaat en in cPC verder op te helderen zou het de moeite waard zijn de rol op te helderen van basale cellen met AR expressie in de homeostase en tumorgenese van de hondenprostaat. Prolifereren ze in antwoord op androgeenstimulatie? Zijn het voorlopers van de snel delende intermediaire/tijdelijk prolifererende cellen? Spelen ze enige rol in de initiatie en ontwikkeling van een androgeenrefractaire kloon in het geval van hormoonrefractaire prostaatkanker? Meer in het algemeen is de rol van de interactie tussen epitheel en stroma in het proces van oncogenese traditioneel onderbelicht gebleven als het om prostaatkanker gaat; het zal een interessante invalshoek bieden om het mysterie van de oncogenese van prostaatkanker te onthullen.

Om de zojuist gestelde vragen op te lossen kunnen we wellicht eerst trachten de basale cellen die CK5 en AR tegelijk tot expressie brengen met immunohistochemie of in situ hybridisatie te lokaliseren. In een primaire kweek zouden de basale cellen kunnen worden geïsoleerd en coculturen zouden kunnen worden opgezet met matrix of prostaatstroma, dat verkregen is uit prostaten van dieren in verschillende toestanden (jong, oud, intact, gecastreerd, normaal, kanker). Door groeifactoren, hormonen of antagonisten die signaalpaden kunnen blokkeren te verstrekken kunnen we mogelijk de betekenis van stroma voor epitheliale proliferatie en differentiatie waarnemen.

Om de mogelijke mechanismen van carcinogenese van cPC op te helderen zouden we verder kunnen kijken naar de verschillen tussen de epitheel-stroma interactie van jonge en oude honden, intacte en gecastreerde honden, of normale honden vs. honden met kanker. Met de moderne krachtige hulpmiddelen zoals microdissectie en microarrays voor genexpressie zouden de profielen van genex-

pressie van de epithelia en het stroma gemakkelijk beschikbaar komen. Door de verschillen in genexpressieprofielen tussen groepen te vergelijken zou inzicht kunnen worden verkregen in de moleculaire veranderingen in de epithelia en het stroma gedurende de carcinogenese en suggesties zouden kunnen worden gedaan voor een potentieel doel voor therapeutische interventie of voor potentiële factoren die celdifferentiatie kunnen opwekken van de cellen die in de proliferatie zijn blijven steken.

Conclusies

Het hiërarchische differentiatiesysteem van epitheliale prostaatcellen wordt zowel bij honden als bij mensen waargenomen. De histopathologische classificatie laat zien dat prostaatkanker van de hond agressiever en van een minder gedifferentieerd type blijkt dan de meest voorkomende humane prostaatkanker. Op basis van de immunohistochemische resultaten suggereren wij dat cPC hoogstwaarschijnlijk uitgaat van de verzamelbuizen. Onze resultaten suggereren ook dat cPC het meest lijkt op de androgeenrefractaire, slecht gedifferentieerde prostaatkanker van de man. Een overwegend cytoplasmatische androgeenreceptor kleuring werd in prostaatkanker van zowel gecastreerde als intacte reuen gevonden. De cytoplasmatische lokalisatie was niet verbonden aan mutaties in het DNA bindende domein van de AR, wat suggereert dat de geringe cytoplasmatische AR kleuring in cPC wellicht verklaard kan worden door mechanismen die leiden tot een verslechterd androgeen-AR signaalpad, of door de afkomst van basale of stamcellen. Bovendien suggereert onze studie naar de CAG repeats van het AR gen dat korte CAG-1 repeats geassocieerd zijn met een toegenomen risico om in de hond prostaatkanker te ontwikkelen. Hoewel COX-2 expressie in cPC werd aangetoond kon voor ontsteking geen potentiële rol in de carcinogenese van cPC worden bewezen omdat er een inverse correlatie tussen ontsteking en COX-2 expressie was. Dit werd verder nog ondersteund door de bevinding dat de cytokines TNF-α en IL-6 en hun betrokken signaalpaden de COX-2 expressie in maligne prostaatcellen van de hond niet stimuleerden.

De meeste sterfte onder mannen met prostaatkanker komt voor in de hormoonrefractaire fase. Een doeltreffende therapie is dus dringend nodig en voor dat doel zou een passend diermodel erg behulpzaam zijn. Afgezien van de hond zijn goedaardige prostaathyperplasie en prostaattumoren bij dieren zeldzaam. Bij knaagdieren worden prostaattumoren gewoonlijk experimenteel opgewekt. Onze resultaten laten zien dat de spontane prostaatkanker van de hond in verschillende histologische en moleculaire opzichten overeenkomt met de late fase van humane prostaatkanker. Daarom kan verder onderzoek met gebruikmaking van spontane prostaatkanker van de hond als model voor de humane equivalent wellicht heilzame resultaten opleveren voor zowel mens als hond.

References

Voor referenties: zie de Reference List van de Engelstalige Summarizing Discussion.

CHAPTER 9

摘要 (Summary)

【摘要】

【序言】

前列腺，或稱攝護腺，為哺乳類雄性動物的附屬性腺，屬外分泌腺體。犬之前列腺體位於膀胱後方並且環繞著尿道，其分泌物形成精液中之主要成份並供給精子營養。

前列腺於人類及狗皆好發數種疾病，包括前列腺炎、膿瘍、囊腫、增生肥大、及癌症。所有物種中，惟人類與狗會自發性地（spontaneously）發生前列腺癌。而演化樹中與人類最接近的靈長類，至目前為止則未發現會自發性地產生前列腺癌。

數十年來，人類前列腺癌的研究根據病理形態學等觀察的結果發現，前列腺上皮細胞可依據其細胞標幟物（cell marker）表現之不同，可分成基底細胞（basal cell），中間過渡細胞（transiently intermediate cell）及分泌性細胞（secretory cell）。研究人員對癌細胞的發生來源提出了幹細胞理論（stem cell theory）。Isaacs 及 Coffey 於 1989 年提出一假設，基底細胞為所有前列腺上皮細胞之幹細胞，即基底細胞能增殖，並逐漸分化成中間過渡細胞，最後分化為成熟的分泌性細胞。自此，即有非常多的猜測關於癌細胞的來源為中間過渡細胞，或是基底細胞。

針對癌症發生（oncogenesis）的長期研究結果指出，癌症的發生絕非單一原因，除了癌細胞的生成之外，細胞與周邊環境交互作用的變化（microenvironment）亦為必須考慮之重要因素。以前列腺癌為例，荷爾蒙的調節（hormonal regulation）及慢性炎症（chronic inflammation）即被發現與前列腺癌的發生有關。而人的前列腺癌症治療，常以荷爾蒙療法（androgen deprivation）來進行，其中有百分之七十的病患於治療初期反應良好，但經過一段時間即復發（reoccur）而成荷爾蒙/雄性素不反應性（hormone irresponsive/ androgen refractory）前列腺癌。因此雄性素受器（androgen receptor）與

癌症的發生與進展亦為長久以來的研究重點。

【目標】

過去，前列腺癌研究所使用的實驗動物皆以嚙齒類為主，經人為誘發或轉殖形成前列腺癌，用作研究所需之活體模式（model）。本研究希望藉由對犬自發性前列腺癌（spontaneous canine prostate cancer）的特性分析，來了解犬前列腺癌細胞可能之發生來源，並對照前列腺癌於人類的研究結果，期為可應用之動物模式提供另一個選擇。

本論文的第一章中簡介現今對人類與犬的前列腺研究所知之基本資訊，包括解剖學、組織學、發生學、荷爾蒙調控及細胞及分子研究等資料。第二章則利用免疫化學法（immunohistochemistry），檢視正常狗之前列腺上皮細胞（epithelial cells）中各種細胞標幟物，包括 cytokeratins、雄性素受器（androgen receptor）及前列腺特異性抗原（PSA 及 PSMA）、小管及尿道上皮特異性蛋白的表現及分佈位置。第三章同樣以免疫化學法，利用同一批標幟物來檢視其於前列腺癌患犬個體中的表現及分佈情況，並與正常狗之結果作比較；此外亦對前列腺癌於狗表現之病理形態進行分型（classification），並探究各病理型態與免疫化學結果是否有相對應之關係。第四章為探討雄性素受器與前列腺癌之發生是否有關，以雷射顯微切割（laser microdissection）方法對患犬的癌細胞分別進行收集，並針對雄性素受器與 DNA 結合區域（DNA binding region of Androgen receptor）之基因進行序列分析。第五章則是探討雄性素受器第一段基因（exon 1 of androgen receptor）上，其 CAG 重覆片段多形性（CAG repeats polymorphism）與犬前列腺癌發生機率之相關性。本論文的第六章則評估炎症反應的存在與 COX-2 表現之關係。COX-2 為一種酵素，與前列腺素之形成有關。於數種人類及犬隻的癌症研究報告中指出有 COX-2 的表現，亦有證據顯示 COX-2 會促進腫瘤的發展。第七章則對所有的結果進行綜合性的討論。

【結果與討論】

與人類相似，正常狗之前列腺上皮細胞依其不同的細胞標幟物可以區分為三群，基底

細胞表現 cytokeratin 5、14 及 HMWCK；分泌性細胞表現 cytokeratin 18 及 PSA；另有一群能同時表現基底細胞與分泌性細胞標幟物 cytokeratin 5、18 及 HMWCK 的細胞，為可能之中間細胞。而負責將前列腺上皮分泌物運送到尿道（prostatic urethra）的前列腺小管（prostatic duct）上皮，則表現 cytokeratin 7 及部份基底細胞與分泌性細胞之性狀。

由病理形態及免疫化學分析顯示，前列腺癌於狗的病理表現為複雜的混合型且多為未分化（undifferentiated）的形態；細胞標幟物表現之分析顯示，極高比例的癌細胞表現出前列腺小管上皮的性狀。此特徵與人類前列腺癌後期經荷爾蒙治療後復發之雄性素不反應性前列腺癌（androgen refractory prostate cancer）極為類似，並且呼應前人研究結果之假設，即犬之前列腺癌細胞來源為前列腺小管上皮具增殖力的基底細胞。

對雄性素受器的研究結果指出，患犬體內雄性素受器與 DNA 結合區域之基因序列與正常狗並無差異，因此癌症病理切片中所觀察到，雄性素受器未進入細胞核與 DNA 結合之原因，可能與訊息傳導機制受阻有關。而關於基因多形性，相較於正常狗，常發現患有前列腺癌的病犬其雄性素受器第一段基因上所帶之 CAG repeats 數目較少。此結果相似於人類前列腺癌的研究發現。於人類的研究已有許多報告顯示，帶有較短 CAG repeats 的族群有較大的機率會發生前列腺癌。而此 CAG repeats 的長短與雄性素受器之轉錄活性（transcriptional activity）有關。然而與人類相比，犬雄性素受器第一段基因上的 CAG repeats 卻是比人類短了許多（犬：10–12 repeats；人：11–31 repeats），儘管前列腺癌在狗的發生機率（約 0.2-0.6%）比人類少上很多（以美國為例，約六人中有一人會發生）。而雄性素受器在狗的轉錄活性仍待進一步的研究。

在 COX-2 的研究上，雖然狗的前列腺癌有 COX-2 的表現，但其 COX-2 表現與炎症反應的存在卻是呈現負相關（inverse correlation），所以炎症反應在犬前列腺癌發生過程中所扮演的角色仍需進一步的探討。

【結論】

在人類，因前列腺癌死亡的病例多處於癌末之荷爾蒙不反應期，故針對此階段找出有效的治療方法為首要之研究目標，而找到合適的動物模式更為研究進行之重大要素。現今有關前列腺癌的研究多以囓齒類動物做為實驗動物，經人為誘導或轉殖而形成的前列腺癌來進行試驗分析。本論文的研究結果指出，犬自發性前列腺癌在各方面，包括組織病理學與分子層面，皆與人類前列腺癌後期的荷爾蒙不反應期極為相似，因此為可運用之動物模式提供另一良好的選擇，並將對前列腺癌研究的進展極有助益

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Curriculum vitae

Curriculum vitae

The author of this thesis was born on December 20, 1972, in Taipei (Taiwan). After finishing High school, she studied veterinary medicine at the Faculty of Veterinary Medicine (Chung-Hsing University). Becoming a veterinarian, she proceeded with a period of 6 months veterinary internship in companion animal medicine. In 1997, she started her master study about characterization of foot-and-mouth disease viral proteins in the Institute of Veterinary Medicine (Chung-Hsing University). After receiving her Master degree, she worked as a research assistant and studied the infection of Kaposi's sarcoma-associated herpesvirus/ human herpesvirus 8 in patient of multiple myeloma and its clinical meaning in Taiwan University Hospital. In 2001, she worked for Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture (Taiwan). She started her PhD in the Faculty of Veterinary Medicine, Utrecht University in 2002 under the supervision of Prof. dr. Jaap van Dijk. The results of the PhD research are presented in this thesis.