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ESTUDIO CLÍNICO-PATOLÓGICO Y MOLECULAR DURANTE LA INDUCCIÓN, DESARROLLO Y REGRESIÓN DE LA HIPERPLASIA BENIGNA DE PRÓSTATA EN PERROS BEAGLE

Tesis doctoral presentada por:

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A mi esposo Antonio
A mi madre Ángela
A mis hijos Tony y Roxana
A la memoria de mi padre Ángel

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ABSTRACT

The relevance of steroid receptors in both normal prostate and in prostatic pathology is well recognized, but tissue-specific distribution of some of them in the different cell compartments of the canine prostate gland is not completely characterized. We analysed the immunohistochemical expression of androgen (AR), oestrogen α (ERα) and β (ERβ) and progesterone (PR) receptors in the different cell types of the canine prostate gland, either normal or with different pathologic conditions (hyperplasia, prostatitis, carcinoma). AR labelling was 100% in the epithelial cells of normal and hyperplastic tissue, 74% in prostatitis, and 65% in carcinomas. ERα was 85% in normal cases, 35% in hyperplastic cases, 22% in inflamed cases, and 12% in neoplastic glands. ERβ expression was 85% in normal cases and around 70% in all of the pathological conditions. On the other hand, PR expression was weak and less common in normal tissue (44%), when compared to hyperplasia and other diseases. Overall, the expression of AR, ERα and ERβ was highest in normal glands, and decreased in hyperplasia, prostatitis, and cancer. In dogs, the combined administration of oestrogen and androgens synergistically increases prostate weight, and continued treatment leads to the development of glandular hyperplasia. The aim of the present study was to examine the immunohistochemical expression of AR, ERα, ERβ and PR in a model of experimentally induced canine prostatic hyperplasia. Five male Beagle dogs were castrated and treated with 25 mg of 5α-androstane-3α, 17β-diol and 0.25 mg 17β-estradiol for 30 weeks. Prostate specimens were surgically obtained every 6 weeks (experimental stages M0 to M6). The control group consisted of three non-castrated dogs treated with vehicle, from which specimens were only taken at the time points M0, M1, M4 and M6. Immunohistochemical data revealed high AR and ERα expression in the epithelial and stromal cell nuclei of all the experimental and control specimens. The suspension of hormone treatment led to a significant reduction in the expression of both receptors. On the contrary, ERβ was only expressed in epithelial cell nuclei, with no significant differences in the percentages of stained nuclei between control and hormonally treated or atrophic prostates. Weak staining for PR was observed in a small proportion of epithelial cell nuclei but not in stromal cells. Results indicate that AR, ERα, ERβ and PR are differently expressed in canine prostate tissue. In addition, the

present study has been to compare the ultrastructural findings in this model of experimentally induced canine prostatic hyperplasia with those of the spontaneously developed changes in untreated dogs. Changes in the control groups were similar but of lower intensity compared to those of the experimental groups. In luminal cells, crowding with papillary projections, prominent, branching microvilli, and abundant, often compartmentalized granules were observed. The most striking change was the previously unreported finding of caveolae in basal cells. The basal cell compartment contains the transient amplifying sub-population of epithelial cells. It is therefore conceivable that it plays a role in the development of BPH. Other purpose of the present study was to determine, by means of ultrastructural morphometry, if the number of caveolae was related to the stages of hormonally induced BPH. An increase in the number of caveolae in basal cells was noted from M0 even M5. The difference between these four stages and the initial ones was statistically significant ($p<.05$). At M6 stage, after hormonal treatment withdrawal, caveolae diminished dramatically in number (18.4%). The most remarkable finding in this model of experimental BPH was the significant increase in the number of caveolae in basal cells throughout the stages of hormonal treatment, and the dramatic reduction after hormonal withdrawal. This finding suggests that basal cells are either a primary target of or are induced by the hormonal stimuli. Finally also we have evaluated the analysis of gene expression associated with BPH. Canine specific Affymetrix microarray analysis performed on sequential biopsies obtained in a beagle dog dynamic model, characterized a number of genes altered during BPH. Transcriptome analysis performed on a dynamic animal model that accurately mimics the human clinics, permitted us to characterize a gene expression pattern associated with the onset of BPH.

Key words: Prostatic hyperplasia, dog, immunohistochemistry, caveolae.

RESUMEN

La relevancia de los receptores de esteroides tanto en la próstata normal como en las diferentes patologías prostáticas es bien reconocida, pero la distribución específica de algunos de ellos en los tejidos en los diferentes compartimientos celulares de la glándula prostática canina no ha sido completamente caracterizada. Nosotros analizamos la expresión inmunohistoquímica del receptor de andrógenos (AR), receptor de estrógenos alfa (ERα), receptor de estrógenos beta (ERβ) y receptor de progesterona (PR) en los diferentes tipos de células de la glándula prostática canina tanto normal como con diferentes condiciones patológicas (Hiperplasia, prostatitis, carcinoma). La expresión de AR fue del 100% tanto en tejido hiperplásico como normal, 74% en prostatitis y 65% en carcinomas. La expresión de ERα fue del 85% en casos normales, 35% en casos de hiperplasia, 22% en prostatitis y sólo 12% en glándulas neoplásicas. La expresión de ERβ fue del 85% en casos normales y alrededor del 70% en todas las condiciones patológicas estudiadas. Por otro lado, la expresión de PR fue débil y menos común en el tejido normal (44%) cuando la comparamos a la hiperplasia y otras enfermedades. En resumen podemos decir que la expresión de AR, ERα y ERβ fue más alta en las glándulas normales y disminuyó en hiperplasia, prostatitis y cáncer. En perros, la administración combinada de estrógenos y andrógenos incrementa de forma sinérgica el peso de la próstata y un tratamiento continuado da lugar al desarrollo de hiperplasia glandular. El objetivo del presente estudio fue examinar la expresión inmunohistoquímica de AR, ERα, ERβ y PR en un modelo de hiperplasia prostática canina inducido experimentalmente. Cinco perros machos de raza Beagle fueron castrados y tratados con 25 mg de androstanediol y 0,25 mg de 17β estradiol durante 30 semanas. Se obtuvieron mediante cirugía biopsias de próstata cada seis semanas en el grupo experimental (estadios experimentales M0 a M6). El grupo experimental consistió en tres perros no castrados y tratados únicamente con el vehículo utilizado para disolver las hormonas aplicadas al grupo experimental y a los cuales sólo se les tomaron cuatro biopsias (M0, M1, M4 y M6). Los resultados de inmunohistoquímica revelaron una alta expresión de AR y ERα en el núcleo de las células epiteliales y estromales de todas las biopsias tanto del grupo experimental como del grupo control. La supresión del tratamiento hormonal se tradujo en una reducción significativa en la

expresión de ambos receptores. Por el contrario la expresión de ERβ sólo se observó en el núcleo de las células epiteliales y no hubo diferencias significativas en el porcentaje de núcleos teñidos entre el grupo control y el grupo tratado con hormonas o las próstatas atróficas. Débil tinción para PR fue observada en una pequeña proporción del núcleo de células epiteliales, pero no en células estromales. Los resultados indican que AR, ERα, ERβ y PR son diferencialmente expresados en el tejido prostático canino. Además, el presente estudio también ha comparado los hallazgos ultraestructurales observados en este modelo de hiperplasia canina inducida experimentalmente con los cambios desarrollados espontáneamente en los perros no tratados. En las células luminales se observó apelotonamiento con abundantes proyecciones papilares, ramificación de microvellosidades, y la presencia de compartimentación de los gránulos de secreción. El cambio más llamativo, no reportado anteriormente, fue el hallazgo de caveolas en las células basales. El compartimiento de las células basales contiene la población de células epiteliales transitoriamente amplificante y podemos concebir que dichas células desempeñen un rol en el desarrollo de la BPH. Otro propósito del presente estudio fue determinar mediante morfometría ultraestructural si el número de caveolas estaba relacionado con los estadios de la hiperplasia benigna de próstata inducida hormonalmente. Un incremento en el número de caveolas en las células basales fue notado de M0 a M5. La diferencia entre estos cuatro estadios y el inicial fue estadísticamente significativa ($p \leq 0.05$). En el estadio M6, después de retirar el tratamiento hormonal, las caveolas disminuyeron drásticamente en número (18,4%). Por tanto el hallazgo más notable en este modelo de BPH experimental fue el incremento significativo en el número de caveolas en las células basales a través de los estadios del tratamiento hormonal y la dramática reducción después de retirar el tratamiento. Estos hallazgos sugieren que las células basales podrían ser el blanco primario o ser inducidas por el estímulo hormonal. Finalmente, también hemos evaluado el análisis de expresión de genes asociado con el inicio de la BPH. El análisis microarray específico para caninos de Affymetrix fue realizado en las biopsias secuenciales obtenidas en este modelo dinámico del perro del beagle y caracterizó un número de genes alterados durante BPH.

Palabras clave: Hiperplasia prostática, perro, inmunohistoquímica, receptores hormonales.

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Capítulo I

INTRODUCCION Y OBJETIVOS

INTRODUCCIÓN

Investigaciones realizadas por diversos autores durante los últimos 60 años demuestran que el agrandamiento prostático debido a hiperplasia benigna de la próstata (HBP) que ocurre en perros machos intactos y viejos, es un trastorno que puede ser utilizado como modelo para el estudio de esta misma enfermedad en humanos (Berry *et al.*, 1986b; Brendler *et al.*, 1983; DeKlerk *et al.*, 1979; Huggins and Clark, 1940; Leav *et al.*, 2001c; Mahapokai *et al.*, 2000; Trachtenberg *et al.*, 1980; Walsh and Wilson, 1976; Winter *et al.*, 1995). Si bien es cierto que el perro no es el modelo animal ideal para los estudios de comparación con el humano, también lo es el hecho de que es el único animal que desarrolla con frecuencia y de forma espontánea esta patología y ante la perspectiva del incremento de la morbilidad de esta enfermedad en el humano está plenamente justificada su utilización como modelo experimental.

Estudios realizados en diferentes animales muestran la presencia de receptores hormonales en diferentes tejidos (Bonkhoff *et al.*, 1999; Bratka-Robia *et al.*, 2002; De Bosschere *et al.*, 2002; De Cock *et al.*, 1997; Dhaliwal *et al.*, 1999; Fixemer *et al.*, 2003; Geraldes *et al.*, 2000; Hiramatsu *et al.*, 1996; Leav *et al.*, 2001c; Linja *et al.*, 2003; Murakoshi *et al.*, 2000c; Nie *et al.*, 2002; Nieto *et al.*, 2000; Papadimitriou *et al.*, 1992; Pelletier *et al.*, 2000; Schulze and Barrack, 1987b; Vermeirsch *et al.*, 2001). Estos receptores muestran diferencias en su expresión en los distintos tejidos y/o en su patrón de distribución en las diferentes patologías que puedan afectar el tracto reproductivo de ambos sexos (De Bosschere *et al.*, 2002; Dhaliwal *et al.*, 1999; Fixemer *et al.*, 2003; Papadimitriou *et al.*, 1992; Saunders *et al.*, 2000) lo cual es un reflejo de las diferencias en el número de receptores contenidos dentro de cada célula y en la actividad de las células dentro del ciclo celular (Bergeron *et al.*, 1988; Papadimitriou *et al.*, 1992).

Los diversos estudios realizados hasta ahora parecen indicar la existencia de una relación entre los cambios presentados en el patrón de distribución de estos receptores y la presentación de determinadas patologías, al menos en el tejido prostático de humanos y del perro (Leav *et al.*, 2001c). Por otra parte el estudio de estos receptores hormonales mediante técnicas inmunohistoquímicas ha mostrado ser efectivo en su utilización como marcadores pronósticos en las lesiones tumorales (Bonkhoff *et al.*, 1999; Fixemer *et al.*, 2003).

El continuo avance de las técnicas inmunohistoquímicas ha hecho posible su utilización en bloques de tejido fijados en formalina e incluidos en parafina, hecho que supone el inicio de un nuevo camino en el estudio de múltiples patologías (Shi *et al.*, 2001; Taylor *et al.*, 1996). Hasta el momento, la mayoría de las investigaciones que utilizan estas técnicas en tejidos incluidos en parafina a nivel de tracto reproductivo en la especie canina han centrado su interés en el tracto genital femenino y en los testículos (De Bosschere *et al.*, 2002; Dhaliwal *et al.*, 1999; Geraldes *et al.*, 2000; Nie *et al.*, 2002; Vermeirsch *et al.*, 2002). Como hemos mencionado anteriormente, desde hace varias décadas, el perro ha sido utilizado como modelo animal para el estudio de las dos patologías prostáticas que más afectan al hombre después de los cincuenta años, la hiperplasia benigna y el cáncer prostático. Por tanto, es lógico pensar que trabajos realizados en la próstata de estos animales presenten un gran interés práctico al permitir la experimentación y reproducción de la hiperplasia benigna inducida mediante tratamientos hormonales tanto en animales sexualmente intactos como en los castrados (Leav *et al.*, 2001c; Mahapokai *et al.*, 2000; Schulze and Barrack, 1987a).

La relevancia de poder determinar la distribución de los receptores hormonales en próstatas de perros sanos y en aquellos que presenten patologías prostáticas podría proporcionar una posible definición de pautas de tratamiento más eficaces que las existentes en la actualidad tanto para la HBP como para el cáncer prostático.

Los continuos avances de la medicina no se limitan a la utilización de técnicas inmunohistoquímicas, sino que incluyen otras técnicas mucho más sofisticadas, tales como la microscopía electrónica, extracción de RNA, PCR, microarrays de cDNA, etc. La microscopía electrónica permite la descripción de la ultraestructura de cualquier tejido, incluyendo el tejido prostático, permitiendo visualizar los cambios que se producen en las organelas celulares entre un tejido normal y otro en el cual se ha desarrollado una patología (Ichihara *et al.*, 1985; Leav *et al.*, 1978; Merk *et al.*, 1980; Razani *et al.*, 2002).

La aplicación de técnicas de Biología Molecular tiene en la actualidad una gran relevancia al permitirnos adentrarnos en el mundo de los posibles genes involucrados en la aparición de la HBP. Mediante la confección de microarrays de cDNA podemos identificar tanto los genes que se sobre-expresen como aquellos que se expresen por

debajo del nivel normal en las diversas patologías prostáticas (DiLella *et al.*, 2001; Luo *et al.*, 2002).

OBJETIVOS

Así, basandonos en las consideraciones anteriores, los objetivos de esta tesis doctoral fueron:

1. La identificación de los tipos celulares prostáticos que expresan los receptores esteroideos (de andrógenos, de estrógenos α y β, de progesterona).
2. El establecimiento de la correlación entre los niveles de expresión de los receptores esteroideos mencionados y las distintas patologías prostáticas.
3. El establecimiento de la correlación entre los niveles de expresión de los receptores esteroideos mencionados y fases del desarrollo de la hiperplasia inducida hormonalmente.
4. El estudio detallado de la ultraestructura de la glándula prostática durante la instauración y desarrollo de la hiperplasia, con especial importancia al rol desempeñado por las caveolas en las células basales.
5. La utilización de técnica de microarrays de cDNA para analizar las variaciones en la expresión de varios genes.

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Capítulo II

REVISIÓN BIBLIOGRÁFICA

2.1 ÓRGANOS SEXUALES DEL PERRO MACHO

El tracto reproductivo del perro macho está formado por los testículos, epidídimos, conductos deferentes, uretra, próstata y pene (Evans and Christensen, 1979).

Los testículos son órganos de forma ovoide constituídos por dos compartimentos bien diferenciados, llamados intersticio y túbulos seminíferos. Los primeros, los intersticios, están constituidos principalmente por las células de Leydig que son de estirpe mesenquimal y producen la mayor parte de las hormonas masculinas o andrógenos. Alternándose con estos intersticios, se encuentran los túbulos seminíferos separados de aquellos por una membrana basal y por una capa de células mioides. A continuación se hallan las células de Sertoli, verdaderas sostenedoras de la estructura tubular, y, entre éstas, las células germinales totipotenciales, a saber, las espermatogonias primarias. Estas irán madurando progresivamente nutriéndose de las células de Sertoli, para acabar convirtiéndose en espermatozoides maduros que, una vez en el centro de la luz tubular, se desplazarán hacia el epidídimo para completar su maduración. Estos espermatozoides serán los responsables de la fertilización de un ovocito y por tanto, de completar una de las funciones principales del testículo que es la de la perpetuación de la especie. La otra función principal es la ya mencionada de producción de andrógenos necesarios para funciones múltiples entre ellas la de la correcta diferenciación sexual masculina y la de soporte de la función reproductiva. Los epidídimos están situados a lo largo de cada testículo y constan de tres porciones: cabeza, cuerpo y cola. A partir de la cola del epidídimo arrancan los conductos deferentes que se continúan a lo largo del canal epididimario junto a los vasos sanguíneos y nervios que lo acompañan en su recorrido formando el cordón espermático que desemboca en la uretra. La uretra es un conducto musculoso que comienza en el cuello vesical y termina en el orificio uretral externo ubicado en el ápice del pene. El pene es el órgano copulador y está constituido por el cuerpo del pene, la uretra y el glande (Evans and Christensen, 1979).

2.1.1. LA PRÓSTATA

2.1.1.1 Anatomía

La glándula prostática es la única glándula sexual accesoria en el perro macho. Es una estructura músculo glandular bilobulada con un septo en medio de su superficie dorsal.

Se encuentra ubicada en el espacio retroperitoneal, en la base del cuello de la vejiga de la orina, rodeando completamente a la uretra proximal (Evans and Christensen, 1979). Entre el nacimiento y la pubertad se producen muy pocas modificaciones en el tamaño de la próstata. Sin embargo, a partir de la llegada de la pubertad, la próstata experimenta un rápido incremento de tamaño que continúa hasta llegar a la etapa adulta de la vida (Berry *et al.*, 1984). Histológicamente, está formada por gran cantidad de acinos de células epiteliales secretorias que drenan a través de varios conductos dentro de la uretra prostática y están rodeados por un estroma de tejido conjuntivo y fibras musculares lisas; todas las estructuras están contenidas dentro de una cápsula de tejido fibro-muscular. El peso aproximado de esta glándula en perros Beagle es de 12-14 gramos (Barsanti and Finco, 1989). Esta glándula no es esencial para la vida y se cree que tampoco lo es para la reproducción, aun cuando debemos recordar que en el perro provee más del 90% del volumen del líquido seminal (Olson *et al.*, 1987). La posición de la glándula dentro del abdomen varía con la edad del animal y el grado de distensión de la vejiga urinaria. En perros jóvenes o castrados a edad temprana, la próstata se encuentra ubicada dentro del canal pélvico. En perros viejos, con hiperplasia o cuando la vejiga de la orina está completamente distendida la próstata se desplazará cranealmente dentro del abdomen (Barsanti and Finco, 1989).

La irrigación arterial de la próstata proviene de la arteria urogenital, rama del tronco visceral de la arteria ilíaca interna. La arteria urogenital emite dos ramas a la próstata: la arteria prostática vesical y la arteria prostática uretral, las cuales a su vez se ramifican dando unas pequeñas ramas colaterales hacia la superficie glandular, la uretra prostática y unas colaterales hacia el cuello vesical.

La inervación de esta glándula corre paralela a la irrigación sanguínea. La inervación simpática es aportada por el nervio hipogástrico y la inervación parasimpática llega a través del nervio pélvico que se origina en el segundo nervio sacro (Evans and Christensen, 1979).

2.1.1.2 Histología

Según Cooney y colaboradores (1992), la próstata canina está compuesta de dos áreas glandulares: el cuerpo glandular y la parte diseminada de la próstata. El cuerpo o porción externa está constituido por lóbulos simétricos separados por el septo medio-

dorsal y el área diseminada forma parte de la pared ventral de la uretra y está envuelta por el músculo uretral.

Este órgano es una glándula túbulo-alveolar formada por varios acinos glandulares y un estroma de tejido conectivo y músculo liso, rodeados por una cápsula fibromuscular**.** El epitelio es de tipo cilíndrico en las glándulas y cambia a tipo transicional en los conductos excretores que desembocan dentro de la uretra. Las células epiteliales pueden dividirse en dos tipos: basales y luminales. En el perro, las células basales se localizan esporádicamente a lo largo de la membrana basal y son las que dan origen a las células epiteliales secretoras. El estroma prostático consiste en un conjunto de células de músculo liso y fibroblastos sumergidos en una matriz de colágeno con sus respectivos vasos sanguíneos y nervios. Antes del inicio de la pubertad, predomina el componente estromal y a partir de la instauración de la madurez sexual predomina el componente epitelial (Root-Kustritz and Klausner, 2001). En comparación con la próstata humana, la próstata canina normal tiene mayor porcentaje de epitelio y menos estroma. La unidad funcional de la próstata incluye tanto el componente epitelial como el componente estromal (Cunha *et al.*, 1987).

2.1.1.3 Fisiología prostática

La función de la próstata es secretar el líquido seminal, líquido que proporciona el ambiente óptimo para la supervivencia y la motilidad de los espermatozoides durante la eyaculación. En condiciones normales, es una secreción clara, casi transparente, serosa y representa la primera y la tercera fracción del eyaculado de los perros. Su pH varía desde 6 a 7.4. Es una glándula hormono-dependiente de la testosterona para su crecimiento durante la pubertad y para mantener su tamaño y estructura en la vida adulta (Barsanti and Finco, 1989; Ewing *et al.*, 1984; Huggins and Clark, 1940). Durante la erección y bajo estímulo parasimpático, la próstata acelera su ritmo de producción y bajo control simpático, impulsa la salida del líquido durante la eyaculación. La próstata participa en el control de la eliminación de la orina a través de la vejiga y en la secreción del fluido seminal durante la eyaculación.

Las secreciones prostáticas son producidas por las células epiteliales de la glándula bajo la influencia de los andrógenos secretados por los testículos y otras partes corporales. La castración hace casi desaparecer la secreción de líquido prostático (Barsanti and Finco,

1989).

O'Shea (1962) sugirió que la glándula prostática de los perros pasa por tres etapas a lo largo de la vida del animal. La primera fase consiste en el crecimiento normal observado en animales jóvenes. Una segunda fase durante la edad adulta en la cual predomina el crecimiento hiperplásico y una tercera fase descrita como involución senil.

2.1.1.4 Patologías prostáticas

Las enfermedades prostáticas son comunes en los perros viejos. Con el envejecimiento, la glándula prostática incrementa en tamaño, pero su función secretora disminuye paulatinamente a medida que pasan los años. Todas las enfermedades prostáticas pueden presentarse en todas las razas de perros. Sin embargo, los perros de razas grandes tienen una prevalencia más alta de este tipo de trastornos, especialmente de hiperplasia y de neoplasias.

2.1.1.4.1 Hiperplasia benigna de la próstata

La HBP consiste en el crecimiento anormal de la glándula y se caracteriza por una proliferación difusa de células epiteliales de la periferia terminal y que se expande hacia la uretra (Oesterling *et al.*, 1988). Es la enfermedad prostática más común en el perro y puede ser asintomática o presentar varios signos relacionados con el aumento de tamaño de la glándula, la cual aumenta tanto en peso como en volumen (Dorfman and Barsanti, 1995). Los síntomas más comunes son: hematuria, constipación y disuria presentándose con menor frecuencia infertilidad y alteraciones en la locomoción de miembros posteriores (Hornbuckle *et al.*, 1978). En el perro Beagle, la próstata no completa su crecimiento normal hasta los dos años de edad, luego comienza un incremento de tamaño causado por la hiperplasia glandular.

En un intento por explicar la etiología de la HBP, en humanos y también en perros se han propuesto tres teorías:

La **primera** teoría se basa en el hecho de que unos testículos funcionales favorecen la acumulación prostática de 5α dihidrotestosterona (DHT) durante la etapa senil. Tomando en cuenta que el desarrollo y el crecimiento prostático normal y patológico en ambas especies depende de la existencia de un eje funcional de señalización de andrógenos, este eje incluye varios componentes: a) Síntesis de testosterona en los

testículos y glándulas adrenales, b) Conversión de la testosterona en DHT, por acción de la enzima 5α-reductasa c) Transporte de la DHT a los tejidos en los que actúa y d) Unión de la DHT a sus receptores diana con la consecuente modulación de genes. Así, el complejo resultante de la unión de la DHT al receptor de andrógenos desencadena la cascada de eventos necesarios para la formación de los factores encargados de regular el crecimiento celular (Carson and Rittmaster, 2003). La DHT es el andrógeno más activo a nivel prostático, ya que presenta una afinidad por el receptor de andrógenos (AR) cinco veces superior a la de la testosterona y al mismo tiempo una tasa de disociación cinco veces inferior (Ewing *et al.*, 1984). El rol de la DHT consiste en mantener el balance entre la proliferación y la muerte celular en la próstata normal.

Estudios realizados en humanos y animales sugieren que el desarrollo de la HBP se presenta cuando se rompe el balance de la homeostasis de la DHT entre la proliferación y la muerte celular por inhibición o disminución de los procesos apoptóticos (Carson and Rittmaster, 2003). Aunque la próstata es un tejido andrógeno-dependiente, los estrógenos influyen, tanto en las funciones normales como en los cambios patológicos de esta glándula (Weihua *et al.*, 2002). La actuación sinergística de andrógenos y estrógenos a nivel de tracto reproductor del macho ha sido reconocida desde hace varias décadas por los investigadores (Hess, 2003). Los estrógenos están presentes en altas concentraciones en el fluido seminal de varios mamíferos, incluyendo el hombre y son reconocidos como una hormona importante en la reproducción del macho (Hess *et al.*, 2001). Los receptores de estrógenos (ER) son abundantes en el tracto reproductivo del perro macho (Nie *et al.*, 2002) y también en el de los humanos, donde actúan como reguladores del crecimiento y la diferenciación del epitelio prostático en conjunto con los AR (Chang and Prins, 1999; Weihua *et al.*, 2001). De hecho, se ha sugerido también que los estrógenos podrían incrementar el número de AR en el tejido prostático (Root-Kustritz and Klausner, 2001). La HBP canina se caracteriza por un aumento de la sensibilidad de este órgano a los andrógenos y una disminución del índice de muerte celular y estos cambios podrían estar relacionados con modificaciones internas de la próstata durante su envejecimiento, como por ejemplo, la pérdida de un sistema biológico de freno en el estroma que facilite un crecimiento no regulado o una alteración de la relación andrógenos: estrógenos (Walsh, 1986).

Se han postulado otras hipótesis sobre la relación estrógenos/andrógenos para tratar de

explicar el crecimiento de la glándula prostática, pero los múltiples estudios efectuados han dado resultados contradictorios (Berry *et al.*, 1986b; Chevalier *et al.*, 1991; Lee, 1996), lo cual podría deberse al hecho de que en una misma glándula prostática podemos encontrar áreas de hiperplasia glandular, entremezcladas con áreas de atrofia del epitelio e incluso acompañados de la formación de quistes prostáticos y concurrente inflamación. En las áreas atrofiadas se observa un aumento de los componentes estromales (colágeno y músculo liso) y todo este conglomerado de cambios, provoca muchas alteraciones en la arquitectura normal de la próstata (Olson *y cols*, 1987).

La **segunda** teoría para explicar la etiología de la HBP está basada en la interacción entre el estroma y el epitelio celular en el crecimiento y mantenimiento de la próstata.

La próstata se compone de una variedad de tipos de células especializadas, las cuales podemos clasificar como epiteliales o estromales. En la próstata adulta, los AR están presentes tanto en células epiteliales como en las estromales (Lee *et al.*, 1995). El posible rol de la inducción estromal-epitelial en la patogénesis de la HBP fue sugerido por Reischauer en 1925. Esta sugerencia se basaba en observaciones de dicho investigador acerca de que la lesión inicial de la HBP en el humano era un nódulo estromal que inducía la posterior migración de células epiteliales dando lugar a la formación de nuevos elementos glandulares (acinos).

Los factores de crecimiento son potentes mediadores de la proliferación, diferenciación y muerte celular. Algunos de estos factores de crecimiento son considerados como los efectores proximales de la acción de los andrógenos a través de la interacción estromal-epitelial. Los factores de crecimiento que han sido implicados en la patogénesis de la HBP son: *Epidermal growth factor* (EGF), *Transforming growth factor alfa* (TGFα) y beta (TGFβ) y el *Fibroblastic growth factor* (FGF). En condiciones normales la homeostasis prostática es mantenida por un equilibrado balance de todas las fuerzas que influyen sobre la proliferación y la muerte celular, siendo los factores de crecimiento uno de los componentes de estas fuerzas que interactúan sobre las células. Cuando ocurre una alteración en la producción o la acción de alguno de los factores de crecimiento se traduce en un crecimiento anormal de la próstata (Lee *et al.*, 1995).

La **tercera** teoría propuesta para intentar explicar la etiología de la HBP patológica se ha denominado la hipótesis de las células madre.

La hiperplasia benigna de la próstata podría ser considerada como una enfermedad de las células madre totipotenciales (o *stem cells*) resultante de una anormalidad en la regulación y maduración de los procesos de renovación celular, ya que, aunque en la hiperplasia hay un incremento del tamaño de la glándula, no existe incremento de la síntesis de DNA, el cual sería el indicador de un aumento en la replicación celular (Barrack and Berry, 1987; Walsh, 1994). La próstata normal vista al microscopio muestra un alto grado de organización celular, donde morfológicamente pueden distinguirse dos tipos de células dentro del epitelio secretor, las células basales y las células luminales. Estas células difieren no solo en su apariencia sino también en sus propiedades biológicas; las células luminales están completamente diferenciadas, son dependientes de los andrógenos, tienen baja capacidad proliferativa y un alto índice apoptótico. Por el contrario, las células basales son, generalmente, indiferenciadas y andrógeno independientes, tienen alta capacidad proliferativa y un bajo índice apoptótico, características éstas que son atribuibles a las células madres o *stem cells*. Intercaladas entre el epitelio prostático también encontramos algunas células neuroendocrinas. Un modelo propuesto por Isaacs y Coffey (1989) también identificó una población celular intermedia entre la población indiferenciada de las *stem cells* de la capa basal y las células altamente diferenciadas del epitelio luminal, a las cuales asignaron el nombre de células transitoriamente proliferantes/amplificantes. Mediante el uso de técnicas inmunohistoquímicas es posible identificar el patrón de expresión de citoqueratinas en cada una de estas poblaciones de células. En experimentos realizados *in vitro*, cultivando células con fenotipo de células madres se evidenció que estas células podían dar lugar a nuevo tejido glandular en el que todos los tipos celulares estaban representados (Schalken and van Leenders, 2003).

La hiperplasia benigna de la próstata es la enfermedad prostática más común en hombres mayores de 50 años. Entre los animales domésticos, el perro es la única especie que desarrolla esta patología de forma espontánea y con elevada frecuencia (Juniewicz *et al.*, 1990; Murakoshi *et al.*, 2002). En ambas especies, esta patología es un proceso andrógeno-dependiente asociado con el envejecimiento. La HBP en el perro se caracteriza por una proliferación difusa del epitelio glandular en toda la glándula, con menor afectación estromal, mientras que en el humano esta enfermedad se desarrolla específicamente dentro del tejido periuretral, en la llamada zona de transición y el tejido

hiperplásico está compuesto de mayor proporción de tejido estromal en relación al glandular (Laroque *et al.*, 1995). Aunque la morfología de la HBP humana sea frecuentemente referida en la literatura como una proliferación de células estromales, tanto la HBP canina como la humana se caracterizan por un grado variable de proliferación de células epiteliales glandulares acompañadas de elaboradas ramificaciones de los alvéolos con un incremento de las proyecciones papilares (Banerjee *et al.*, 2001). Independientemente de estas diferencias, hay suficientes similitudes en esta enfermedad entre las dos especies que permiten la utilización del perro como modelo para el estudio de la HBP humana (Brendler *et al.*, 1983). Según un estudio realizado por Juniewicz y colaboradores, el perro representa el mejor modelo animal disponible para el estudio de la HBP en humanos no sólo porque en ambas especies esta enfermedad ocurre espontáneamente, sino porque además puede ser experimentalmente inducida mediante la utilización de hormonas esteroides tanto en los perros sexualmente intactos como en los castrados (Juniewicz *et al.*, 1990).

Según el estudio realizado por Berry e Isaacs (1984), el 61% de los hombres mayores de 40 años presentó evidencia histológica de HBP y este porcentaje fue incrementando progresivamente a medida que aumentaba la edad de los sujetos estudiados (Garraway *et al.*, 1991). De igual manera, estudios realizados en perros han demostrado que el porcentaje de perros con HBP también incrementa con la edad, ya que, según el estudio realizado por Brendler y colaboradores (1983) en perros Beagle se puede observar que el 40% de los animales mayores de dos años y medio, presentan evidencia histológica de HBP, elevándose la incidencia a más del 95% en perros mayores de 9 años de edad (Lowseth *et al.*, 1990).

Numerosos estudios mencionan que más del 80% de los perros machos intactos, mayores de 5 años presentan evidencia microscópica de hiperplasia benigna de próstata (DeKlerk *et al.*, 1979; Kawakami *et al.*, 2001) y que a la edad de 8 años este porcentaje se acerca al 100 % de la población de perros machos sexualmente intactos (Berry and Isaacs, 1984; Ewing *et al.*, 1985; Isaacs and Coffey, 1984; James and Heywood, 1979; Zirkin and Strandberg, 1984). La presencia de testículos funcionales es requisito indispensable para el desarrollo de hiperplasia benigna de la próstata en perros (Coffey and Walsh, 1990; Huggins and Clark, 1940), pero no para la presentación de neoplasias prostáticas, ya que estas pueden aparecer en perros castrados (Bell *et al.*, 1995; Leav *et*

al., 2001c). Aún cuando la patogénesis de la hiperplasia benigna de la próstata en el perro no es conocida en su totalidad, se ha sugerido que su presentación está asociada con cambios o anormalidades en la secreción de andrógenos y estrógenos en dicha glándula (Berry *et al.*, 1984; Berry and Isaacs, 1984; Leav *et al.*, 2001c; Oesterling *et al.*, 1988).

La frecuencia de presentación de HBP tanto en humanos como en perros ha experimentado un fuerte incremento en la última década, especialmente en países desarrollados, debido principalmente al aumento de la esperanza de vida que conlleva al incremento del envejecimiento poblacional. Del mismo modo, la utilización, por parte de los veterinarios, de nuevos tratamientos y cuidados en los perros ha permitido incrementar su esperanza de vida y en consecuencia, ha incrementado el número de animales que padecen la HBP clínica.

Los diversos tratamientos médicos y quirúrgicos llevados a cabo en hombres, con el objeto de obtener mejoría en la calidad de vida del paciente, representan un elevado coste económico para la salud pública, especialmente en países como España donde un elevado porcentaje de la población son hombres mayores de 50 años. Según cifras obtenidas del último censo de población realizado en España, la población actual española ronda los 41 millones de habitantes, entre los cuales, la población mayor de 65 años es el 17% del total y de este porcentaje casi un 50% son hombres que padecen HBP. De ese mismo censo se desprende que existe un millón y medio de habitantes mayores de 80 años, y uno de cada tres personas de este grupo son hombres (Censos 2001, Instituto Nacional de Estadística), hecho que nos permite deducir la fuerte inversión económica que debe hacer el estado en estos tratamientos.

En el transcurso de los últimos 50 a 60 años se han postulado muchas teorías acerca de los diferentes factores relacionados con el desarrollo de HBP tanto en humanos como en perros llegándose a la conclusión de que indudablemente su etiología es multifactorial. A pesar de ello solo la presencia de testículos funcionales y el envejecimiento han estado invariablemente ligados a la aparición de esta enfermedad. Sin embargo, los procesos exactos mediante los cuales el envejecimiento y los factores endocrinos testiculares se relacionan con el inicio y progreso de la HBP no han podido ser completamente dilucidados (Steiner *et al.*, 1999). En resumen, podemos decir que los

factores etiológicos pueden dividirse en factores extrínsecos y factores intrínsecos. Los factores intrínsecos están relacionados con la interacción entre células estromales y epiteliales, mientras que los extrínsecos se relacionan con los andrógenos, estrógenos y sus respectivos receptores, así como diversos factores ambientales y una predisposición genética.

2.1.1.4.2 HBP inducida por esteroides en perros

El hombre y el perro son los únicos mamíferos en los cuales la hiperplasia prostática se desarrolla de manera espontánea con elevada frecuencia. Por este motivo el perro ha sido ampliamente utilizado en estudios experimentales destinados al incremento del conocimiento de la etiología de dicha patología (Murakoshi *et al.*, 2001b; Winter *et al.*, 1995).

La inducción experimental de la HBP requiere la inoculación de andrógenos y estrógenos (Funke *et al.*, 1982; Merk *et al.*, 1982; Merk *et al.*, 1986; Trachtenberg *et al.*, 1980; Winter *et al.*, 1995). El tratamiento con 17ß-estradiol sólo induce hiperplasia estromal y glandular, metaplasia escamosa con el consiguiente descenso de la función secretora de las células epiteliales metaplásicas y un incremento en el número de receptores de estrógenos intranucleares (Funke *et al.*, 1982; Trachtenberg *et al.*, 1980). Los perros tratados únicamente con andrógenos, 3α-androstanediol, muestran una proliferación glandular así como evidencia histológica de HBP leve (Merk *et al.*, 1986; Moore *et al.*, 1979). Sólo los perros tratados con ambos 17ß-estradiol y 3α-androstanediol desarrollan HBP con metaplasia escamosa y una considerable hiperplasia y hipertrofia de las células epiteliales prostáticas (Funke *et al.*, 1982; Merk *et al.*, 1986; Moore *et al.*, 1979; Trachtenberg *et al.*, 1980; Winter *et al.*, 1995). La hipotéticas razones para la necesidad de ambas hormonas para la inducción de la HBP incluyen un crecimiento andrógeno-dependiente de las células epiteliales prostáticas perjudicado por metabolitos de estrógenos con radicales libres activos (Winter and Liehr, 1996) o bien un incremento de la sensibilidad de la glándula a los cambios mediados por estrógenos debido a una papel más bien permisivo que inductivo de la elevadas concentraciones de DHT intraprostáticas (Ewing *et al.*, 1983).

Según los resultados obtenidos por Jacobi *y cols* (1978) el crecimiento de la próstata cuando es inducido por la administración de esteroides exógenos no continúa

indefinidamente, sino que se ralentiza o se detiene después de alcanzar el estado de hipertrofia, aproximadamente a los 6 meses del inicio del tratamiento.

Los datos experimentales hacen pensar en dos fases distintas durante el proceso hiperplásico. La primera es la fase inicial observada en perros menores de cinco años de edad, caracterizada por hiperplasia glandular con aumento simétrico de la próstata. La segunda se caracteriza por el aumento de las células luminales o secretoras en número y tamaño y su proliferación en la fase final, denominada hiperplasia compleja, que se presenta en perros de más de cinco años de edad y en la cual se presenta un aumento asimétrico de la próstata con áreas de hiperplasia entremezcladas con áreas de atrofia (DeKlerk *et al.*, 1979).

2.1.1.4.3 Neoplasias prostáticas

Las neoplasias prostáticas son patologías poco comunes que se presenta de forma insidiosa en perros senescentes. Puede aparecer tanto en animales sexualmente intactos como en perros castrados (Bell *et al.*, 1991). El tipo de neoplasia más común en la próstata de los perros es el adenocarcinoma, aunque el carcinoma indiferenciado o el carcinoma de células transicionales también pueden presentarse. En el perro, a diferencia del humano, no hay una región anatómica específica que sea más frecuentemente afectada por los tumores. Estos son generalmente difusos y pueden extenderse al cuello de la vejiga. Leav y Ling (1968) identificaron tres tipos histológicos de adenocarcinomas, a saber: 1) proliferativo intraalveolar; 2) mal diferenciado y 3) acinar pequeño. El tipo proliferativo intraalveolar es el que se presenta con mayor frecuencia.

De la misma forma que en patología humana, ha sido descrito en el perro la lesión precancerosa llamada Neoplasia Prostática Intraepitelial (PIN, por sus siglas en inglés). Esta se presenta en el 55% de los perros de 7 años sexualmente intactos (Waters and Bostwick, 1997).

La causa de los carcinomas prostáticos en perros es desconocida, pero la castración y el envejecimiento parecen incrementar considerablemente el riesgo de aparición (Root-Kustritz and Klausner, 2001). El promedio de edad de aparición del adenocarcinoma es de 9-10 años y es frecuente la presentación de metástasis al momento del diagnóstico. Los sitios más frecuentes de aparición de metástasis son: huesos, pulmón, nódulos

linfáticos ilíacos, vejiga, colon y recto. Las próstatas carcinomatosas suelen ser grandes, irregulares y con presencia de quistes (Gobello and Corrada, 2002; Root-Kustritz and Klausner, 2001).

2.1.1.4.4 Prostatitis

La prostatitis es una infección bacteriana de la próstata que se presenta generalmente como resultado de infecciones ascendentes de la flora normal de la uretra (Ling and Ruby, 1979). Las prostatitis son, en su mayoría, secundarias a algún desorden primario, tales como hiperplasia, neoplasia o metaplasia, porque todas estas patologías predisponen a la glándula a las infecciones al alterar los mecanismos normales de defensa que impiden el movimiento retrógrado de bacterias. Los microorganismos más comúnmente involucrados en las prostatitis caninas son: *E. coli, Staphylococcus* sp, *Streptococcus* sp, *Klebsiella* y *Pseudomonas*. La próstata infectada puede o no estar aumentada de tamaño. En presencia de abscesos prostáticos, puede presentarse prostatomegalia asimétrica severa (Root-Kustritz and Klausner, 2001).

2.2 HORMONAS ESTEROIDEAS Y SUS RECEPTORES

Las hormonas esteroideas (también llamadas esteroides) son moléculas hidrofóbicas relativamente pequeñas (peso molecular aproximado de 300) producidas por diversos órganos del tracto reproductor (testículo, ovario y placenta principalmente) y por las glándulas suprarenales, a partir del metabolismo del colesterol y que, a causa de su peso molecular, atraviesan la membrana plasmática por difusión simple. Estas hormonas se desplazan por la circulación general desde su lugar de origen hasta los tejidos diana donde ejercerán su acción. Tanto, los glucocorticoides como los mineralocorticoides y los esteroides sexuales tienen capacidad para modificar el crecimiento, diferenciación y metabolismo celular. Entre estos últimos, los andrógenos, los estrógenos y la progesterona intervienen, afectan y/o modifican los procesos fisiológicos que ocurren en el tracto reproductivo y por tanto, en la próstata de los machos adultos. Las hormonas esteroideas circulan unidas con alta afinidad a sus proteínas específicas de transporte (*sex-hormone binding globulin* o SHBG) o también unidas con baja afinidad pero alta capacidad a proteínas inespecíficas, entre las que la más importante es la albúmina. Para ejercer su acción, sólo la fracción libre de los esteroides difunde directamente a través de la membrana plasmática de las células diana para unirse a sus proteínas receptoras

intracelulares o receptores de esteroides. En los últimos años, se ha postulado un segundo mecanismo de acción de los esteroides que iría asociado a un *binding* específico de la SHBG y su ligando a un receptor de membrana que, a partir de aquí y posiblemente asociado a una ciclasa, desencadenaría un proceso de transmisión de señal hacia el citoplasma. En cualquier caso, el mecanismo clásico de acción de los esteroides es la entrada del esteroide al citoplasma, la unión de la hormona a su receptor y la consecuente activación del complejo hormona-receptor, inicialmente a través de un cambio en la conformación de dicho complejo. A continuación, este complejo atravesará la membrana nuclear y una vez dentro del núcleo, se unirá a unas secuencias específicas de algunos genes llamadas elementos de respuesta a las hormonas (o HRE por las siglas en inglés de *hormone-responsive elements*). Dicha unión será la responsable de la regulación directa de la transcripción de aquellos determinados genes. Estos receptores tienen estructuras relacionadas entre si y constituyen la superfamilia de receptores de hormonas esteroideas (Alberts *et al.*, 1996).

La respuesta celular a las diferentes hormonas esteroideas (andrógenos, estrógenos y progesterona) es mediada a través de los receptores nucleares existentes para cada una de ellas, es decir, que la acción de los andrógenos en la próstata, así como en otros tejidos, requiere la presencia de los AR para mediar la actividad transcripcional (Bremner *et al.*, 1994). En el transcurso de las últimas décadas se han producido importantes avances acerca del conocimiento de las propiedades moleculares de los receptores de hormonas esteroides y su acción a nivel genómico. Todos estos descubrimientos nos han permitido saber que los receptores de hormonas esteroideas pertenecen a una superfamilia de moléculas que poseen similar localización nuclear de señales, un dominio común de unión al DNA y un sitio específico de unión al esteroide. Las semejanzas moleculares entre los diferentes miembros de esta superfamilia de receptores, sugiere, no solo un ancestro común, sino además, la posibilidad de variadas e importantes interacciones entre los diversos receptores o entre sus productos genómicos (Brenner *et al.*, 1990). Podemos decir entonces, que los distintos miembros de la superfamilia de receptores nucleares actúan como moduladores transcripcionales e incluyen, además de los receptores de hormonas esteroides, los receptores para vitamina D, los retinoides y los llamados receptores huérfanos, porque se desconoce su ligando (Chang and Prins, 1999). La unión de las hormonas esteroideas a sus receptores

específicos induce una modificación de la conformación del receptor, la cual implica la separación del receptor de su proteína chaperona citoplasmática y la exposición de la secuencia de localización nuclear (Simoncini and Genazzani, 2003).

Una célula diana típica contiene aproximadamente 10000 receptores esteroideos, cada uno de los cuales se unirá específicamente con una molécula de hormona esteroidea de forma reversible y con una elevada afinidad (Alberts *et al.*, 1996).

Para lograr un entendimiento completo de la acción de las hormonas esteroideas se requiere integrar el conocimiento sobre la superfamilia de receptores de esteroides con la información disponible sobre la producción, comportamiento y rol genómico de los distintos factores de crecimiento que sirven como comunicadores célula a célula (Brenner *et al.*, 1990). Es sabido que la próstata está constituida por dos compartimentos principales, a saber, el estroma, formado por células musculares lisas, fibroblastos y colágeno, y a donde llegan los capilares sanguíneos y nervios; y las glándulas formadas por las células epiteliales secretoras, responsables de liberar a la luz glandular los productos necesarios para el funcionamiento prostático y las células basales, responsables del recambio de las células maduras secretoras. Entre ambos compartimentos se produce un intercambio de moléculas mediadoras de funciones diversas que con toda probabilidad tendrán gran relevancia en los procesos fisiológicos y patológicos de la próstata (Bonkhoff *et al.*, 1994; Isaacs and Coffey, 1989).

Desde hace varias décadas se han venido realizando diversos experimentos *in vivo e in vitro* con el objeto de conocer el metabolismo de las hormonas esteroideas en la próstata, debido a los numerosos indicios que hacen sospechar que una alteración de la actividad en el metabolismo esteroideo podría ser responsable de la patogenia de algunas patologías prostáticas (Habib, 1991). También se han recopilado evidencias de que la próstata canina es funcional y bioquímicamente muy heterogénea, en contraste con su apariencia histológica, la cual se considera relativamente homogénea y uniforme (DeKlerk *et al.*, 1979; Schulze and Barrack, 1987b).

2.2.1. RECEPTORES DE ANDRÓGENOS (AR)

El mantenimiento y desarrollo de la función secretora de la próstata depende del mantenimiento adecuado del nivel de andrógenos circulantes (Coffey and Walsh, 1990; Isaacs and Shaper, 1985), es decir, que tanto la estructura como la actividad funcional

dependen de la circulación de esta hormona. De allí, la importancia de la testosterona en el desarrollo de la próstata normal y en el curso de la evolución de algunas patologías prostáticas, tanto en el perro como en el humano. La producción de andrógenos es regulada por el eje hipotalámico-pituitario-gonadal (Debes and Tindall, 2002). Los efectos de los andrógenos en los distintos tejidos son mediados a través de los AR. En ausencia de andrógenos, los AR inactivos son secuestrados en el citoplasma como complejos de multiproteínas. Después de la unión del andrógeno al receptor, este último es liberado del complejo multiproteínico para poder iniciar los cambios conformacionales que permitirán que el ligando unido al AR entre en el núcleo (Chatterjee, 2003). Los AR están organizados estructural y funcionalmente dentro de dominios que se encargan de efectuar la translocación nuclear, la dimerización, la unión al DNA y la activación transcripcional y pueden, por tanto, ser detectados por inmunohistoquímica en el núcleo de las células fibro-musculares del estroma y en las del epitelio glandular tanto en humanos como en perros (Murakoshi *et al.*, 2001b). Estos receptores de AR pueden existir dentro de las células diana en estado inactivado y, después de unirse al ligando los receptores ya activados, pueden interactuar con los elementos de respuesta a hormonas (Yeh *et al.*, 1998). Existen de 5000 a 20000 receptores de andrógenos por cada célula (Coffey, 1988).

La presencia de AR tanto en células epiteliales como estromales confirma estudios previos efectuados en ratas acerca de la influencia androgénica sobre ambos componentes celulares (Pelletier *et al.*, 2000; Sar *et al.*, 1990). Las células del epitelio glandular prostático que muestran alta actividad secretora poseen mayor cantidad de AR que las células inactivas (Takeda *et al.*, 1990). Se han efectuado numerosos trabajos sobre los receptores de hormonas esteroideas ante la posibilidad de que, su medición pudiera servir de ayuda para predecir la respuesta de los pacientes cancerosos a la hormonoterapia ya que se asume que los niveles de AR podrían ser pronósticos de respuesta hormonal en el carcinoma prostático en humanos (Pertschuk *et al.*, 1995; Walsh, 1975). Sin embargo, en otras publicaciones más recientes, los autores expresan la opinión de que estos receptores por si solos son insuficientes para predecir la respuesta al tratamiento endocrino. Estas opiniones podrían ser el resultado de las dificultades encontradas para desarrollar estudios fiables sobre los receptores androgénicos (Habib, 1991).

El rol de la señalización entre andrógenos y AR en la etiología de la hiperplasia de próstata y en las displasias que progresan hacia adenocarcinomas ha sido evidenciado en humanos a través de varias líneas de estudio. Primero porque los varones castrados antes de la pubertad no desarrollan ni hiperplasia ni cáncer de próstata. Segundo, porque bloqueando la producción de DHT en la próstata se logra un tratamiento exitoso de los síntomas de HBP y una tercera línea de evidencia se deriva de los estudios realizados con modelos animales de carcinogénesis prostática, ya que en el modelo de cáncer de próstata de ratas Lobund-Wistar se requiere del suministro de andrógeno para el desarrollo del adenocarcinoma prostático después de la administración de un potente carcinógeno químico. En resumen, podemos decir que las señales entre andrógenos/AR desempeñan un papel crítico en la biología de la próstata, ya que en respuesta a ciertas condiciones patológicas los andrógenos y/o los factores de crecimiento pueden promover la hiperestimulación de las células epiteliales de la próstata rompiendo de esta forma el balance entre proliferación y apoptosis de estas células (Chatterjee, 2003).

Utilizando métodos inmunohistoquímicos los AR han sido localizados en una amplia variedad de tejidos humanos y animales, incluyendo tejidos del tracto reproductivo de ambos sexos (Bremner *et al.*, 1994; Goyal *et al.*, 1998; Kadi *et al.*, 2000; Sajjad *et al.*, 2004; Shirasaki *et al.*, 2004; Torlakovic *et al.*, 2005; Vermeirsch *et al.*, 2002). En la glándula prostática, dichos receptores se han observado en el núcleo de las células epiteliales y/o estromales de varias especies (Banerjee *et al.*, 2001; Brolin *et al.*, 1992; Harper *et al.*, 1998; Leav *et al.*, 2001b; Murakoshi *et al.*, 2000b; Pelletier *et al.*, 2000; Prins *et al.*, 1991; Sar *et al.*, 1990; Yamashita, 2004).

Estudios de fluorescencia en células vivas han revelado la localización de AR no unidos a ligando, predominantemente en el citoplasma, pero que después de unirse a los andrógenos son translocados hacia el núcleo de la célula en un período de 10 a 60 minutos (Tyagi *et al.*, 2000). En general, existe muy poca literatura actualizada acerca de la detección inmunohistoquímica de AR en la próstata de perros. Según el estudio de Murakoshi y colaboradores (2001b) la expresión de AR en la próstata de perros se ubica sólo en el núcleo de las células epiteliales y estromales.

2.2.2. RECEPTORES DE ESTRÓGENOS (ER)

La presencia de receptores de esteroides específicos para los estrógenos ha sido

documentada en la próstata canina (Frenette *et al.*, 1982; Robinette *et al.*, 1978; Schulze and Barrack, 1987a; Schulze and Barrack, 1987b). También se ha descrito su presencia en otras especies (Danzo and Eller, 1979; Fisher *et al.*, 1997; Goyal *et al.*, 1997; Hess *et al.*, 1997; Iguchi *et al.*, 1991; Kwon *et al.*, 1997; Nie *et al.*, 2002; Saunders *et al.*, 2001; Schleicher *et al.*, 1984; West and Brenner, 1990). Hace más de 40 años que Jensen y Jacobsen (1962) llegaron a la conclusión de que los efectos de los estrógenos sobre los órganos en los cuales actúan eran mediados a través de una proteína receptora. A partir de este descubrimiento esta proteína fue extensamente estudiada hasta lograr la clonación de este receptor de estrógenos, dándole el nombre de ER. En 1995, con el descubrimiento de la segunda isoforma de los ERs, los primeros en ser descubiertos pasaron a llamarse ERα y los segundos, ERβ. Estudios realizados en próstatas humanas obtuvieron como resultado la detección de ERα en las células estromales de glándulas normales y raramente en algunas células basales (Leav *et al.*, 2001c). En estudios similares realizados en la glándula prostática de perros utilizando técnicas inmunohistoquímicas, los ERα sólo fueron visualizados en células estromales y epitelio de los ductos periuretrales (Schulze and Barrack, 1987a; Schulze and Barrack, 1987b). Cabe mencionar que la expresión de ERβ en células epiteliales luminales de próstata de ratas y de monos ha sido demostrada por los estudios de Saunders y colaboradores (1997) y Pelletier y colaboradores (1999).

Los estrógenos son sintetizados en el tracto reproductivo del macho por tres tipos de células: las células de Sertoli, las de Leydig y las células germinales y actúan como importantes moduladores de las funciones reproductivas normales. Los estrógenos están implicados tanto en el crecimiento normal de la próstata como en los cambios hiperplásicos que se desarrollan en dicha glándula. La acción de los estrógenos, igual que la de los otros esteroides, es mediada por receptores específicos en las células sobre las cuales actúan ya que al unirse al ligando provocan cambios conformacionales que dan como resultado la dimerización y la subsiguiente asociación con regiones específicas dentro de las regiones reguladoras de los genes diana. Estudios experimentales han demostrado que los ERs son importantes en la biología del tracto reproductivo del macho (Zhou *et al.*, 2002). Aunque los ERα y los ERβ son similares en estructura, difieren en los dominios de transactivación C-terminal y N-terminal (Nie *et al.*, 2002). Desde el descubrimiento de los ERβ en el año 1995, se han realizado

numerosos estudios sobre los ERs que han permitido demostrar que esta isoforma de ER se expresa ampliamente en el organismo lo que ha permitido replantear la acción de los estrógenos en los distintos tejidos. Algunos receptores de esteroides son capaces de dimerizar con otros miembros dentro de esta superfamilia para formar heterodímeros (Chang and Prins, 1999) y por tanto existe la posibilidad de formación de heterodímeros entre los ERα y los ERβ, que pueden tener efectos diferentes sobre la activación de genes (Saunders, 1998). Ogawa y colaboradores (1998) sugirieron que los ERβ podrían funcionar como un inhibidor dominante negativo sobre la actividad transactivadora de los ERα. Es importante investigar el papel de los ERβ en la próstata y entender cómo estos modulan la relación autocrina dentro del estroma y la relación paracrina entre el estroma y el epitelio (Ito *et al.*, 2000).

Todos los miembros de la familia de receptores de esteroides comparten seis dominios de función estructural, denominados desde la A hasta la F. El rol funcional de estos dominios de los receptores de esteroides ha sido definido en algunos experimentos, en los cuales algún dominio individual de los receptores ha sido cambiado o donde la actividad de los receptores ha sido modificada por mutagénesis dirigida (Saunders, 1998; Signoretti and Loda, 2001). Aunque la función biológica precisa de las dos isoformas de ER en la próstata de los machos permanece aún indefinida, en un estudio con ratones a los cuales faltaban los ERβ ocurrió el desarrollo de hiperplasia prostática relacionada con la edad, lo cual sugiere que este receptor podría actuar inhibiendo el crecimiento anormal de esta glándula (Krege *et al.*, 1998; Leav *et al.*, 2001b).

Los ERβ se expresan en la próstata normal y sus niveles parecen disminuir en el cáncer de la próstata. Tomando en cuenta que la pérdida de ERβ se asocia a la proliferación del epitelio prostático en ratones experimentales, parece posible que dichos receptores pudieran ser el blanco para el tratamiento de las enfermedades proliferativas de la próstata (Weihua *et al.*, 2002).

2.2.3. RECEPTORES DE PROGESTERONA (PR)

La importancia de los receptores de progesterona (PR) en la fisiología de la hembra ha sido ampliamente estudiada (Dhaliwal *et al.*, 1997; Flores *et al.*, 2001; Geisert *et al.*, 1994; Ing and Tornesi, 1997; Lessey *et al.*, 1988). Recientemente estos estudios han podido ampliarse mediante la utilización de ratones *knock-out* lo que ha permitido

mostrar que las hembras que tienen mutaciones de los PR presentan defectos en todos los tejidos del tracto reproductivo, incluyendo el útero (Conneely *et al.*, 2001). Sin embargo, la importancia de los PR en la fisiología o patologías del tracto genital del macho ha recibido poca atención por parte de los investigadores.

En humanos, se han realizado estudios en próstatas patológicas que demuestran la presencia de receptores de progesterona en la mayor parte de los casos de hiperplasia benigna de la próstata (Bashirelahi *et al.*, 1983; Hiramatsu *et al.*, 1996; Mobbs and Liu, 1990) y en el cáncer de próstata (Bonkhoff *et al.*, 2001; Ekman *et al.*, 1979). Los resultados obtenidos por Bashirelahi han conducido a formular la hipótesis acerca de que la presencia de PR en la hiperplasia prostática podría representar un efecto secundario a cambios en los niveles hemáticos de andrógenos/estrógenos (Habib, 1991).

Se conoce muy poco acerca de la función de la progesterona en la próstata de humanos y otros mamíferos (Hiramatsu *et al.*, 1996) y los pocos estudios realizados hasta el momento para demostrar la localización inmunohistoquímica han arrojado resultados contradictorios. En el estudio realizado por Hiramatsu y colaboradores (1996), la expresión de PR fue observada en el núcleo, tanto en células epiteliales como estromales en las muestras con hiperplasia. En cambio, Wernert y colaboradores (1988) sólo detectaron expresión en células estromales en casos de hiperplasia. De igual forma, el estudio de Bonkhoff y colaboradores (2001) en tumores de próstata demostró la expresión de PR únicamente en células estromales. Hasta el momento no existen estudios sobre la expresión inmunohistoquímica de los receptores de progesterona en la próstata canina.

2.3 ULTRAESTRUCTURA DE LA GLÁNDULA PROSTÁTICA

Las técnicas de ultraestructura han sido aplicadas en el estudio de las enfermedades proliferativas que afectan comúnmente el tracto urogenital del hombre. La HBP y el cáncer de próstata no han sido inmunes al escrutinio del microscopio electrónico. Estudios de microscopía electrónica de transmisión y de *scanning* han permitido el entendimiento de la estructura, función e interrelación entre la vejiga y la próstata (Bostwick *et al.*, 1997; Srigley *et al.*, 1988). Tomando en cuenta que el perro ha sido utilizado como modelo de la HBP del humano, resulta lógico el hecho de que se hayan realizado estudios de ultraestructura en la glándula prostática del perro. De tal manera

que en la década de los 60s y los 70s fueron realizados varios estudios de microscopía electrónica en esta especie (Merk *et al.*, 1980; Merk *et al.*, 1982; Seaman and Winell, 1962). En el estudio realizado por Bartsch y Rohr (1980) fueron descritos los hallazgos observados al microscopio electrónico en perros sin patología prostática y con hiperplasia espontánea de próstata, haciendo especial referencia a la presencia de numerosos gránulos de secreción electrondensos, ubicados en la porción apical de las células epiteliales luminales.

2.3.1. CÉLULAS LUMINALES

En los acinos de la próstata de perros intactos, se observan dos tipos de poblaciones celulares, las células basales de reserva y las células epiteliales glandulares. Las células del epitelio glandular o células luminales tienen forma cuboidal o cilíndrica y su núcleo ocupa la porción basal de la célula y muestra nucleolos prominentes. Un poco por encima del núcleo, puede observarse el aparato de Golgi y un bien desarrollado retículo endoplasmático (RE) rugoso así como una gran cantidad de ribosomas dispersos en el citoplasma y abundantes gránulos secretorios electrondensos en la porción apical de la célula (Bartsch and Rohr, 1980). La membrana plasmática está localizada hacia la base de las células luminales y en algunas partes de los acinos está fuertemente asociada con las células de músculo liso. Las mitocondrias están localizadas en el área perinuclear o en la región basal de la célula (Seaman and Winell, 1962). Colocadas entre las células luminales vecinas podemos localizar algunas células basales, así como la presencia de los complejos de unión propios de las estructuras glandulares.

2.3.2. CÉLULAS BASALES

La ultraestructura de las células basales en la próstata del perro fue someramente descrita por Seaman y Winell en 1962, pero sin atribuirles ninguna función (Seaman and Winell, 1962).

Las células basales están situadas por fuera y entre células luminales secretorias vecinas y, en contraste a ellas, las basales no llegan hasta el lumen de los acinos. Por lo general su forma es aplanada o cúbica y frecuentemente se encuentran en cercana asociación con los vasos sanguíneos y su orientación es paralela al eje longitudinal de la lámina basal. El núcleo de estas células, ocupa una gran parte de la célula y frecuentemente es invaginado. Los contornos laterales y apical de las membranas de la célula basal se

introducen e interdigitan con las membranas de las células secretoras adyacentes. Se describió la presencia de vesículas de pinocitosis en la membrana plasmática de las células basales (Timms *et al.*, 1976). En la actualidad, se sabe que se trata de estructuras denominadas caveolas.

2.3.2.1 Caveolas

En la superficie de muchas células, existen minúsculas invaginaciones de la membrana llamadas caveolas. Las caveolas son estructuras dinámicas de la membrana que están abiertas para recibir material o cerradas para poder procesarlo, almacenarlo o liberarlo. Las células son constantemente bombardeadas por estímulos mecánicos provenientes tanto de las células que las rodean como de la matriz extracelular. Estos impulsos podrán ser correctamente interpretados si la célula mantiene una relación apropiada con sus células vecinas en el tejido. La forma y maleabilidad de la célula esta controlada por un citoesqueleto que está compuesto, entre otros, por filamentos de actina (Anderson, 1993). Las caveolas son microdominios especializados que aparecen como invaginaciones en forma de bolsillo en la superficie de muchas células (Liu *et al.*, 1997a). Numerosos estudios morfológicos han documentado que los caveolas median el movimiento de moléculas a través de las células endoteliales (Simionescu, 1983).

Yamada, en 1955 propuso el nombre de caveolas (pequeñas cuevas) intracelulares para definir "una vesícula, un bolsillo, una cueva o una hendidura pequeña que se comunicaba con el exterior de la célula" en las células epiteliales de la vesícula biliar (Anderson, 1998; Yamada, 1955).

Las caveolas se conocen sobre todo por su capacidad para transportar las moléculas a través de las células endoteliales, pero las técnicas celulares modernas han ampliado drásticamente nuestro conocimiento acerca de ellas. Forman un compartimento endocítico y exocítico único en la superficie de la mayoría de las células y son capaces de importar moléculas y de entregarlas a las localizaciones específicas dentro de la célula, así como de exportar las moléculas al espacio extracelular, y de compartimentar una variedad de señales (Anderson, 1998).

Tres genes de las caveolinas han sido identificados en los mamíferos. La caveolina-1 es una proteína integral de la membrana que está presente en el material estriado que adorna la superficie interna de la membrana de las caveolas de fibroblastos y células

endoteliales. Una función potencial de la caveolina 1 es suplir colesterol a las caveolas. La integridad estructural de las caveolas de los fibroblastos depende del colesterol y, por otro lado, tanto el colesterol como los ácidos grasos facilitan la oligomerización de la caveolina 1 (Liu *et al.*, 1999).

En base únicamente a su aspecto ultraestructural como invaginaciones de la membrana plasmática, al principio se pensaba que las caveolas funcionaban como vesículas de transporte macromolecular. Inicialmente, la única función propuesta estaba limitada al proceso del pinocitosis. Sin embargo, con el advenimiento de nuevas herramientas, se ha podido estudiar mejor su función y su papel como transportador vesicular se ha ampliado para incluir tanto la endocitosis como la transcitosis, la homeostasis del colesterol y diversos procesos de transducción de señales (Cohen *et al.*, 2004).

Tradicionalmente se ha pensado que las hormonas esteroides actúan exclusivamente por unión a los receptores nucleares, los cuales posteriormente llevan a cabo la transcripción de los genes diana. Recientemente han aparecido evidencias que apoyan acciones rápidas, también llamadas no genómicas, de los esteroides; más aun, se han detectado receptores de estrógeno (ER) en la membrana plasmática de las células (Razandi *et al.*, 2000). En general, los efectos no genómicos de los esteroides se caracterizan por: 1. Ocurrir demasiado deprisa para implicar cambios en el RNAm y la síntesis de proteínas (desde segundos a pocos minutos). 2. Observarse incluso en células altamente especializadas que no realizan síntesis de proteínas o de RNAm (por ejemplo, los espermatozoides). 3. Desencadenarse incluso por esteroides unidos a sustancias de alto peso molecular que no pasan a través de la membrana plasmática y no entran a la célula. 4. No ser bloqueados por los inhibidores de RNAm o de la síntesis de proteínas. 5. No ser bloqueados por los antagonistas de los receptores de esteroides genómicos. y 6. Ser altamente específicos, pues esteroides con una estructura química muy similar, pero no idéntica, muestran varios grados de potencia en ejercerlos. Existen evidencias que indican que en varios tipos de células del tracto reproductivo, los estrógenos ejercen unos efectos fisiológicos tempranos que son demasiado rápidos para que puedan ocurrir por medio de la secuencia de activación genómica. Los tipos celulares en los cuales estas respuestas celulares rápidas a los estrógenos han sido interpretadas como resultado de la transducción de una señal esteroide de la superficie de la célula son las células endometriales, células de la granulosa, ovocitos y espermatozoides. Este mismo tipo de

efectos fisiológicos también ocurre con los andrógenos. La mayoría de las acciones no genómicas de los esteroides que han sido descritas involucran el calcio como un segundo mensajero (Revelli *et al.*, 1998).

En resumen, las caveolas constituyen un complejo sistema de membranas cuya función está muy probablemente relacionada, entre otros, con el procesamiento de señales hormonales. Las caveolas constituyen un importante foco de investigación para comprender la organización y actividad celular de las numerosas moléculas que se relacionan con ellas (Anderson, 1998).

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Capítulo III

EXPRESSION OF ANDROGEN, ESTROGEN α AND β, AND PROGESTERONE RECEPTORS IN THE CANINE PROSTATE: DIFFERENCES AMONG NORMAL, INFLAMED, HYPERPLASTIC AND NEOPLASTIC GLANDS

3.1 ABSTRACT

The relevance of steroid receptors in both normal prostate and in prostatic pathology is well recognized, but tissue-specific distribution of some of them in the different cell compartments of the canine prostate gland is not completely characterized. We analysed the immunohistochemical expression of androgen (AR), oestrogen α (ERα) and β (ERβ) and progesterone (PR) receptors in the different cell types of the canine prostate gland, either normal or with different pathologic conditions (hyperplasia, prostatitis, carcinoma). AR labelling was 100% in the epithelial cells of normal and hyperplastic tissue, 74% in prostatitis, and 65% in carcinomas. ERα was 85% in normal cases, 35% in hyperplastic cases, 22% in inflamed cases, and 12% in neoplastic glands. In this study, we have investigated for the fist time the expression of ERβ and PR in the canine prostate. ERβ expression was 85% in normal cases and around 70% in all of the pathological conditions. On the other hand, PR expression was weak and less common in normal tissue (44%), when compared to hyperplasia (70%), prostatitis (62%) and cancer (64%). Overall, the expression of AR, ERα and ERβ was highest in normal glands, and decreased in hyperplasia, prostatitis, and cancer. Conversely, PR expression was lower in normal epithelial cells and tended to increase in pathological conditions. The characteristic expression patterns of steroid receptors support their differential role in prostatic diseases. The present study in canine prostate supports the proven usefulness of this model to investigate steroid physiology and it may be also very helpful in both descriptive and mechanistic studies in this field.

3.2 INTRODUCTION

Among all mammals, only humans and dogs have a propensity to spontaneously develop benign prostatic hyperplasia (BPH) and adenocarcinoma (Berry and Isaacs, 1984; Berry *et al.*, 1986b; Lowseth *et al.*, 1990; McNeal, 1978; Osterling, 1991; Strandberg and Berry, 1987). The prevalence of BPH approaches 100% in dogs above 7 or 8 years of age (Berry *et al.*, 1986b; Lowseth *et al.*, 1990; Strandberg and Berry, 1987). While canine prostate cancer is relatively rare (Bell *et al.*, 1995; Krawiec and Heflin, 1992; Ladds, 1993), carcinoma of the human gland is one of the most frequent causes of death from cancer in older men (Boring *et al.*, 1993).

Although the exact pathogenesis of prostatic disorders is not well understood, hormonal factors are clearly involved. Androgens are involved in the development and physiology of male accessory sex organs, as well as in the function of other organs and tissues (Carson-Jurica *et al.*, 1990). By immunohistochemistry (IHC), androgen receptor (AR) has been localized in a variety of human tissues, in both sexes, including the reproductive organs (Kimura *et al.*, 1993; Ruizeveld de Winter *et al.*, 1994). Similarly, in the rat and the dog, AR has also been localized not only in male, but also in female reproductive tissues (Hirai *et al.*, 1994; Murakoshi *et al.*, 2000c; Pelletier, 2000; Sar *et al.*, 1990; Tetsuka *et al.*, 1995; Vermeirsch *et al.*, 2001).

ERs and PR play a pivotal role in oestrogen target cells and oestrogen-dependent tumours. Although prostatic adenocarcinoma is considered a classical target of androgens, oestrogens may also be involved in its natural history. The recent discovery of the ERα in pre-malignant lesions, as well as in metastatic and androgen-insensitive tumours, suggests that oestrogens can affect prostatic carcinogenesis and tumour progression through a receptor-mediated process (Bonkhoff *et al.*, 1999). The presence of PR in a significant number of metastatic and androgen-insensitive prostatic adenocarcinomas suggests that these tumours harbour a functional ER, mediating the downstream events of oestrogen signalling. This supports the concept that prostate cancer cells can escape androgen deprivation by using oestrogens for their growth and maintenance (Bonkhoff *et al.*, 2001).

ERα has been reported in the male and female reproductive system of several species, including dogs (Schulze and Barrack, 1987b), mice (Krege *et al.*, 1998; Rosenfeld *et al.*, 1998), rats (Kuiper *et al.*, 1996a; Pelletier, 2000; Saunders *et al.*, 1998), rabbits (Danzo and Eller, 1979; Danzo *et al.*, 1983), roosters (Kwon *et al.*, 1997), goats (Goyal *et al.*, 1998; Goyal *et al.*, 1997), monkeys (Fisher *et al.*, 1997) and humans (Linja *et al.*, 2003; Tsurusaki *et al.*, 2003). While the tissue distribution of ERβ and PR in the human prostate has been described in detail (Hiramatsu *et al.*, 1996; Papadimitriou *et al.*, 1992; Pasquali *et al.*, 2001), expression of ERβ and PR in canine prostate has not been studied.

The complex interactions of steroid hormones with the different cell subsets of the prostate are essential in the pathogenesis of prostate diseases, particularly hyperplasia

and cancer. The aim of this work has been to analyse the expression of AR, ERα, ERβ and PR in normal, inflamed, hyperplastic and neoplastic canine prostate glands. The distributions of ERβ and PR in the canine prostate are documented for the first time in the present study.

3.3 MATERIAL AND METHODS

3.3.1. SELECTION OF SAMPLES

Archival paraffin-embedded prostate specimens from the Veterinary Pathology Diagnostic Service of the Universitat Autònoma de Barcelona were used in this study. These tissues were obtained through autopsies or biopsies, from mixed-breed, sexually mature dogs.In addition, prostate samples were obtained from five normal Beagles, aged 18-24 months, used in another project for different purposes. Samples were diagnosed as normal prostate glands (n=8), hyperplasia (n=11), prostatitis (n=11) and carcinoma (n=8). They were fixed in 10 % neutral buffered formaldehyde, dehydrated, and embedded in paraffin. Five-μm thick sections were cut from each block and used for IHC.

3.3.2. REAGENTS

The immunohistochemical detection of AR was performed using a polyclonal antibody (NCL-Arp,) at 1:20 dilution for 30 min. The antibody against the ERα was a monoclonal mouse antibody (NCL-ER-LH2,) at 1:50 dilution for 30 min. For ERβ, a polyclonal rabbit antibody (H-150: sc-8974) was used at 1:200 dilution for 1 hour and for PR, a monoclonal antibody (PR88) at 1:20 dilution for 30 min.

3.3.3. IMMUNOHISTOCHEMICAL STAINING PROCEDURE

Sections for immunohistochemical analysis were placed on positively-charged slides and air-dried for 90 min at 60ºC. After deparaffinization in xylene and rehydration, the sections were pretreated with a sterilizer, heating the slides in antigen retrieval citrate buffer solutionat pH 7.4 for 1 min at 121ºC. The slides were allowed to cool in buffer for 30 min at room temperature, and were placed on autostainer platforms. The immunolabelling was based on the EnVision methodwhich is a simple, two-step visualization system with very high sensitivity, based on a unique, registered compound

consisting of an enzyme-conjugated polymer backbone that, in addition, also carries secondary antibody molecules. The sections were then counterstained with haematoxylin, dehydrated, mounted in DPX, and examined with a light microscope Olympus B40 at x 400 magnification.

Positive and negative external controls were included in each labelling procedure. In all immunostained batches, the omission of the primary antibody and substitution with the diluting solution alone (without adding antibody) served as a negative control. Both cryostat sections of normal canine prostate gland, and paraffin sections of normal human prostate were used as a positive controls. For ER and PR, bitch ovary and uterus were also used as controls.

3.3.4. SCORING OF IMMUNOHISTOCHEMICAL RESULTS

For each dog, one tissue section was labelled for each steroid receptor expression. In every labelling procedure, both positive and negative controls were included. The receptor expression in each tissue section was examined microscopically, at x 400 magnification by 2 independent observers, who were not informed about the details of the protocol and other clinical data. When there was a discrepant score between the observers, consensus was reached in a third observation, made by both observers at the same time.An intensity score and a proportional score were obtained in every case. The intensity score reflected the intensity of the positive reaction in the cell nuclei or cytoplasm (0: no labelling; 1+: weak labelling; 2+: moderate labelling; 3+: strong labelling; 4+: very strong labelling), whereas the proportional score reflected the percentage of cell nuclei that labelled positive in the different cell aggregates. These two scores were obtained in areas containing a similar amount of glandular epithelium and stromal cells. A minimum of one hundred parenchymal and stromal cells were evaluated in each sample.

3.4 RESULTS

In the present study, the cellular localization of AR, ERα, ERβ and PR expression in the prostate tissue of adult male dogs was investigated. The presence of the receptors was visualized by IHC on paraffin sections, in order to compare the intensity and the topographic distribution of these receptors in cases of normal prostate, hyperplasia,

prostatitis, and cancer. The labelling intensity and percentage distribution for the different receptors in the various disorders studied are shown in figure 1.

3.4.1. ANDROGEN RECEPTOR EXPRESSION

Positive labelling was observed in the nuclei of epithelial and stromal cells and also, to a lesser degree, in the cytoplasm of the epithelial cells. Labelling was absent in all of the negative controls. Results for AR were similar in normal (Fig 2a) and hyperplastic epithelial cells, both in intensity (3-4+) and percentage (≥ 95%). A decrease in the intensity of labelling and the percentage of positive epithelial cells was observed in prostatitis (2+ and ~ 74%, respectively) (Fig 2b). The lowest positive reaction was observed in carcinoma (Fig 2c), with 58% of positive cells. The labelling intensity of tumour tissue was very heterogeneous, with areas showing very high reactivity (3+) and areas with weak or no labelling. In all cases, a small percentage (10-20%) of positive stromal cells was observed.

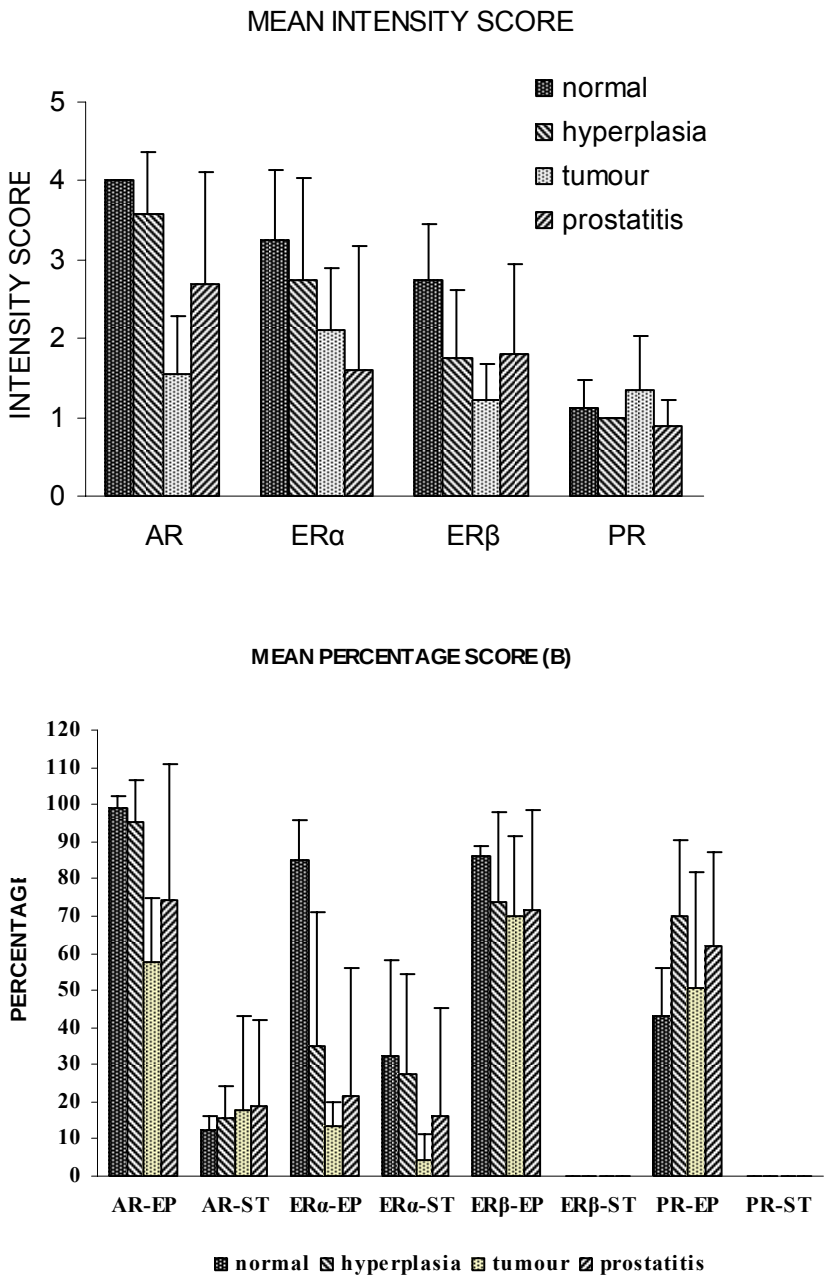

Figure 1. Mean intensity (a) and proportional (b) scores for androgen, estrogen α and β, and progesterone hormone receptors in canine epithelial and stromal cells from histological sections of prostatic canine tissue, classified as normal, hyperplastic, neoplastic, or inflamed.

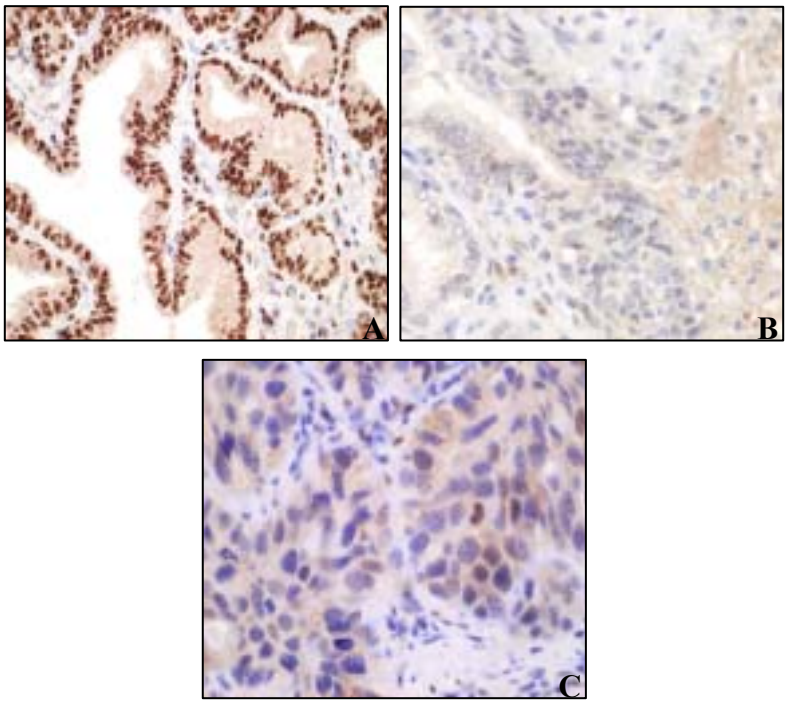

Figure 2. Immunolocalization of AR in normal (A), inflamed (B) and neoplastic (C) canine prostate. A: Strong expression of the receptor was evident in epithelial cells. The presence of cytoplasmic labelling in the epithelial cells was also apparent. Nuclear labelling was evident in stromal cells. B: The intensity of nuclear labelling decreased compared to normal or hyperplasic glands. C: No labelling for AR was found in the majority of epithelial cells. Nuclear labelling was also observed in stromal cells.

3.4.2. A AND β ESTROGEN RECEPTOR EXPRESSION

For ERα and ERβ, labelling was observed only in nuclei. Positive labelling was absent in all of the negative controls. An exclusive nuclear localization of ERα was detected both in epithelial and stromal cells, with 85% of positive epithelial cells with 3+ labelling intensity in normal glands (Fig 3a) and lower percentages in hyperplastic (35%) (Fig 3b), inflamed (22%), and neoplastic (13%) (Fig 3c) glands.

Nuclear ERβ labelling was uniformly present in prostatic epithelial cells, but absent in stromal cells. Labelling was totally absent in the negative controls. The highest percentage of ERβ-positive cells (85%) and labelling intensity (3+) was detected again in normal prostatic tissue, with a similar decrease of around 70% and 1-2+, respectively, in all of the pathological conditions (Figure 4). From the 38 samples, eight were not immunoreactive for ERα and two for ERβ.

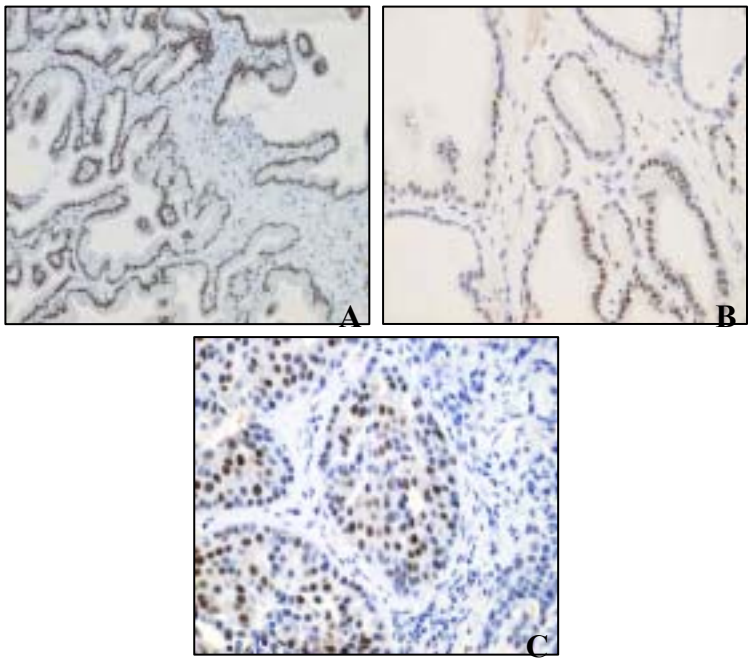

Figure 3. Immunolocalization for ERα in normal (A), hyperplastic (B) and neoplastic (C) canine prostate. A: Strong nuclear labelling was observed in the majority of epithelial cells. Nuclear labelling was also evident in 30% of stromal cells. B: The intensity of nuclear labelling of epithelial cells varied from strong to weak. Nuclear labelling was also evident in 30% of stromal cells. C: Strong nuclear labelling was observed in 12% of epithelial cells. Nuclear labelling was detected in a very low percentage of stromal cells.

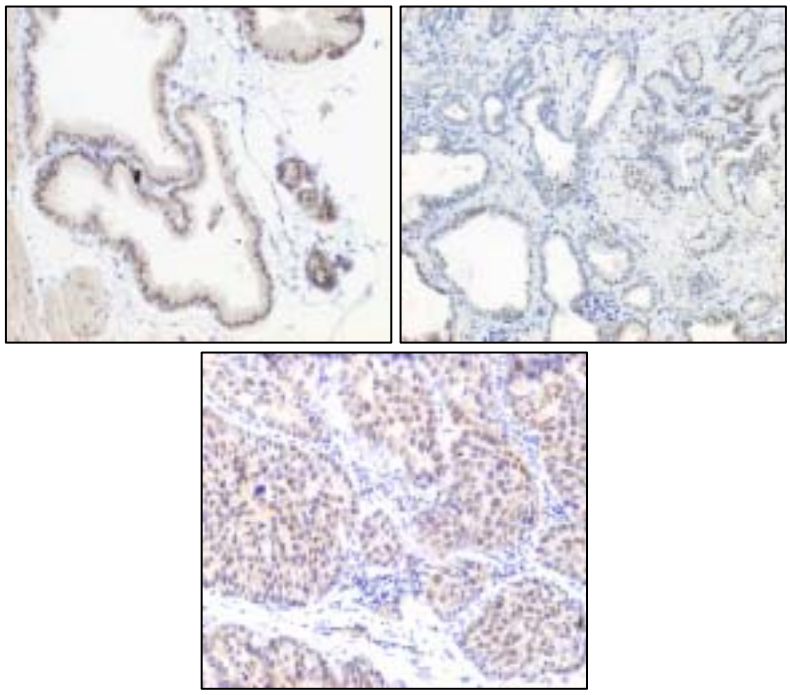

Figure 4. Immunolocalization for ERβ in hyperplastic (A), inflamed (B) and neoplastic (C) canine prostate. An absence of positive labelling for ERβ was observed in stromal cells for all tissues studied. A: Intense nuclear labelling was detected in the majority of epithelial cells. B:

Weak to negative labelling was observed in inflamed areas, whereas the remaining cells were strongly to moderately labelled. C: Note the strong nuclear labelling in the majority of epithelial cells.

3.4.3. PROGESTERONE RECEPTOR EXPRESSION

PR positivity was restricted to the nuclei of epithelial prostatic cells. Nuclei of stromal cells were completely negative. Negative controls showed complete lack of labelling. The percentage of positive epithelial cells varied from 43% in normal areas (Fig 5a) to 70% in hyperplastic (Fig 5b), and 62% in inflammatory areas. PR labelling intensity was weak in 95% of the samples studied. In positive controls (bitch ovary and uterus), labelling intensity varied from weak to strong with the same antibodies and dilutions. In carcinomas, mean PR expression was around 50%, but with marked heterogeneity among different cases, positively percentages varying from 10 to 90% (Fig 5c). Labelling intensity in this subset was usually weak, except in one case corresponding to a prostatic adenocarcinoma with solid and cribriform nodules, in which a moderate labelling intensity was observed.

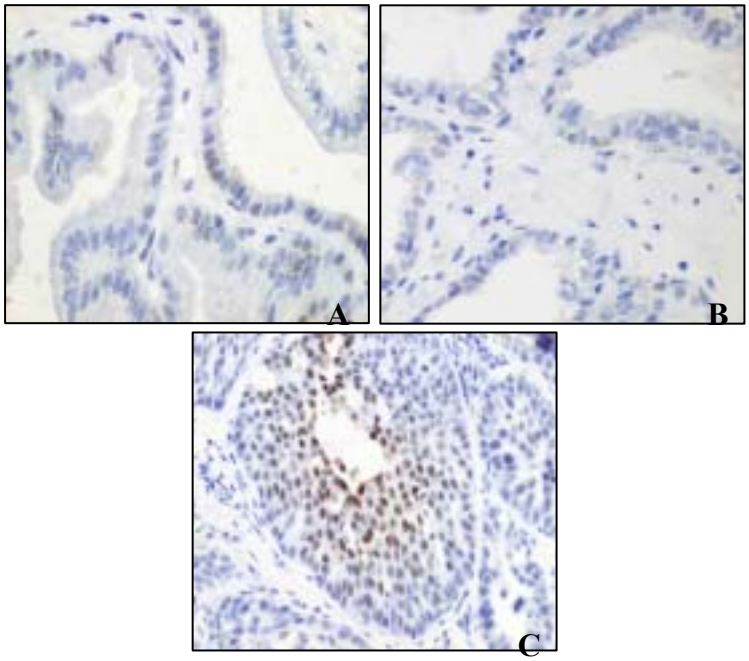

Figure 5. Immunolocalization for PR in normal (A), hyperplastic (B) and neoplastic (C) canine prostate. An absence of positive labelling for PR was observed in stromal cells for all tissues studied. A: Weak labelling was detected in the majority of the nuclei of epithelial cells. B: Weak nuclear immunolabelling was evident in 70% of epithelial cells. C: Strong nuclear labelling was observed in a low percentage of epithelial cells, whereas the remaining cells were weakly to negatively labelled.

3.5 DISCUSSION

In the present descriptive study we used IHC on paraffin sections from prostatic tissue to investigate the normal distribution pattern of AR, ERα, ERβ and PR in healthy dogs, as well as their changes in the different spontaneous canine prostate diseases. To the best of our knowledge, expression of ERβ and PR are reported for the first time in this series. The demonstration of most nuclear receptors in paraffin-embedded tissue sections by IHC is very difficult and usually requires antigen retrieval pre-treatment, in order to unmask the epitopes that become antigenically inert because of fixation and processing (Taylor *et al.*, 1994).

3.5.1. ANDROGEN RECEPTORS

In our study, ARs were located predominantly in the nucleus of the epithelial cells and to a lesser degree in the nucleus of the stromal cells. In normal and hyperplastic canine prostate samples, the percentage of positive epithelial cells was very similar, while it was markedly reduced in the other disorders studied. These results are similar to those reported in canine (Murakoshi *et al.*, 2000b; Murakoshi *et al.*, 2000c) and rat (Pelletier, 2000) tissues, in which AR has been detected in the nuclei of both glandular epithelial and fibro-muscular stromal cells. This localization of AR was noted in both healthy dogs and dogs with BPH (Murakoshi *et al.*, 2000b; Murakoshi *et al.*, 2000c). Similarly, in BPH, human prostate epithelial cells show uniformly intense nuclear labelling for AR (Sar *et al.*, 1990).

3.5.2. OESTROGEN RECEPTORS

Oestrogens are involved in the control of growth and cellular differentiation of the prostate in male animals and take part in the development of canine prostatic disorders. They are believed to play a critical role in the pathogenesis of BPH. Whereas treatment of castrated dogs with androgen alone is not sufficient to induce BPH, the concomitant administration of oestradiol plus androgen (estradiol-17β plus 5α-dihydrotestosterone) does induce BPH (Winter *et al.*, 1995; Winter and Liehr, 1996). To date, two isoforms of ER have been identified, represented by the abbreviations ERα, for the first that was studied, and ERβ, for the last one discovered, with several variants for both ERα and ERβ.

In our study, immunolabelling for ERα was detected predominantly in the nuclei of both glandular epithelial and stromal cells, in all of the samples. One of the most remarkable features of this receptor was its heterogeneous distribution within any given case, independent of the percentage of positive cells and also regardless of the overall intensity of the reaction. Similar observations have been reported in human prostatic tissue (Papadimitriou *et al.*, 1992).

Schulze and Barrack (1987b) reported ER labelling confined to the nuclei of the canine prostatic stroma and the prostatic duct epithelium, but, in contrast with our findings, they did not find any specific labelling in the acinar epithelium. These differences in the location of ERα, with respect to the observations made in our study, could be related to limited specificity of the antibody used, ineffective methods of antigenic retrieval, differences in sample processing, or differences in the sensitivity of the employed technique (Pasquali *et al.*, 2001).

In the diseases included in this series, a decrease in the percentages of ERα-positive epithelial prostate cells was observed. Oestradiol enhances androgen-induced glandular hyperplasia in the canine prostate. If there are less ER in BPH than in normal prostatic epithelial cells, alternative mechanisms that could account for the synergistic effect of oestrogen on the glandular epithelium, including an indirect effect through prostatic stroma, need to be considered (Cunha *et al.*, 1983). Moreover, the observed reduction in AR, but also in ERα and ERβ expression, in the malignant neoplastic epithelial cells in this study, supports the contention that the actions of oestrogens on the prostate are very complex and probably, in combination with androgens, have dual effects in the development of prostate cancer, by means of incomplete answers from their respective receptors (Bonkhoff *et al.*, 1999).

In the canine prostate, ERβ was found to be highly expressed in the epithelial secretory cells, while no ERβ was detected in stromal cells. Similarly, in the human prostate, Enmark *et al*. (1997), also detected ERβ mRNA in epithelial secretory cells, while the stroma was totally unlabelled. In the monkey, Pelletier *et al*. (1999) reported that ERβ mRNA was exclusively expressed in epithelial secretory cells. Similarly, ERβ immunoreactivity was observed in rat prostatic epithelial cells, while the stroma was

unlabelled (Pelletier, 2000). A reduction in ERβ was found in pathologic prostate glands and this is in keeping with recent experimental studies suggesting an antiproliferative effect of this receptors in prostatic epithelium (Krege *et al.*, 1998; Weihua *et al.*, 2002).

3.5.3. PROGESTERONE RECEPTORS

The presence of PR in the prostatic tissue of dogs suggests that they also may play a role in the physiology and pathology of this gland. With the demonstration of PR in prostatic hyperplasia and neoplasia in humans (Hiramatsu *et al.*, 1996), as well as in the canine diseases investigated in the present study, further research is needed to clarify the effect of progesterone on the prostate. This has not been analysed in detail in either men or dogs, but the evidence suggests that it could be quite relevant (Hiramatsu *et al.*, 1996). It is well established that transactivation of the PR gene is enhanced and maintained by oestrogens through an ER-mediated process (Kastner *et al.*, 1990). Therefore, the presence of PR in human tissue is a widely accepted marker for a functional ER pathway. Bonkhoff *et al*. (2001) suggested that the gradual emergence of PR expression during tumour progression obviously reflects the ability of metastatic and androgen-insensitive tumours to use oestrogens through an ERα-mediated pathway. These findings could have therapeutic implications in androgen-insensitive tumours.

The results of the present study show that the expression of AR, ERα and ERβ is highest in normal glands, with a progressive decrease in hyperplasia, prostatitis, and cancer. ERα shows a similar pattern in the stromal cells. On the other hand, the expression of PR in the dog prostate is lower in normal epithelial cells and shows a gradual increase in pathological conditions. Both ERβ and PR, whose distribution in the canine prostate is reported for the first time in this study, are expressed in epithelial but not in stromal cells. The differential patterns of the four receptors support their role in the pathogenesis of prostatic diseases. The canine prostate may provide a valuable model for use in the investigation of prostatic pathology.

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Capítulo IV

IMMUNOLOCALIZATION OF ANDROGEN, OESTROGEN α AND β, AND PROGESTERONE RECEPTORS IN EXPERIMENTALLY INDUCED CANINE PROSTATIC HYPERPLASIA

4.1 ABSTRACT

Benign prostatic hyperplasia (BPH) is an age dependent prostatic disease affecting male humans and dogs. In dogs, the combined administration of oestrogens and androgens synergistically increases prostate weight, and continued treatment leads to the development of glandular hyperplasia. The aim of the present study was to examine the immunohistochemical expression of androgen (AR), oestrogen α (ERα) and β (ERβ) and progesterone (PR) receptors in the different cell types of the prostate gland in a model of experimentally induced canine prostatic hyperplasia. Five male Beagle dogs were castrated and treated with 25 mg of 5α-androstane-3α, 17β-diol and 0.25 mg 17β-estradiol for 30 weeks. Prostate specimens were surgically obtained every 45 days (experimental stages M0 to M6). The control group consisted of three non-castrated dogs treated with vehicle, from which specimens were only taken at the time points M0, M1, M4 and M6. Immunohistochemical data revealed high AR and ERα expression in the epithelial and stromal cell nuclei of all the experimental and control specimens. Weak staining of the cytoplasm was only observed in epithelial cells. The suspension of hormone treatment led to a significant reduction in the expression of both receptors. On the contrary, ERβ was only expressed in epithelial cell nuclei, with no significant differences in the percentages of stained nuclei between control and hormonally treated or atrophic prostates. Weak staining for PR was observed in a small proportion of epithelial cell nuclei but not in stromal cells. Positivity towards PR remained similar during BPH induction and a significant decrease was observed at the end of the hormone regime. Results indicate that AR, ERα, ERβ and PR are differently expressed in canine prostate tissue and that they show specific expression patterns in response to the hormonal induction of BPH.

4.2 INTRODUCTION

The prostate is an androgen regulated exocrine gland of the male reproductive system, which acts as a major glandular source for the non-cellular components of semen. This is particularly true in the dog, since the prostate is the only accessory sexual gland. Although the specific functions of the prostate are not yet fully understood, it is thought to be important in fertility and protecting the lower urinary tract from infection

(Chatterjee, 2003).

Although all male mammals have a prostate gland, only humans and dogs have an age related propensity to develop benign prostatic hyperplasia (BPH) and adenocarcinoma. BPH is common in aging humans and dogs (Berry *et al.*, 1986a; Berry *et al.*, 1986b; Lowseth *et al.*, 1990; Strandberg and Berry, 1985). However, while canine prostatic cancer is relatively rare (Bell *et al.*, 1995; Krawiec and Heflin, 1992; Leav and Ling, 1968), carcinoma of the human gland is the most frequent cause of death from cancer in older men (Boring *et al.*, 1993). In both species, BPH and carcinoma share several morphological and clinical features, but distinct differences emerge when the two diseases are compared in human and dogs (McNeal, 1978; McNeal, 1983).

Steroid hormones regulate innumerable body functions, including male and female reproduction. The biological action of steroid hormones is mediated through hormone-specific receptors located in specific cells in target tissues. Androgens play a central role in regulating the growth of the mammalian prostate gland through the androgen receptor (AR). The secretory epithelial cells of the prostate express AR, requiring chronic androgenic stimulation for their survival and functional integrity. When androgen levels drop below a threshold, such as occurs following surgical (Berry *et al.*, 1986a) or chemical (Forti *et al.*, 1989) castration, secretory cells undergo apoptosis, causing involution of the gland. The essential role of the AR/androgen signalling pathway in the aetiology of BPH remains unclear (McConnell, 1991) but AR localisation has been detected in both healthy and hyperplastic prostates: human (Leav *et al.*, 2001b; Sar *et al.*, 1990); dog (Murakoshi *et al.*, 2000b; Murakoshi *et al.*, 2000c).

Oestrogens are believed to play a critical role in the pathogenesis of BPH. Whereas the treatment of castrated dogs with androgen alone is not sufficient to induce BPH, the concomitant administration of oestradiol plus androgen (5α-androstane-3α, 17β-diol or 5α-dihydrotestosterone) does induce BPH (DeKlerk *et al.*, 1979; Leav *et al.*, 2001c; Walsh and Wilson, 1976). The effects of oestrogens on target tissues are now know to be mediated by two ligand-specific transcription factor receptor proteins termed oestrogen receptors α and β (ERα and ERβ). Both receptors occur in many of the same tissues but differences in organ and tissue distribution as well as in expression levels have been reported for the two isoforms (Kuiper *et al.*, 1997; Kuiper *et al.*, 1996b;

Mosselman *et al.*, 1996). The presence of ERα is confined to connective tissue nuclei both in healthy (human: Leav *et al.*, 2001b; rat: Sar and Welsch, 2000; dog: Schulze and Barrack, 1987b) and hyperplastic (human: Hiramatsu *et al.*, 1996; Leav *et al.*, 2001b) prostate glands. Studies in the different species have localized ERβ in the epithelial cell nuclei of healthy rat prostates (Sar and Welsh, 2000; Pelletier et al., 2000) or in the epithelial and, to a lesser extent, stromal cell nuclei in human healthy and hyperplastic prostate tissue (Fixemer *et al.*, 2003; Pasquali *et al.*, 2001). Similarly, ERβ mRNA was found to be predominant over the ERα isoform in the rat prostate (Kuiper *et al.*, 1996b).

The possible role of progesterone in the development and biological behaviour of BPH has not yet been explored in detail, but it is well established that transactivation of the PR gene is enhanced and maintained by estrogens through an ER-mediated process (Kastner *et al.*, 1990). Several studies have identified the presence of PR in human prostate hyperplasia and cancer (Bonkhoff *et al.*, 2001; Hiramatsu *et al.*, 1996) but as far as we know this issue has not been addressed in canine tissue.

To improve our understanding of the role of androgens, oestrogens and progesterone during the course of induced BPH in dogs, the exact site(s) of action of these sex steroids needs to be established. This study was designed to identify the cell types expressing AR, ERα, ERβ and PR along with expression patterns in canine prostate tissue. We undertook an immunohistochemical study to locate these receptors during the course of BPH in dogs experimentally induced by the administration of 5α-androstane-3α, 17β-diol in combination with 17β-estradiol

4.3 MATERIALS AND METHODS

4.3.1. ANIMALS

Eight male Beagle dogs (1.5/2 years) were used to establish an experimental group, G I (n = 5), and a control group G II (n = 3). The dogs were individually housed, fed a standard commercial diet and provided with water *ad libitum*. The care and treatment of animals was performed according to the guidelines established by the School of Veterinary Sciences of the Autonomous University of Barcelona.

4.3.2. HORMONE TREATMENT

Animals in G I were castrated and intramuscularly injected with 25 mg of 5α-androstane-3α, 17β-diol, and 0.25 mg of 17β-estradiol (Steraloids Inc., Newport, USA). The three G II dogs only received 1 ml of the triolein vehicle. Treatment was administered 3 times weekly (Monday, Wednesday, and Friday) for 30 weeks. The intramuscular injections were alternately administered to the hind legs. Surgical biopsies of the prostate were obtained from alternate quadrants: the first at baseline before castration and treatment, and then at 6-week intervals for 36 weeks. These 7 experimental stages were denoted M0 to M6. As for M0, specimens obtained at the last stage (M6) lacked hormone treatment. Specimens from control animals were only obtained at stages M0, M1, M4 and M6.

4.3.3. SURGICAL PROCEDURES

The dogs were fasted for 24 h before surgery and then premedicated with 0.4 mg/kg morphine sulphate (Morfina, BBraun, Spain) and 0.05 mg/kg acepromazine maleate (Calmo Neosán, Pfizer Salud Animal, Spain). A single dose of 4 mg/kg of propofol (Propofol Lipuro® 1%, BBraun, Spain) was given intravenously for anaesthesia. The trachea was intubated with a 9-10 mm endotracheal tube, and anaesthesia was maintained with 1.5-2% isofluorane (IsoFlo®, Abbott Laboratories, Spain) in 100% oxygen administered through a semi-closed circular anaesthetic system. All animals were allowed to breathe spontaneously. Epidural anaesthesia-analgesia was achieved to by introducing 0.2 ml/kg lidocaine 2% (BBraun, Spain) and 0.1 mg/kg morphine in the lumbosacral space.

The dogs then underwent a laparotomy using aseptic techniques involving an approximate 10-cm midline incision from the pelvic brim to a point below the umbilicus. Measurements of the length, width and depth of the prostate gland, approximately at the midpoint, were obtained in situ using callipers as described previously (Wade *et al.*, 1981) (data not shown). Surgical biopsies were excised from alternating quadrants of the prostate at each interval using a biopsy punch (0.6 cm). Small samples of tissue were immediately fixed in 10% phosphate-buffered formalin and processed for paraffin embedding.

Postoperative care involved treatment with systemic antibiotics (15 mg/kg amoxicilin,

Duphamox ®L.A., Fort Dodge Veterinaria, USA) every 48 h for 15 days. Postoperative analgesia was maintained using fentanyl patches (50µgr/kg/h, Duragesic Matrix, Janssen-Cilag, Spain) placed the day before surgery. The sutures were removed 7 to 10 days postoperatively and the healing of the prostate was monitored by ultrasonography to detect complications.

4.3.4. REAGENTS

The immunohistochemical detection of AR was performed using a polyclonal antibody (NCL-Arp, Novocastra, U.K.) at a 1:20 dilution for 30 min. The antibody against the ERα was a monoclonal mouse antibody (NCL-ER-LH2, Novocastra, U.K.) used at a 1:50 dilution for 30 min. For ERβ, a polyclonal rabbit antibody (H-150: sc-8974, Santa Cruz Biotech, USA) was used at a 1:200 dilution for 1 hour and for PR, a monoclonal antibody (BioGenex, USA) 1:20 was applied for 30 min.

4.3.5. IMMUNOHISTOCHEMICAL STAINING PROCEDURE

Sections for immunohistochemical analyses were placed on positive-charged slides (Dako Corporation, Denmark) and air-dried overnight at 56ºC. Labelling of the four different antibodies was performed using an indirect immunoperoxidase staining procedure, using sections cut at 4 µm, deparaffinized, and rehydrated through a graded alcohol series and subsequently incubated in H_2O_2 (0.3%) to block endogenous peroxidase. To overcome cross-linking by formalin fixation, paraffin-embedded tissue sections were pre-treated by high-temperature antigen retrieval that was performed by incubating the slides in an autoclave to expose antigenic sites. Antigen retrieval was conducted in a solution of 10 mM citrate-buffer, pH 7.4 for 3 min at 121ºC. After pre-treatment, sections were allowed to cool for 20 min at room temperature (RT).

Antibody labelling was detected by the immunohistochemical EnVision method according to the manufacturer's instructions. This simple, high-sensitivity two-step visualization system is based on a unique enzyme-conjugated polymer backbone, which, in addition, also carries secondary antibody molecules. Thus, the slides were first immunostained for AR, ERs and PR using an automated staining system (DAKO Autostainer, DakoCytomation, Denmark). The sections were incubated with primary antibodies at RT for 30 min, and then washed three times with the washing buffer. Briefly, the bound primary antibodies were detected by 30 min incubation with the

dextran polymer reagent conjugated with peroxidase and a secondary antibody (DAKO EnVision+, DakoCytomation, Denmark). The tissues were then washed three times in buffer solution. Colour was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAKO DAB Liquid System, DakoCytomation, Denmark) for 10 min. The tissues were counterstained with Mayer's haematoxylin (DakoCytomation, Denmark) at RT for 2 min. After this, sections were dehydrated, mounted in DPX (Probus, Spain) and examined under an Olympus B40 light microscope (Olympus, Japan).

Positive and negative external controls were included in each labelling procedure. In all immunostained batches, the omission of the primary antibody and substitution with the diluting solution alone served as a negative control. Both cryostat sections of normal canine prostate gland, and paraffin sections of human hyperplastic prostate were used as positive controls. For ERs, ovary and uterus tissue were also used as controls; and for PR, canine adenocarcinoma was also used.

4.3.6. SCORING OF IMMUNOHISTOCHEMICAL RESULTS

For each dog, one tissue section was labelled with each steroid receptor antibody. Receptor expression in each tissue section was examined at a magnification of x400 by 2 independent observers, who were not informed about the details of the protocol or clinical data. When there was a discrepant score between the observers, consensus was reached in a third observation, made by both observers at the same time. An intensity score and a proportionality score were obtained in each case. The former reflected the intensity of the positive reaction in the cell nuclei or cytoplasm (0: no labelling; 1+: weak labelling; 2+: moderate labelling; 3+: intense labelling; 4+: very intense labelling), whereas the proportionality score indicated the percentage of positively labelled cell nuclei in the different cell aggregates. These two scores were obtained in areas containing a similar amount of glandular epithelium and stromal cells. At least, 100 parenchymal and stromal cells were examined per tissue sample.

4.3.7. STATISTICAL ANALYSIS

All statistical analyses were conducted by ANOVA implemented in the MIXED SAS procedure (v. 9.1, SAS Institute Inc., USA). Experimental stage was the repeated measure subjected to the experimental unit, or animal under a given treatment. The animal was considered a random effect, and treatment and the interaction treatment x

experimental stage were the fixed effects. Since sample numbers were not the same for the two treatment groups, samples lacking at any given period were considered as missing values and analyzed using an unbalanced design with the autoregressive order 1 as covariance structure. Means were computed by least square means procedures and differences between means were separated by pairwise *t*-tests. All variables expressed as percentages were square root-arcsine transformed before analysis and their means and standard errors were back-transformed for the presentation of data. The level of significance was set at $P < 0.05$.

4.4 RESULTS

4.4.1. HISTOPATHOLOGY

Throughout the experimental period, prostate size increased only in the treatment group. At the end of the 30-week experimental period, all samples from M1 to M5 showed histological evidence of prostatic hyperplasia, compared to those from control dogs or M0 samples (Figure 1A). The extent of hyperplasia increased during hormonal treatment until sample M4 and remained similar in the M5 sample. When prostatic hyperplasia was grossly identified, an obvious increase in secretory epithelial cell numbers was observed (Figure 1B). Compared to the normal gland, each lobule was larger and showed more elaborate branching. The alveoli were also larger and contained more cells. As a result, papillary projections of the secretory epithelium into the alveoli were also more elaborate. In addition, secretory epithelial cells were larger due mainly to an increased amount of cytoplasm. In almost all tissues from both the hormone-treated and control groups, lymphoplasmacytic interstitial infiltrates were observed. At the end of hormone treatment, the glands became completely atrophic, exhibiting a marked loss of acinar folds, diminished epithelial height and abundant surrounding stromal tissue. The stroma was extensively infiltrated by dense collections of lymphocytes and plasma cells, which replaced the glandular component in many areas. Haemorrhagic foci and dilated blood vessels containing abundant polymorphonuclear leukocytes were also observed (Figure 1C).

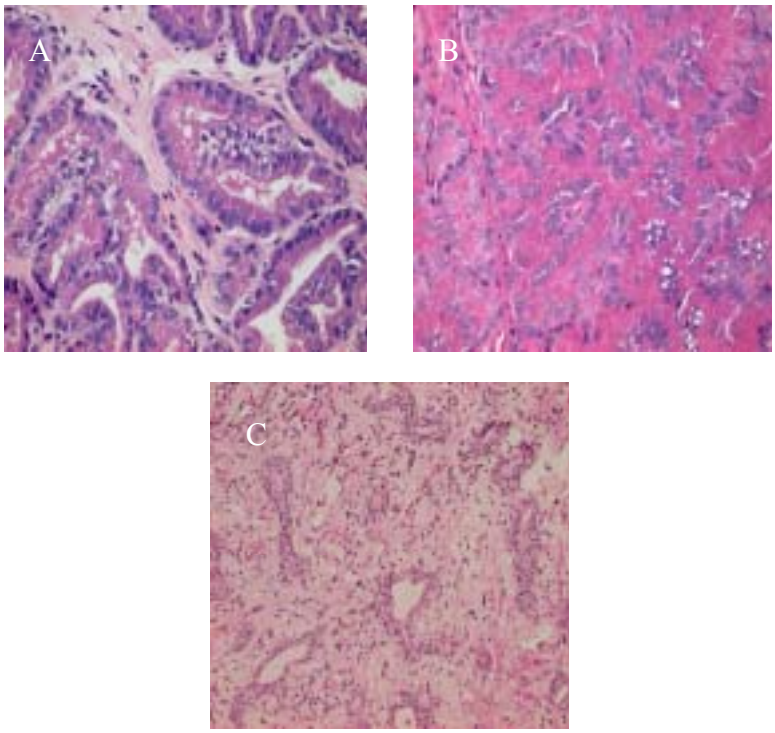

Figure 1. A) Histologically normal prostate gland with well-developed secretory epithelium forming papillary infoldings into the alveoli. The interlobular stroma is relatively scant (400x). B) Glandular prostatic hyperplasia showing enlarged alveoli and increased papillary infolding. Secretory cells are also larger than normal. The amount of stroma varies and does not show a proportional increase (400x). C) Severe atrophy corresponding to the hormone depletion stage after treatment. Note the diminished acinal branching and reduced height of epithelial cells, along with a more abundant fibro-muscular component (200x).

4.4.2 Immunohistochemistry

Our aim was to compare the expression of different hormone receptors in successive prostate tissue specimens taken from the experimental group and control group dogs in an attempt to correlate our findings with the specific changes provoked by the induction (samples M0 to M5) and regression (sample M6) of benign prostatic hyperplasia by 5α-androstane-3α, 17β-diol and estradiol.

4.4.2.1 Androgen receptor

Figures 2 and 3 show the changes in AR immunoreactivity undergone by the canine prostate gland during the hormonal induction and regression of BPH. Nuclear immunostaining for AR was detected in the nuclei of both glandular and fibro-muscular stromal cells. Weak specific staining for AR was also evident in the cytoplasm of secretory cells. Proportionality and intensity scores and labelling intensities observed in control and hormone-treated animals were very high, and often 100% of the epithelial

cells showed very intense labelling (4+) (Figure 4A). Only the M6 sample taken from the experimental dogs, corresponding to the hyperplasia regression stage was assigned significantly lower proportionality (4%) and intensity (0.2+) scores (Figure 4B) than experimental samples M0-M5. In addition, immunostained cell percentages and intensities recorded for the experimental group M6 samples were significantly lower when compared to control group M6 scores (100% and 4+, respectively in the latter group).

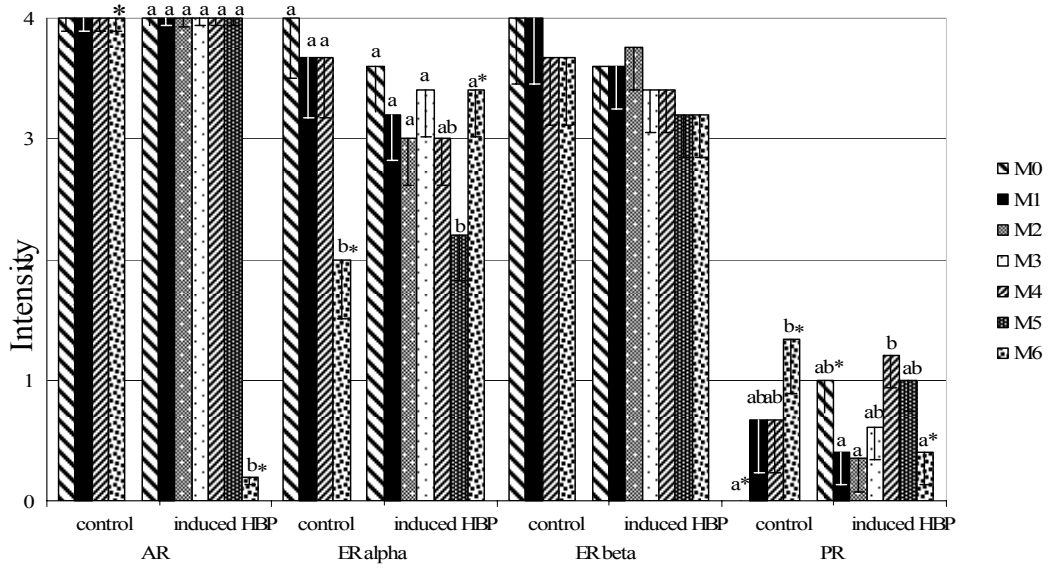

Figure 2. Mean nuclear staining intensities for AR, ERα, ERβ and PR in the dog prostate gland and standard errors of the means. a, b: Significant differences recorded during hormone treatment. * Significant difference with respect to the control value.

Proportionality scores for stromal cells in control specimens were significantly higher for M1 (53%) compared to M0 (30%), while scores for M4 (49%) and M6 (44%) failed to differ from those obtained for M0 or M1 (Figure 3). No positive staining was observed in negative controls. When comparing experimental specimens, M2 (50%) and M5 (59%) showed significantly higher stromal cell proportions than M3 (31%). M6 showed the lowest percentage of stromal cell staining (4%) when compared to the remaining experimental samples or the control M6 sample (44%).

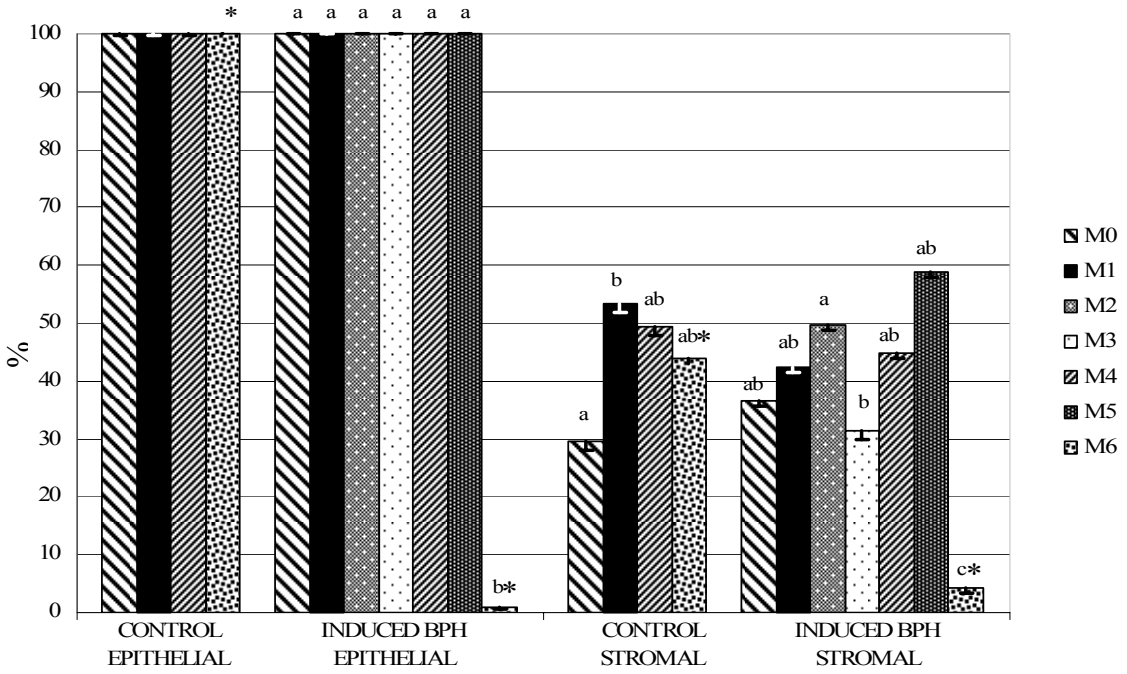

Figure 3. Proportionality nuclear staining scores for AR in the dog prostate gland and standard errors of the means. a, b, c: Significant differences recorded among the experimental stages. * Significant difference with respect to the control value.

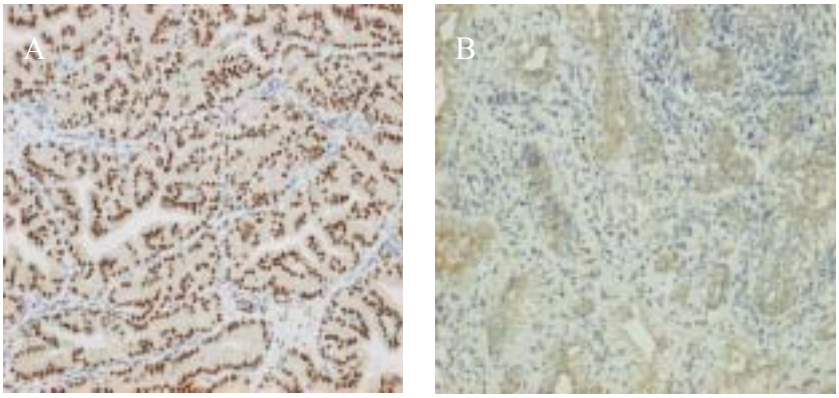

Figure 4. A) Androgen receptor localization in the prostate of a dog with induced BPH. The glandular epithelial cells show uniformly intense immunostaining for nuclear AR. AR also localizes in the nuclei of stromal cells and the cytoplasm of secretory cells (200x). B) Canine prostate specimen taken at experimental stage M6, 6 weeks after the suppression of hormone treatment. The immunoreaction for AR is very weak in both glandular epithelial cells and fibro-muscular stromal cells (200x).

4.4.2.2 Oestrogen α receptor

Nuclear immunohistochemical staining for ERα was observed in the prostate of the male dog during the hormonal induction and regression of BPH (Figures 2 and 5).

Specific ERα antibody staining was observed in the nuclei of epithelial cells, smooth muscle cells and fibroblasts. Weak specific staining was also evident in the cytoplasm of epithelial cells.

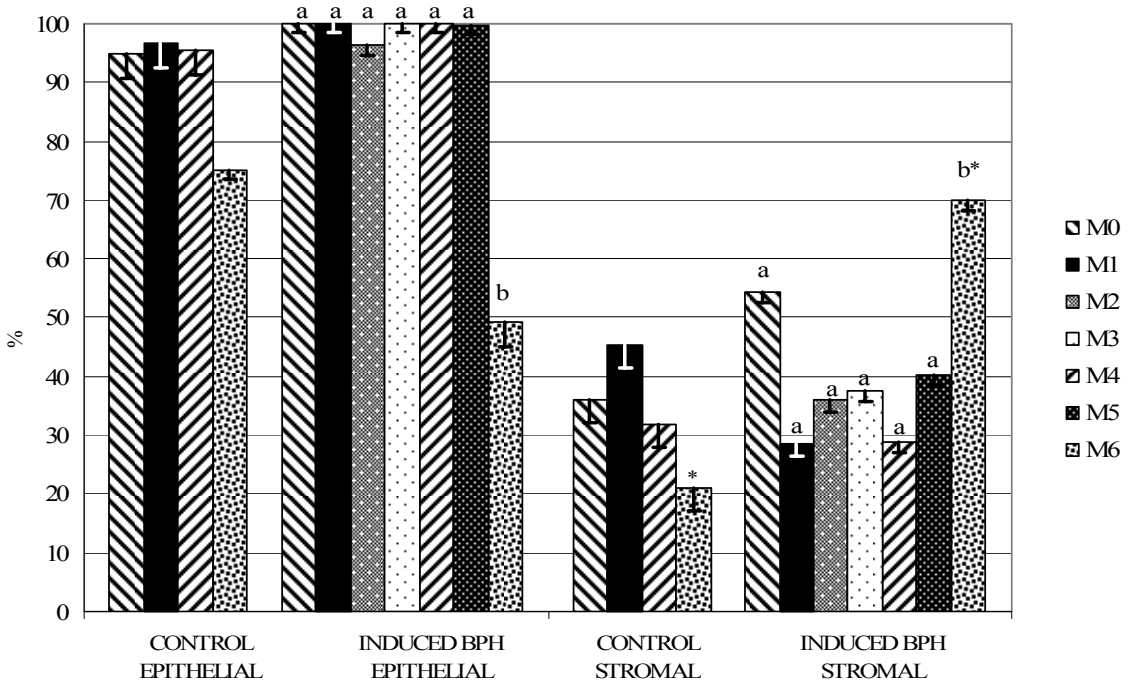

Figure 5. Proportionality scores of nuclear staining for ERα in the dog prostate gland and standard errors of the means. a, b: Significant differences among the experimental stages. * Significant difference with respect to the control value.

When we analysed the specimens from control animals, significantly lower intensity scores were obtained for stage M6 (2+) compared to M0 (4+), M1 (3.6+) or M4 (3.6+). Similarly, ERα positivity was detected in higher percentages of epithelial and stromal cells at stages M0, M1 and M4 compared to M6, although these differences were not significant.

In the experimental dogs, percentages of positive epithelial cells were significantly lower 6 weeks after the end of hormone treatment (M6: 50%) when compared to the remaining specimens (~98%) (Figure 6A). However, in stromal cells, both proportionality and mean intensity scores were significantly higher for M6 (70%, 3.4+, respectively) (Fig 6C) when compared to the sample obtained just before hormone treatment suspension (M5: 40%, 2.2+, respectively) (Figure 6B). Mean intensity and

proportionality scores for experimental M6 specimens (3.4+, 70%) were significantly higher than those recorded for the control M6 specimens (2+, 21%).

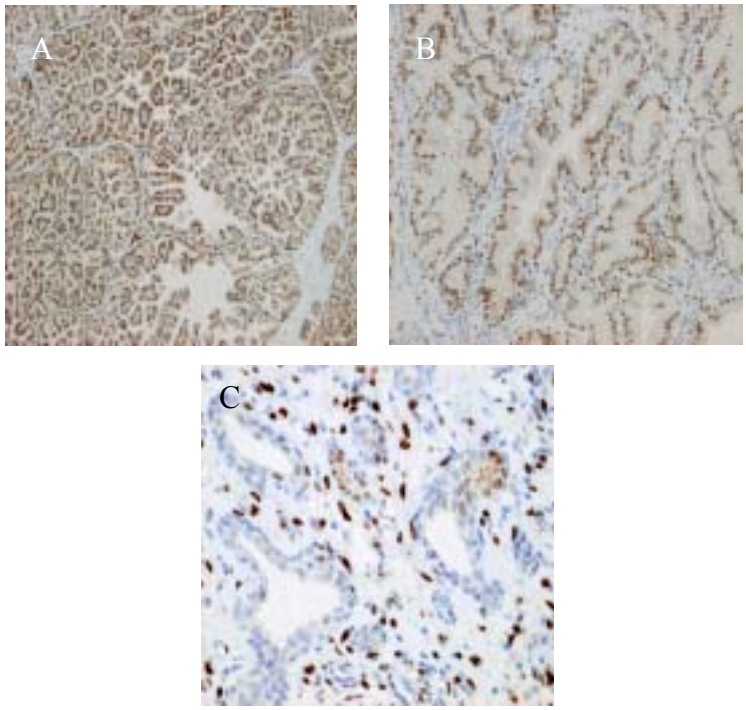

Figure 6. A) ERα immunohistochemistry in an M3 specimen taken from a dog with experimentally induced prostatic hyperplasia. Glandular epithelial cells show uniformly intense immunostaining for nuclear ERα, while only 38% of stromal cells showed positive staining for ERα. ERα also localizes in the cytoplasm of secretory cells (200x). B) Prostate specimen (stage M5) from a dog with induced BPH. Note the reduced mean nuclear staining intensity in both epithelial and stromal cells (200x). C) ERα localization in the canine prostate 6 weeks after the end of hormone treatment (stage M6). Percentages of positive epithelial cell nuclei are reduced while stromal cell proportionality scores and mean intensity scores are higher than for the M5 stage samples (400x).

4.4.2.3 Oestrogen β receptor

Glandular epithelial cells showed uniformly intense immunostaining for nuclear ERβ. Immunoreactivity was evident both in control and experimental canine prostate specimens and no significant differences where observed among the different samples (M0-M6) or between control and experimental dogs. No nuclear staining was detected in smooth muscle cells and fibroblasts (Figure 2, 7 and 8).

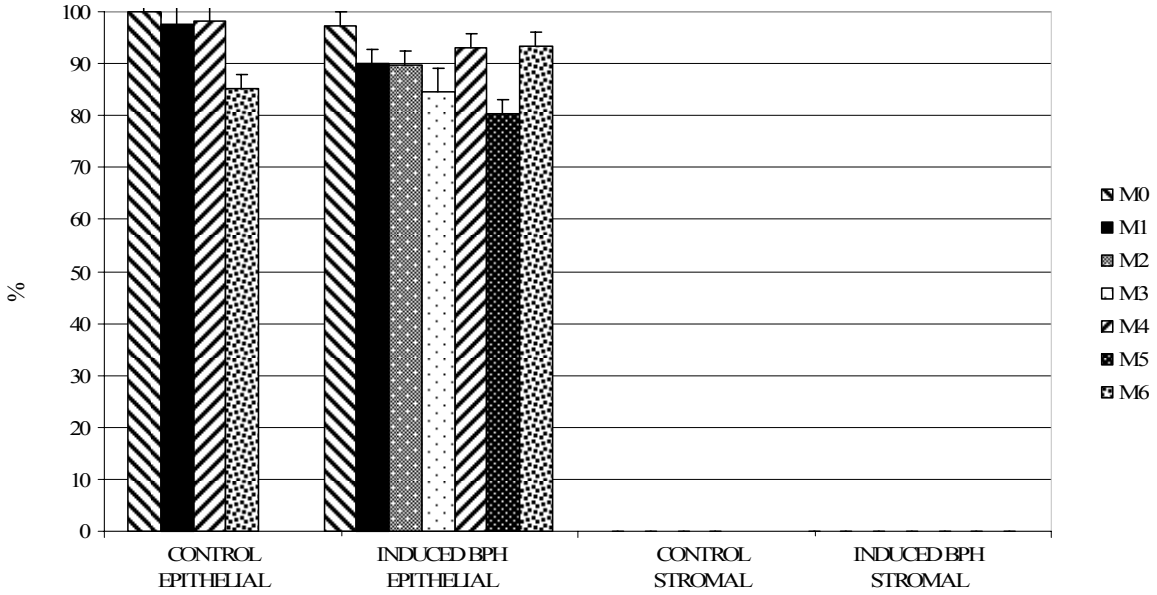

Figure 7. Proportionality scores (and standard errors of the means) obtained for nuclei showing ERβ staining in the dog prostate gland.

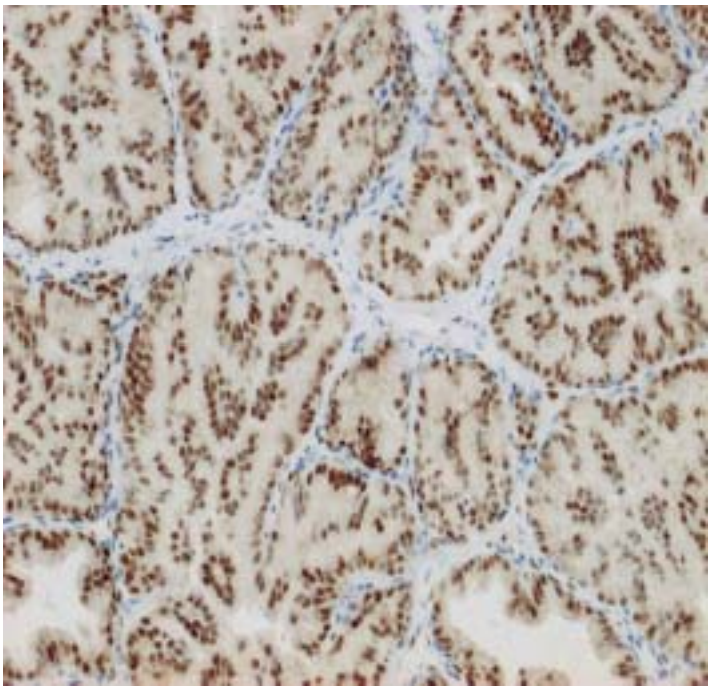

Figure 8. Prostate tissue 18 weeks after initiation of hormone treatment (stage M3). Intense immunoreactivity (intensity score=3.4) towards the ERβ antibody can be observed in 85% of the epithelial cell nuclei. Specific nuclear immunostaining was absent from stromal cells (200x).

4.4.2.4 Progesterone receptor

The changes in PR immunoreactivity observed in the canine prostate gland during the hormonal induction and regression of BPH are shown in Figures 2, 9 and 10. Glandular epithelial cells showed weak to absent nuclear immunoreactivity for PR while no positive labelling could be detected in the nuclei of stromal cells. In control dogs, proportionality and intensity scores increased from M0 (absence) to M6 (8% and 1.3+, respectively). Hormone treatment led to a significant increase in the proportions of positive epithelial cell nuclei after 30 weeks of treatment (M5, 4.5%) (Figure 10A), compared to the proportions observed in samples M1 and M2 but M5 failed to differ from those obtained for M0, M3 or M4. After the withdrawal of hormone treatment, a significant decrease in the proportionality score was again observed (Figure 10B).

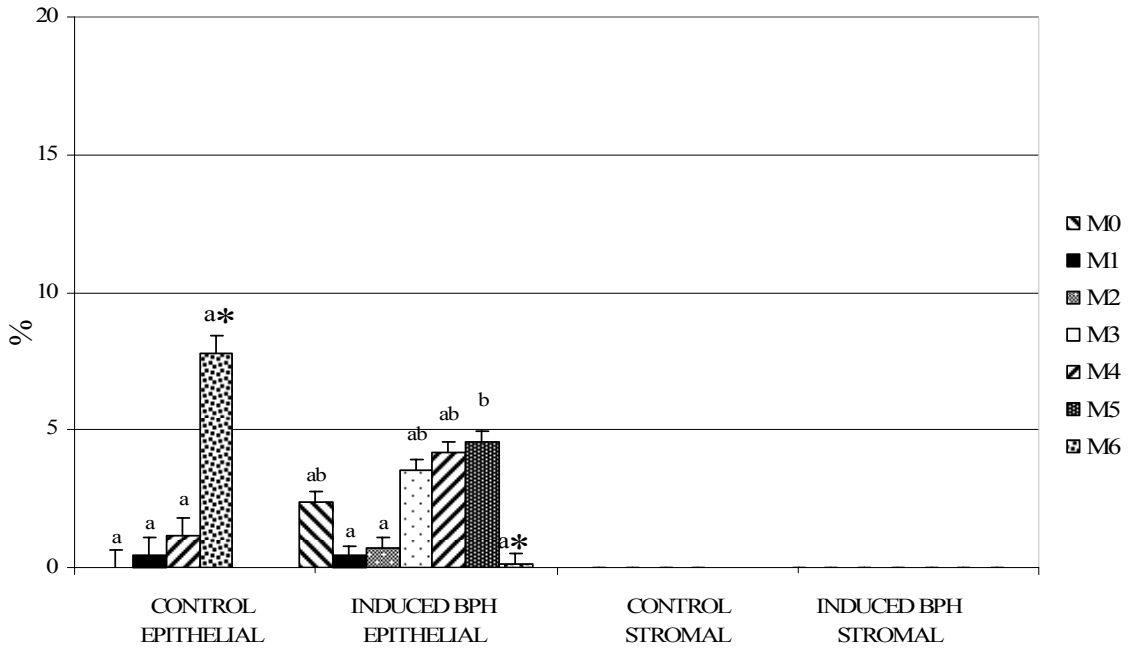

Figure 9. Proportionality scores for nuclear staining for PR in the canine prostate and standard errors of the means. a, b: Significant differences among the experimental stages. * Significant difference with respect to the control value.

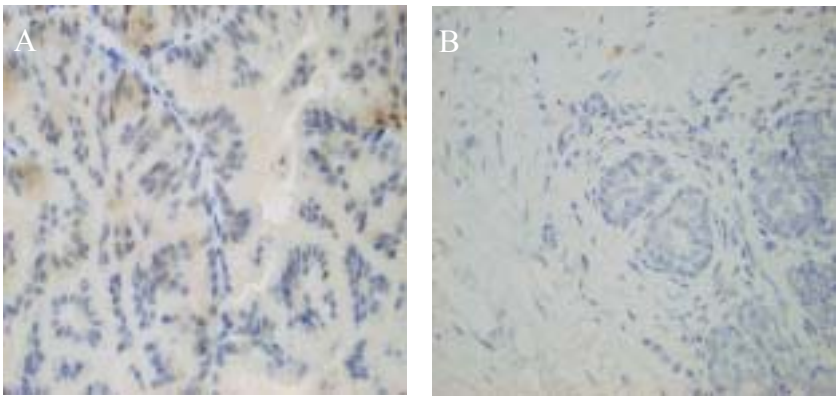

Figure 10. A) Prostate tissue 30 weeks after the onset of hormone treatment (M5). The immunoreaction for PR is very weak in glandular epithelial cells. No positive staining is observed in the nuclei of fibro-muscular stromal cells (400x). B) Immunohistochemistry results 6 weeks after the suspension of hormonal treatment (stage M6). Immunoreactivity for PR is negative in the epithelial cells while no positivity is observed in the stromal cells (200x).

4.5 DISCUSSION

Previous studies have demonstrated that canine prostatic hyperplasia can be successfully induced in castrated young dogs by the administration of either dihydrotestosterone or 5α-androstane-3α, 17β-diol in combination with estradiol (DeKlerk *et al.*, 1979; Leav *et al.*, 2001c; Walsh and Wilson, 1976). However, while spontaneous prostatic hyperplasia in the Beagle progresses with age from a glandular to a cystic histological appearance, a state of cystic hyperplasia is not reached when young animals are treated. It is unlikely that this failure is attributable to the duration of treatment, since continued administration in a study by Walsh and Wilson (1976) of 5α-androstane-3α, 17β-diol and 17β-estradiol for an entire year failed to induce the cystic condition. In contrast, old age does not seem to be a requirement for the induction of glandular hyperplasia. It is possible, however, that the cystic condition can only be achieved in aging dogs.

In the present study, nuclear staining for AR was detected in both glandular epithelial and fibro-muscular stromal cells. These results are similar to those reported for canine (Murakoshi *et al.*, 2000b; Murakoshi *et al.*, 2000c) and rat (Pelletier *et al.*, 2000) tissues, in which AR have been detected in the nuclei of both glandular epithelial and fibro-muscular stromal cells. This localization of AR has been noted in both healthy dogs and dogs with BPH (Murakoshi *et al.*, 2000b; Murakoshi *et al.*, 2000c). Similarly, in normal and BPH human prostate tissue, intense nuclear staining for AR has been

observed in epithelial and stromal cell nuclei (Leav *et al.*, 2001b; Sar *et al.*, 1990).

In contrast to reports by other authors, we observed positive staining for AR in the cytoplasm of prostrate epithelial cells. There were no indications that this cytoplasmic staining was non-specific, since the stromal cell cytoplasm or negative control sections showed no staining for the receptor. In effect, the high hormone doses used in our experiment may have induced high levels of AR synthesis in the rough endoplasmic reticulum, thus giving rise to the non-specific localization of these receptors in the cytoplasm (Simoncini and Genazzani, 2003). In addition, previous studies in which nuclear and cytosol extracts were separately analysed, have shown both nuclear and cytosol AR contents in normal, spontaneous hyperplastic and hormonally induced hyperplastic canine prostrates (Trachtenberg *et al.*, 1980).

In this study, intense nuclear immunohistochemical staining for AR was observed in prostrate tissues from both control animals and those with hormonally-induced prostatic hyperplasia, with no significant differences observed among specimens obtained during the course of BPH in terms of positive epithelial and stromal cell proportions or staining intensities. When Trachtenberg et al. (1980) analyzed cytosol and nuclear prostatic AR contents in experimentally induced canine prostatic hyperplasia (using androstenediol plus estradiol), results showed that nuclear, but not cytosolic, prostatic AR levels were significantly higher compared to levels in the prostates of age-matched control dogs. Moreover, prostatic cytosolic and nuclear AR contents remained low in untreated castrated dogs. In our study, withdrawal of hormone treatment led to a significant reduction in epithelial and stromal AR signaling, similar to the observed as a result of castration or treatment with a gonadotropin-releasing hormone agonist (Forti *et al.*, 1989), or antiandrogenic agents such as cyproterone acetate (Huang *et al.*, 1985) or chlormadinone acetate (Murakoshi *et al.*, 2000c).

Although prostate tissue is androgen-dependent, oestrogens influence both normal functions and pathological changes in this gland. It has been established that oestrogens are both tumour promoters and suppressors depending on the dose and the type of oestrogenic agent used. This dual action may be attributed, at least to some extent, to the existence of two oestrogen receptors, ERα and ERβ (Weihua *et al.*, 2002). In this study, immunolabelling for ERα was detected in the nuclei of both glandular epithelial

and stromal cells. Schulze and Barrack (1987b) reported ER labelling confined to the nuclei of the canine prostatic stroma and the prostatic duct epithelium, but, contrary to our findings, they detected no specific labelling in the acinar epithelium. Similarly, other authors have reported no ERα reaction in epithelial cells and the presence of ERα was confined to connective tissue nuclei both in healthy (human: Leav *et al.*, 2001a; rat: Sar and Welsch, 2000) and hyperplastic (human: Hiramatsu *et al.*, 1996; Leav *et al.*, 2001b) prostate tissue. Pelletier et al. (2000) were unable to detect ERα expression in normal rat prostate tissue by immunohistochemistry or in situ hybridisation. These differences in the location of ERα with respect to the observations made in our study, could be related to limited specificity of the antibody used, ineffective methods of antigenic retrieval, differences in sample processing, or differences in the sensitivity of the technique employed (Pasquali *et al.*, 2001). The administration of oestradiol to castrated dogs is known to cause squamous metaplasia in the prostatic urethra and prostatic ducts (Huggins and Clark, 1940; Tunn *et al.*, 1979) and increases prostatic stromal volume (Bartsch *et al.*, 1987). However, it is also well documented that oestrogen treatment enhances androgen-induced glandular hyperplasia in the canine prostate (DeKlerk *et al.*, 1979; Jacobi *et al.*, 1978; Walsh and Wilson, 1976). Here, we show using immunohistochemical methods that ERα occur in cell types known to undergo histological changes in response to the administration of oestrogens. It is, therefore, likely that the immunoreactive ERα localized in our study represent biologically functional receptors.

During the hormonal induction of BPH, we observed uniformly intense immunostaining of epithelial cells for ERα and the proportions of stained cells decreased significantly after hormone withdrawal. This behaviour was also observed for AR and is probably caused for the involution of the gland. In contrast, positive stromal cell staining percentages and mean intensity scores increased significantly 6 weeks after the end of hormone treatment, when compared to the tissue samples obtained just before the hormone withdrawal. A possible explanation for the increased ERα expression detected in stromal cells at stage M6 (after withdrawal of hormonal treatment) could be related to the intense inflammatory process associated with several consecutive surgical procedures. Previous studies conducted in other species, have shown that inflammatory cells express oestrogen receptors (Stygar *et al.*, 2006). Thus it could be that this stromal

expression could be related, at least in part, to interspersed stromal cells.

We could also hypothesize that this increase in ERα expression could be the result of epithelial-stromal interactions. Indeed, in other study included in this thesis (Chapter VI), we observed significantly more caveolae in basal cells, along their basal aspect and in close relationship with the basal membrane and prostatic connective tissue. The presence of ERα in the caveolae of various cell types has been well documented (Chambliss *et al.*, 2002; Chambliss *et al.*, 2000; Chen *et al.*, 1999; Hnasko and Lisanti, 2003). Caveolae may directly communicate with the extracellular space or selectively fix and store molecules that can then be released into the stroma in response to different stimuli. These structures could therefore be involved in the trafficking, processing and signalling mediated by ERα.

Using a polyclonal antibody which identifies a post-transcriptionally modified form of the long-form of ERβ, here we immunolocalized the ERβ in epithelial cell nuclei but not in stromal cells. There are no data for comparison in the literature because, to our knowledge, our group is the first to address ERβ expression by immunohistochemistry in the healthy and hyperplastic canine prostate gland. Studies performed in different species have identified ERβ in the epithelial cell nuclei of healthy rat prostates (Pelletier *et al.*, 2000; Sar and Welsch, 2000) as well as epithelial and, to a lesser degree, stromal cell nuclei in human healthy and hyperplastic prostate tissue (Fixemer *et al.*, 2003; Pasquali *et al.*, 2001). Intense nuclear staining of ERβ was observed during the hormonal induction and regression of BPH, and no significant differences were observed in percentages of positive epithelial and basal cells or in staining intensities. Moreover, no significant differences were identified with respect to the normal prostates of age-matched dogs.

The epithelium of the prostate is maintained by two functional compartments. The secretory epithelium constitutes the differentiation compartment, which is androgen dependent but has a limited proliferative capacity. In contrast, the basal cell layer consists of generally undifferentiated and androgen independent basal cells that proliferate under oestrogen stimulation and show a low apoptotic index (Bonkhoff and Remberger, 1996; Bonkhoff *et al.*, 1994). These basal cells may express nuclear oestrogen and progesterone receptors (Isaacs and Coffey, 1989; Wernert *et al.*, 1988).

Following hormone inhibition or castration, studies in animal models have suggested that basal cells are resistant to castration-induced apoptosis and are still able to proliferate following androgen repletion (Evans and Chandler, 1987). In the present study, we noted a persistently high percentage of ERβ-positive cells after the withdrawal of hormone treatment, contrary to what was observed for the other receptors. Hence, we could speculate that ERβ labelling might be attributed to the presence of basal cells that are still active despite the withdrawal of hormone treatment. This hypothesis, however, requires experimental confirmation.

The biological effects that oestrogens may exert on these functional compartments could also depend on interplay between both oestrogen receptors (ERα, ERβ) to form homo- or heterodimeric signalling complexes. Coexpressed with the nuclear androgen receptor in luminal cells, ERβ may interact with AR to control differentiation, as described recently in the rat ventral prostate gland (Weihua *et al.*, 2001). In the basal cell layer, ERβ may interfere with cell proliferation by interacting with the ERα or other non-steroidal growth factor pathways. Experimental data obtained in the ventral prostate of the rat suggest that the ERβ mediates antiproliferative effects on the prostatic epithelium (Weihua *et al.*, 2001). In fact, it has been shown that ERβ knockout mice develop BPH with age, indicating that a functional ERβ protects the prostatic epithelium from hyperplastic changes (Krege *et al.*, 1998), in contrast to the stimulated proliferation thought to occur with ERα (Fujimoto *et al.*, 2004). Further, Jiang and co-authors (2005) found ERβ mRNA expression in both prostatic epithelial and stromal cells, except for stromal cells from 4-year-old dog prostates. This decrease in the expression of ERβ in prostatic stromal cells with age could reflect reduced negative control over AR, and could thus be associated with the overgrowth of canine prostatic stromal cells, further inducing the development of canine BPH.

The discovery of ERα in metastatic and androgen-insensitive tumours has led to the suggestion that oestrogens could also affect prostate cancer progression through a receptor-mediated process (Bonkhoff *et al.*, 1999). This newly recognized pathway is related to downstream events of oestrogen signalling in prostate cancer cells. Among the oestrogen-regulated proteins, PR is one of the most important markers of oestrogen-regulated growth, as suggested in clinical studies (Allred *et al.*, 1998). In the present

study, a very weak signal for PR was observed in only a small percentage of epithelial cells, while intense epithelial nuclear staining was observed in external positive controls (human BPH and canine adenocarcinoma). The results of previous PR immunohistochemical localization studies in human prostate tissue have been inconsistent. Immunoreactivity for PR has been variably identified in several studies: in stromal cells of BPH but not in those of prostatic carcinoma (Wernert *et al.*, 1988), in stromal cells of patients with both BPH and prostatic carcinoma (Mobbs and Liu, 1990), and in the stromal and epithelial cells of patients with BPH and prostatic carcinoma (Hiramatsu *et al.*, 1996). In the dog, we previously observed weak PR positivity restricted to the nuclei of prostate epithelial cells (Chapter III). Percentages of positive epithelial cells were estimated in healthy, hyperplastic, inflamed and neoplastic dog prostates. In prostate carcinoma, mean PR expression was around 50%, but with marked heterogeneity among individuals, and positivity percentages ranged from 10 to 90%. Labelling intensity in this subset was usually weak, except in three cases corresponding to prostatic adenocarcinomas with solid nodules, in which moderate labelling intensity was observed.

Our present results also indicate slightly increased PR-labelled cell proportions during hormone treatment although labelling decreased 6 weeks after treatment suspension. It is well established that transactivation of the PR gene is enhanced and maintained by oestrogens through an ER-mediated process (Kastner *et al.*, 1990). These last authors demonstrated that human PR forms A and B are encoded by separate mRNAs, which are transcribed from two distinct promoters, both under oestrogen control. In fact, PR is a widely accepted marker of a functional ER pathway in human tissue. Our results indicate that the presence of PR in hyperplastic tissue is invariably associated with high levels of detectable ERα. This provides circumstantial evidence that up-regulation of the PR in canine prostate hyperplastic cells involves the ERα pathway. Further studies are also necessary to confirm this hypothesis.

In summary, the canine prostate tissue examined here showed uniformly nuclear and cytoplasmic staining for AR and ERα in glandular epithelial cells. Both receptors were also localized in the nuclei of fibro-muscular stromal cells. While AR and ERα levels remained elevated in both control and hormone-treated dogs, cell positivity decreased

after treatment. ERβ were localized only in epithelial cell nuclei and, regardless of the hormone treatment, high positivity percentages were observed; these percentages were similar to those recorded in age-matched dogs. PR immunoreactivity was very weak in epithelial cells and negative in stromal cells. The hormone treatment of castrated dogs led to a slight increase in PR positivity, with a subsequent reduction at the end of treatment. These observations indicate that AR, ERα, ERβ and PR are differentially expressed in prostate tissue and that they react differently during the hormonal induction of BPH. The experimental model used here is a potentially valuable tool for investigating the respective roles of epithelial and stromal hormone receptors. These receptors are critical for understanding the mechanisms through which hormones regulate prostatic epithelial growth, differentiation and function, and in turn, for identifying the paracrine mediators involved in these regulatory cell-to-cell interactions.

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Capítulo V

ULTRASTRUCTURAL CHANGES IN PROSTATE CELLS DURING HORMONE INDUCED CANINE PROSTATIC HYPERPLASIA

5.1 ABSTRACT

Benign prostatic hyperplasia is a very prevalent disease that has received relatively little attention in spite of its morbidity and remarkable social impact. There are few animal models of prostatic hyperplasia. The dog is the only specie, along with humans, in which prostatic hyperplasia develops spontaneously and almost universally with age. The aim of the present study has been to compare the ultrastructural findings in a model of experimentally induced canine prostatic hyperplasia with those of the spontaneously developed changes in untreated dogs. An experimental group of 5 male Beagle dogs were castrated and treated with combined steroids (three weekly doses for over 40 weeks). Prostate samples were surgically obtained every 6 weeks (experimental stages M0, through M6). The control group consisted of three non-castrated dogs that were treated with vehicle and in which samples were taken only at stages M0, M1, M4 and M6. Changes in the control groups were similar but of lower intensity compared to those of the experimental groups. In luminal cells, crowding with papillary projections, prominent, branching microvilli, and abundant, often compartmentalized granules were observed. The most striking change was the previously unreported finding of caveolae in basal cells. They were mostly located in the basal aspect of basal cells and were more prominent in the experimental group and in advanced stages of treatment. These ultrastructural findings have not been previously reported in canine or human prostatic hyperplasia and merit further research. The model of experimentally induced canine prostatic hyperplasia provides an adequate setting for the understanding of this disease.

5.2 INTRODUCTION

Hyperplasia of the prostate gland (BPH) is a very prevalent disease, affecting most elderly men in different degrees and with variable morbidity. Despite of intensive research, its pathogenesis is not fully understood. Experimental models provide a unique background to advance in the knowledge of this disease. Canine prostatic hyperplasia has been extensively investigated and constitutes the best model for comparative studies with its human counterpart (Bartsch *et al.*, 1987; Merk *et al.*, 1980; Merk *et al.*, 1982). Nevertheless, there are important differences in the features of BPH between the two species (Bartsch and Rohr, 1980; DeKlerk *et al.*, 1979).

In a number of mammalian species, electron microscopy has established that the lining of prostatic glandular acini contains two distinct epithelial cell types, the columnar, secretory or acinar epithelial cells (SECs), and the flattened, polygonal or triangular shaped basal cells (BCs). The latter surround more or less completely the acinar SECs, resting on the acinar basal lamina (Ichihara *et al.*, 1985; Srigley *et al.*, 1988).

The present study describes the sequential ultrastructural changes in the basal and luminal cells of the dog prostate during the induction of prostatic hyperplasia by hormonal treatment. Although the ultrastructural changes in the dog's prostate gland have been extensively studied, the fine structure of the early phases of experimentally induced glandular hyperplasia, and its progression in time have not been documented.

5.3 MATERIALS AND METHODS

5.3.1. EXPERIMENTAL ANIMALS AND TREATMENT PROTOCOLS

Eight male beagle dogs (11/2-2 years) in two groups (n = 5, experimental group, G I) and (n = 3, control group, G II) were used. They were housed individually, fed a standard commercial diet, and provided with water ad libitum. G I animals were castrated and were injected intramuscularly 25 mg of 5α-androstane-3α, 17β-diol, and 0.25 mg 17β-oestradiol. G II dogs received only 1 ml of the triolein vehicle. Treatment was administered 3 times weekly (Monday, Wednesday, and Friday) for 30 weeks. The intramuscular injections were given alternately in the hind legs. Surgical biopsies of the prostate were obtained from alternate quadrants, the first one before castration and without treatment, and then at 6-week intervals for 36 weeks, resulting in 7 experimental stages, labeled M0 through M6, the latter again without hormonal treatment. Samples from the control group were obtained only at stages M0, M1, M4 and M6. All animals were treated and cared for in accordance with the Autonomous University of Barcelona School of Veterinary guidelines for the treatment of experimental animals.

5.3.2. TISSUE SAMPLES

Surgical biopsies of the prostate were obtained via a caudal midline celiotomy and were excised from alternating quadrants of the prostate at each interval. Small samples of tissue were fixed glutaraldehyde 2% and paraformaldehyde 2% 0.1M, in phosphate

buffer, and then post-fixed osmium tetroxide 2% in 0.1M phosphate buffer. The specimens were embedded in Epoxy resin. Semithin sections were stained with toluidine blue. Thin sections of selected blocks were stained with uranyl acetate and lead citrate, and examined with a Philips CM100 transmission electron microscope.

5.4 RESULTS

Overall, the ultrastructural examination of most cases disclosed that the cytoplasm of secretory epithelial cells (SEC) was filled with abundant electron-lucent vacuoles, numerous mitochondria, abundant secretory granules, scattered lysosomes, and well developed Golgi complexes. Nuclei were typically round with diffuse, finely granular chromatin. Nucleoli were usually present but not prominent or irregular. The cells often displayed features similar to those of apocrine secretion, including the presence of cytoplasmic blebs and microvilli on the luminal surface. The plasma membranes showed irregular infolding with occasional desmosomes, hemidesmosomes, and attached tonofibrils. The basal cells formed a thin, discontinuous layer at the periphery, and were surrounded by basal lamina.

The secretory acini of the intact prostates were lined by a typical pseudo stratified columnar epithelium with secretory epithelial and basal cells, surrounded by connective tissue with small capillaries and a thin layer of smooth muscle cells. The nuclei of normal prostatic epithelial cells were basally located, with uniformly dispersed euchromatin and a small amount of heterochromatin condensed along the nuclear envelope. A distinct nucleolus was often seen. Basal cells, either parallel or perpendicular to the basement membrane, had moderately abundant caveolae mostly in their outer or basal aspect (Fig. 1), and often displayed long, slender, thin cytoplasmic processes underlying secretory epithelial cells.

In each experimental animal, seven samples representing different time points in the induction of hyperplasia were obtained. In the first sample (normal prostate, before castration) glands showed luminal cells with abundant apical microvilli and abundant secretory granules (Fig. 2). In some cases, apoptotic cells could be identified. On the other hand, basal cells had an often indented nucleus with small nucleolus, scanty profiles of rough endoplasmic reticulum, small Golgi complexes, mitochondria and

occasional glycogen pools, and also some caveolae along the cell membrane of their basal domain. Capillary vessels were often closely apposed to the glands, and the stroma contained smooth muscle and fibroblastic cells, variable amounts of collagen, as well as larger blood vessels (Fig. 3). Basal and luminal cells were joined by desmosomes. Although they were not easily found, profiles of neuroendocrine cells containing abundant neurosecretory granules could be observed in some glands (Fig. 4).

Upon starting of hormonal treatment, and already from the first post-treatment sample (6 weeks), the most remarkable change in basal cells is a prominent increase in the number of caveolae (Fig. 5). In luminal cells, cell aggregates and papillae bulging into the glandular lumina are observed (Fig. 6). In addition, individual cells also show protrusion of their apical cytoplasm towards the glandular lumina. In these cells, electrondense secretory granules are packed in the central cytoplasm (Fig. 7), while most peripheral, apical granules show a clearing effect, by which the electrondense secretory cores appear to subdivide or fragment in smaller ones inside each granule (Figs. 7 and 8). Between these granules, the apical domain shows variable proportions of long, slightly tortuous, sometimes branching microvilli. Very often, the hyperplastic process resulted in cell narrowing and elongation, with more frequent papillary infoldings towards de glandular lumen. Rarely, mitotic figures could be found.

In the successive samples (12, 18, 24, and 30 weeks for M2, M3, M4, and M5 experimental stages, respectively), glandular cell packing, increased number of capillary vessels next to, and sometimes partly surrounded by glandular cells (Fig. 9), as well as persistent images of granule fragmentation and increasing complexity of microvilli were observed, although a clear quantitative increase was difficult to assess from stage to stage (Fig. 10). In basal cells, increased numbers of caveolae were detected (Fig. 11), although again it was more difficult to observe differences between stages, as compared with the initial increase from M0 to M1. Inflammatory cells in the stroma, and sometimes permeating and infiltrating the glands were often found in these stages.

In the last sample (36 weeks, stage M6), taken after withdrawal of the hormonal stimulation, at the end of the 9-month experimental period and after several surgical procedures, there was usually extensive atrophy, with predominance of dense stroma with reduced smooth muscle, along with small, collapsed glands, with basal cells that

did not show the prominent caveolae of the previous stages (Fig. 12). Secondary lysosomes were sometimes found in these cells.

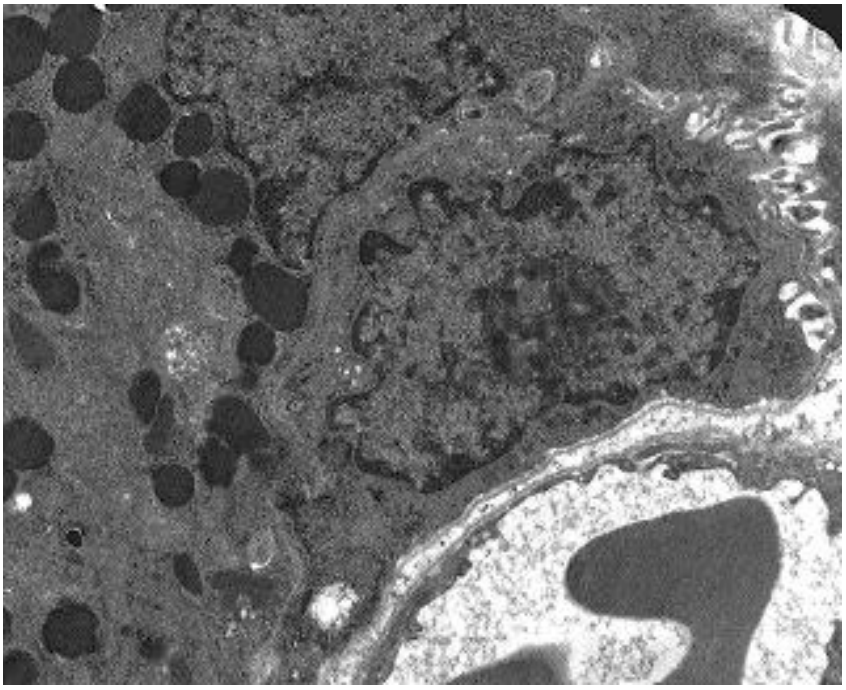

Figure 1. Ultrastructural appearance of a basal cell, with few organelles and scanty caveolae along its basal aspect.

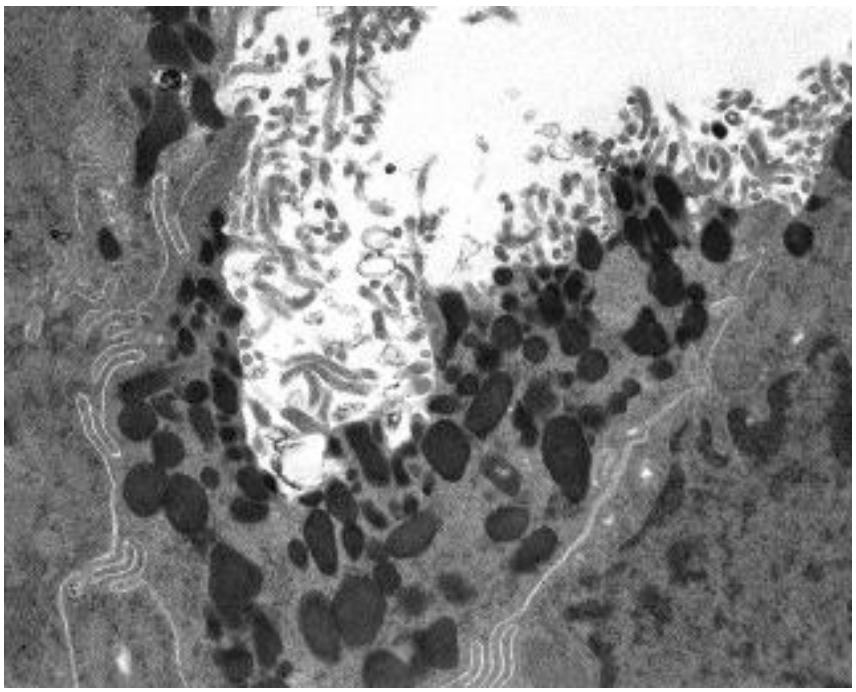

Figure 2. Luminal cells with numerous, irregular microvilli, and abundant secretory granules.

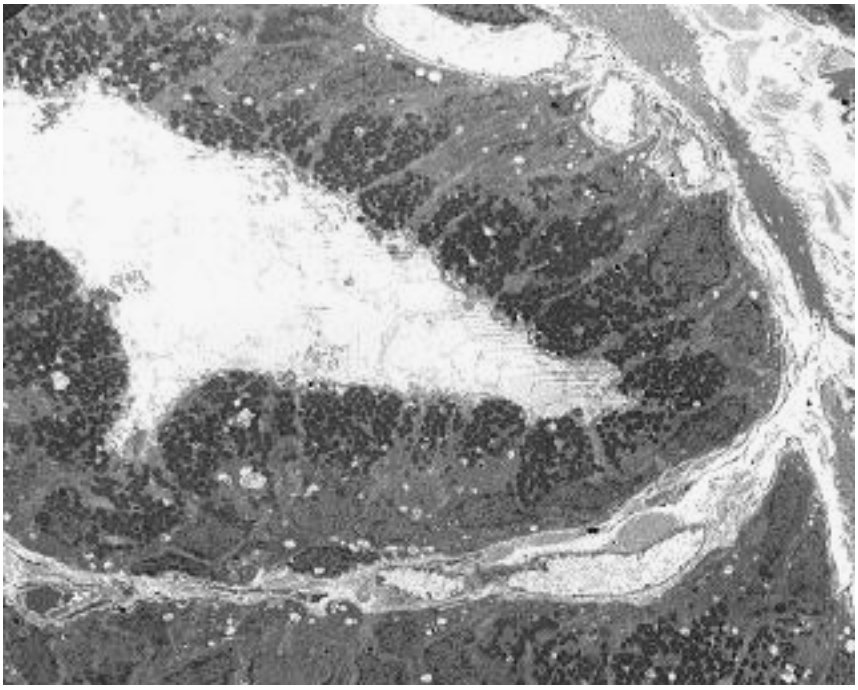

Figure 3. Low power view of a baseline prostatic gland, with adjacent stroma showing smooth muscle cells and capillary blood vessels.

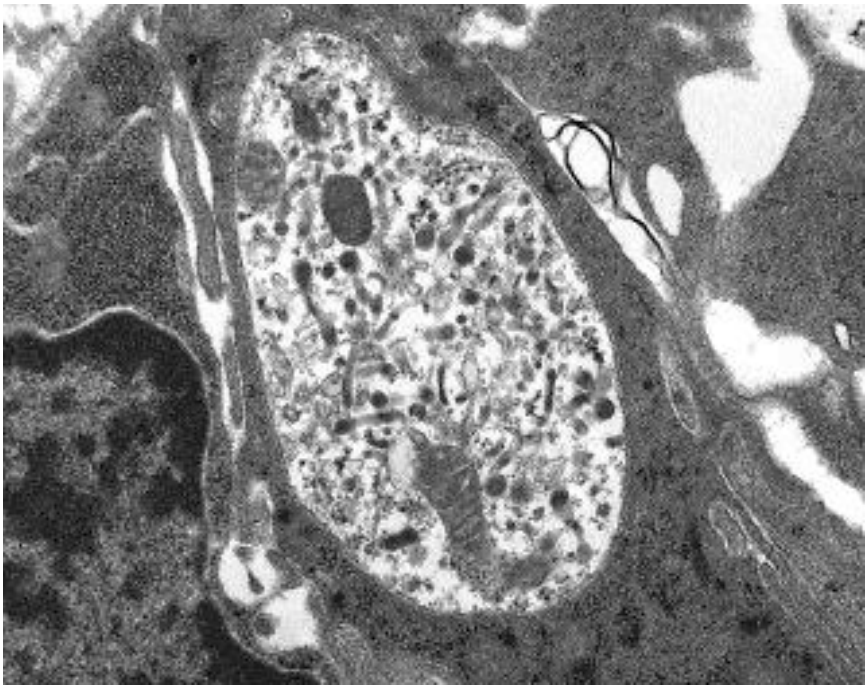

Figure 4. Cytoplasmic profile of a neuroendocrine cell with abundant, mostly elongated neurosecretory granules.

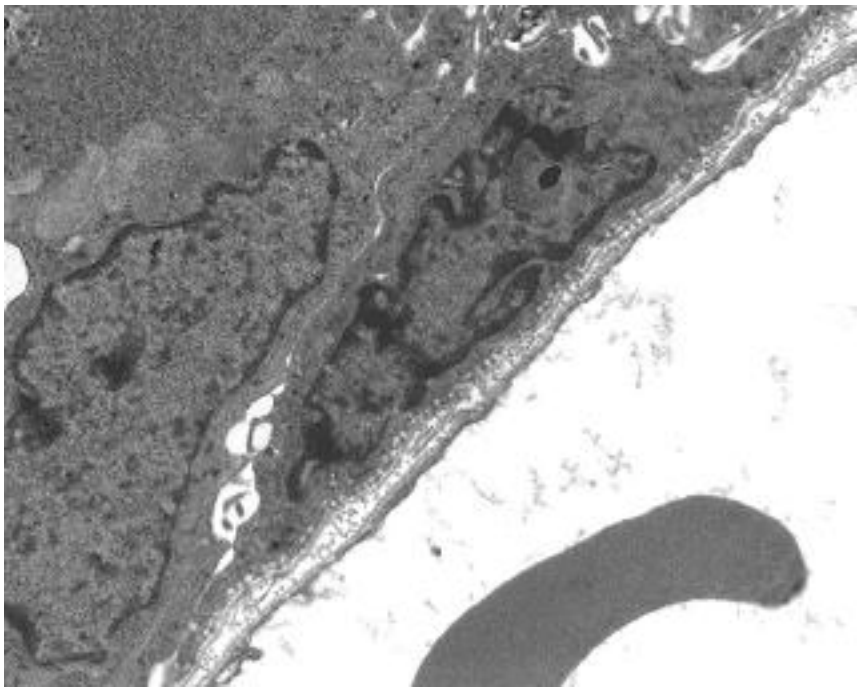

Figure 5. A prominent increase in the number of caveolae in the basal aspect of basal cells was apparent from early steps of hormonal treatment.

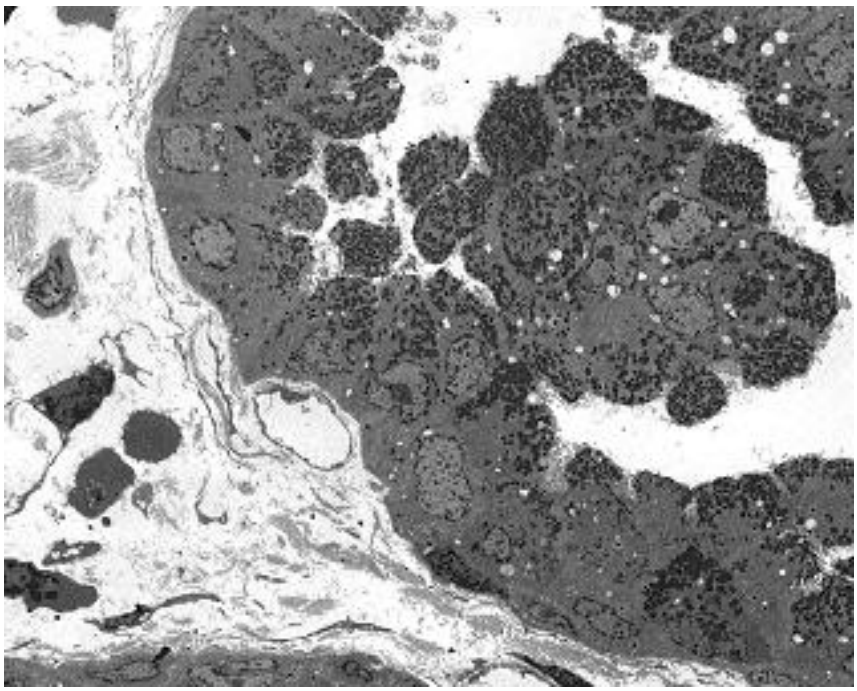

Figure 6. The experimentally induced hyperplastic process often resulted in the infolding of cell clusters or papillae towards the glandular lumen

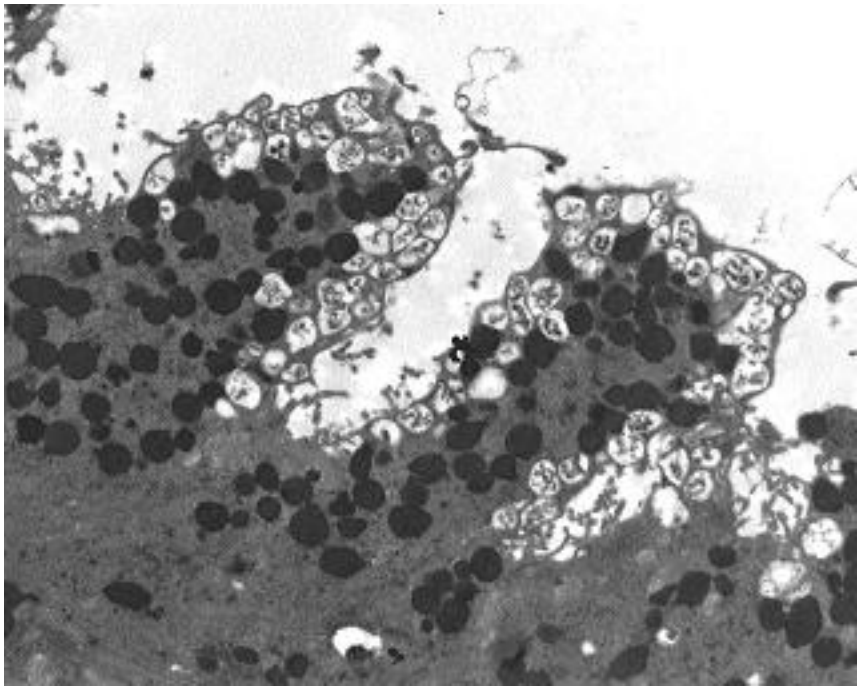

Figure 7. Individual cells in these hyperplastic glands also showed protrusion of their apical cytoplasm towards the lumina, and two appearances were observed in secretory granules: more central or internal electron dense granules, and fragmented granules with clearing effect towards the apical subplasmalemmal cytoplasm.

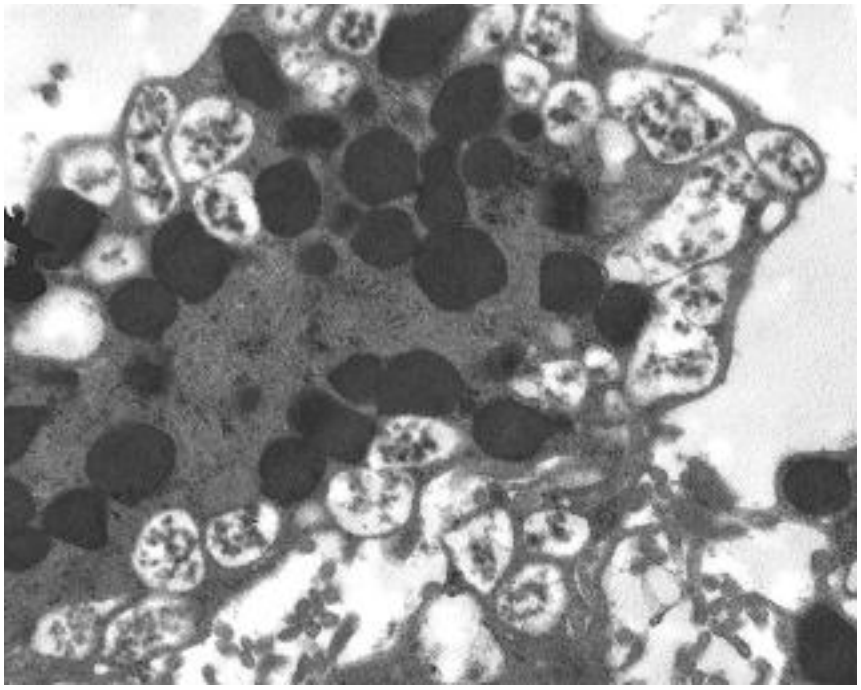

Figure 8. Close up view of the two granule appearances.

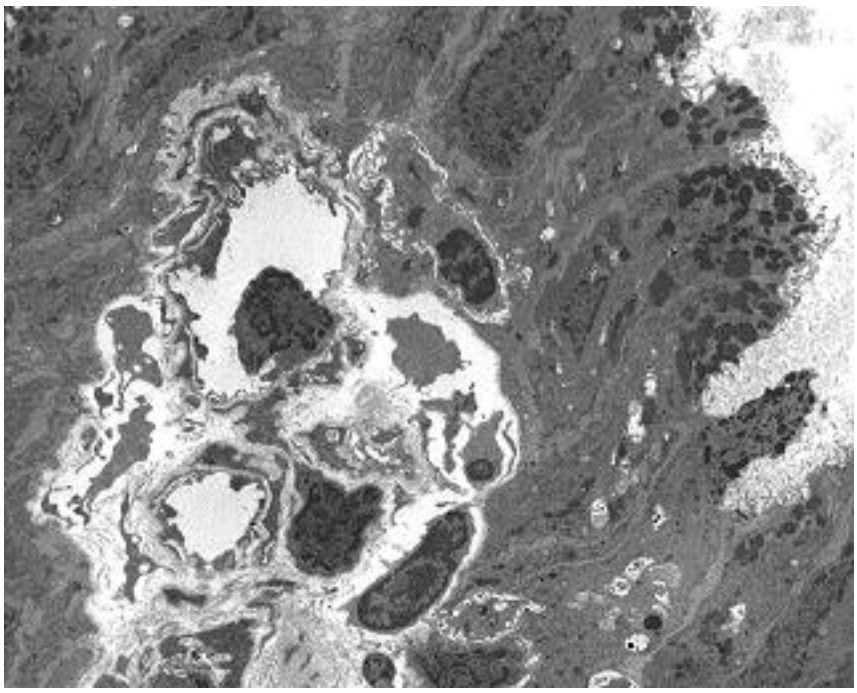

Figure 9. Hyperplastic gland with papillary epithelial infoldings surrounding a markedly developed network of capillary blood vessels

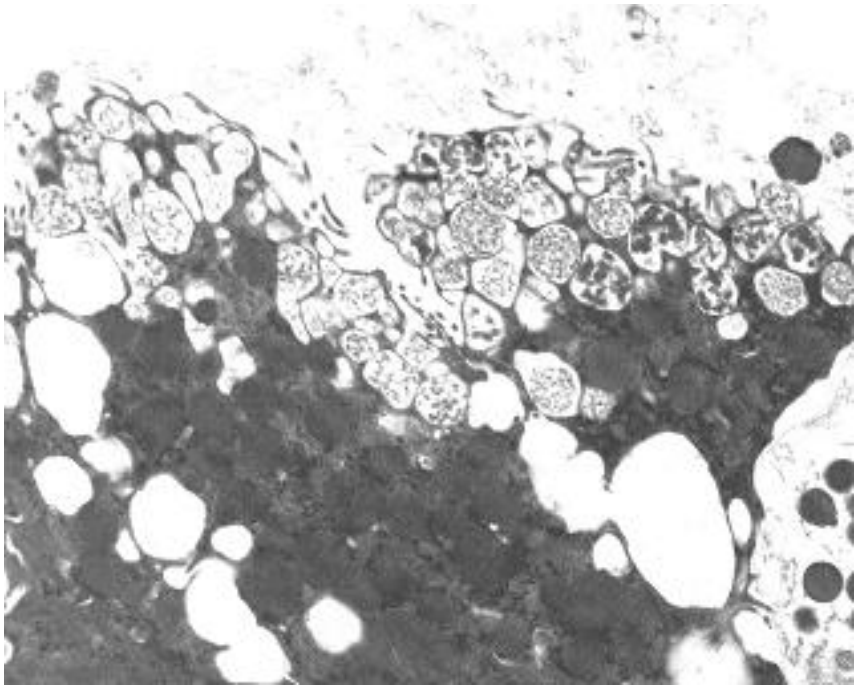

Figure 10. Complex microvilli and granules with several degrees of matrix fragmentation in glandular epithelial cells at M3 (18 weeks) experimental stage.

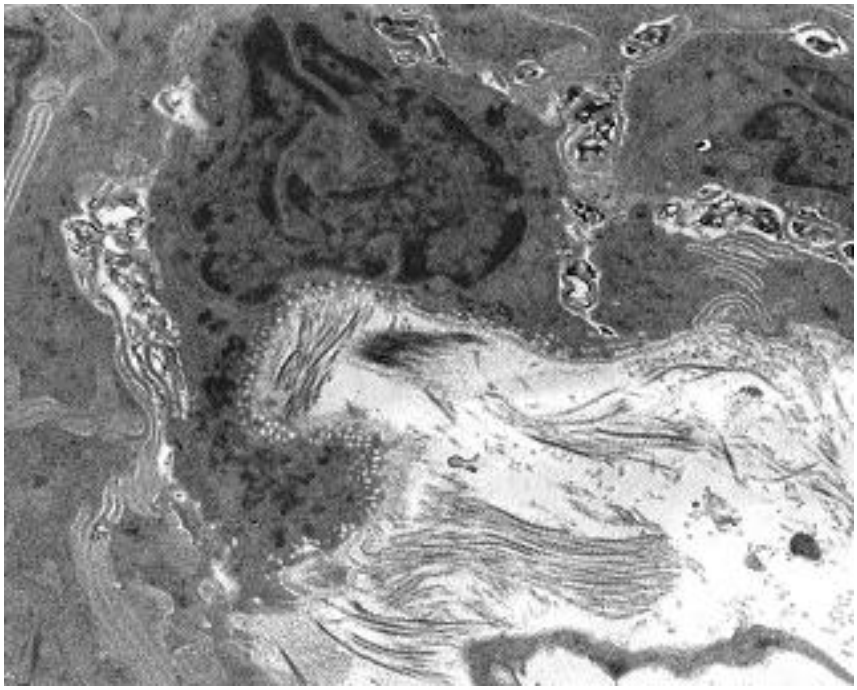

Figure 11. Abundant caveolae in a tangentially sectioned basal cell at M4 (24 weeks) experimental stage.

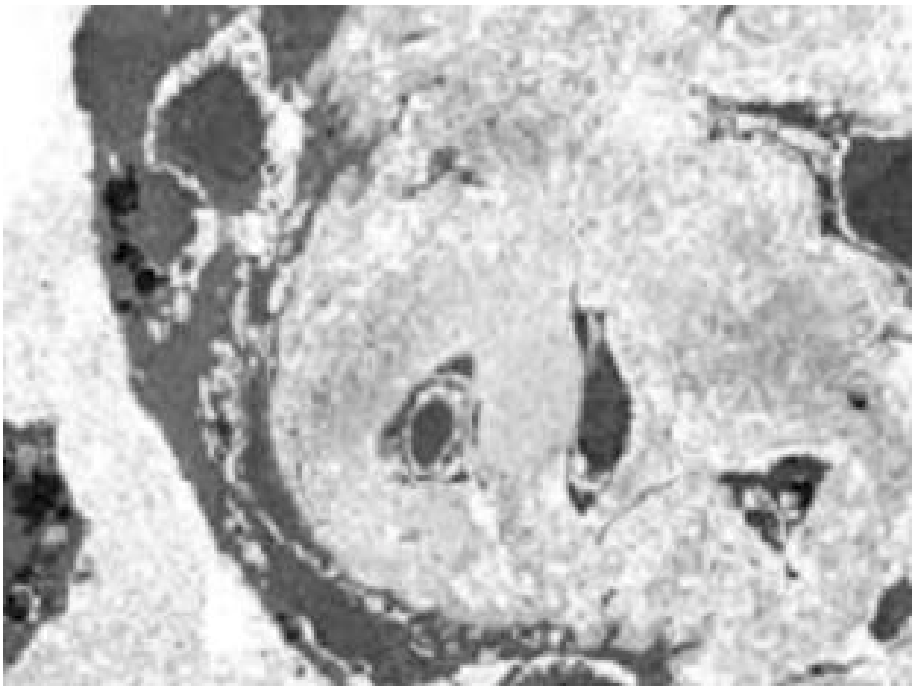

Figure 12. After interrupting the hormonal treatment, severe glandular atrophy was observed in the experimental group. Basal cell caveolae were markedly reduced in number.

5.5 DISCUSSION

The prostate gland has classically been considered the prototype of an androgen-dependent organ. Androgens are necessary for maintenance of its normal structure and function. Nevertheless, the prostate also responds to the influence of estrogens. The sensitivity of the prostatic epithelium to the action of either sex hormone suggests that both of them may be acting in a coordinated fashion at physiologic levels in the regulation of prostate homeostasis (Leav *et al.*, 1978).

The ultrastructure of the normal prostate of the dog was investigated by several authors during the 1960s and 1970s (Seaman and Winell, 1962; Timms *et al.*, 1976).

Similar to the human prostate, two main cell types are well documented in the canine prostatic acini: the basal or reserve cells and the luminal or secretory cells. The basal cells are located immediately on the basal lamina and are considered to be responsible for renewal of the luminal, highly differentiated cells. Basal cell nuclei are relatively small, but with a higher nuclear/cytoplasmic ratio than luminal cells, and with a usually inconspicuous nucleolus. Their cytoplasm contains polyribosomes, and occasional glycogen pools.

They often have elongated, thin, slender cell processes underlying luminal cells. The latter are usually endowed with protein secretion machinery, including rough endoplasmic reticulum, Golgi complex and electron-dense secretory granules, as well as a large nucleus with predominant euchromatin and prominent nucleolus. Prostate stroma is characterized by a mixture of cell types, most notably smooth muscle cells, and fibroblasts, as well as some macrophages and well developed blood vessels (Bartsch *et al.*, 1987; Ichihara *et al.*, 1985; Seaman and Winell, 1962).

The ultrastructural changes in prostatic hyperplasia have been reported in the human prostate: abundant rough endoplasmic reticulum with enlarged cisternae, studded with ribosomes, a prominent Golgi complex have been cited as the most common findings, and some authors note a paradoxical reduction in the number of secretory granules (Bartsch *et al.*, 1979; Bartsch and Rohr, 1980; Biagini *et al.*, 1982; Montorsi *et al.*, 1992; Yang *et al.*, 2003). It is well known that the canine prostate has many similarities with its human counterpart, mainly because of the spontaneous development of

hyperplasia and cancer in both species. The model of induced canine prostatic hyperplasia has been reported by Walsh and Wilson (1976) as well as by Mahapokai and colleagues (2000), and allows to study the pathogenesis of this highly prevalent disease from different standpoints.

There have been no previous reports on the fine structure of the sequential changes along the process of experimentally induced canine prostatic hyperplasia. In the present study, the main findings relate to the sudden increase in the number of caveolae in the basal aspect of basal cells, a feature observed from the early stages of hormonal induction and that is maintained in a relatively steady condition along the experimental stages. Caveolae (little caves) are specialized lipid raft microdomains forming 50–100 nm flask-shaped vesicular invaginations of the plasma membrane, which serve as a scaffold for signaling molecules related to cell adhesion, growth, and survival. Caveolae are composed of cholesterol, sphingolipids, and structural proteins termed caveolins; functionally, these plasma membrane microdomains have been implicated in signal transduction and transmembrane transport (Anderson, 1998; Lisanti *et al.*, 1994; Liu *et al.*, 1997a; Liu *et al.*, 1997b; Podar *et al.*, 2003). Since their initial discovery in the early 1950s, caveolae, with their unique flask-shaped morphology, have elicited multiple speculations as to their functional significance. Although detailed morphological examinations have provided some insight into their function, it was not until the discovery of the caveolae coating proteins, caveolins, that the true nature and importance of these organelles was realized. Since that time, the exponential growth of the caveolae field has provided numerous clues as to the physiological functions of caveolae and their caveolins. Caveolae were simultaneously identified in capillary endothelial cells and epithelial cells from the mouse gall bladder. Since then, caveolae have been identified in a wide variety of tissues and cell types (Cohen *et al.*, 2004). Their occurrence in prostatic basal cells has not been specifically documented in the literature as a relevant finding. It could be a cell response to increased hormonal stimuli, and could perhaps be related to the processing and internalization of these hormonal signals in the basal cells. Thus, the basal cells would seem to be the primary target of hormonal stimuli leading to the hyperplastic process, probably through proliferation and differentiation into luminal cells.

Although in this ultrastructural study we have not calculated the relative ratios of the different cell compartments, it is apparent from simple inspection that there is an increase in luminal cells. While an increase in the number of basal cells is not apparent, it will be necessary to apply quantitative measurements to investigate this possibility more precisely. The increased number of luminal cells results in crowding and higher complexity of the glandular structure, with more prominent infoldings of the glandular epithelium. Also, the apical portion of the cells tends to bulge towards the lumen, resulting in an increased surface for granule secretion. Secretion granules in the canine prostate, and particularly in the latter stages of the experimental induction, may be subdivided into two categories: the homogeneously electron-dense granules usually located more internally, and the heterogeneous granules with a similar size but with fragmentation of the secretion products. The exact meaning of this finding is not clear from our observations, but it would seem to be a sort of elution prior to the voiding of the granules into the glandular lumen. The microvilli in prostatic epithelial cells tend to be long, tortuous, and occasionally dichotomized.

In summary, the results of our study indicate a central role for basal cells in the early phases of induction of prostatic hyperplasia in the dog. Progressively, the changes are more prominent in luminal cells. This process tends to stabilize in spite of continued hormonal treatment, suggesting either a saturation effect or the need for progressive increases in treatment dosages to produce sustained increases in proliferation and differentiation of glandular cells. This is a preliminary, descriptive study, of a series of unique, previously not reported findings both in basal and luminal cells. Further studies with different techniques will be required to draw more precise conclusions about the biological meaning of these findings. An important difference between canine and human prostatic hyperplasia is the much greater involvement of the fibromuscular stroma in the latter (Bartsch and Rohr, 1980). Taking this fact into account, this model of induced canine prostatic hyperplasia provides an excellent opportunity for understanding its human counterpart and, eventually, for designing better treatment strategies for this highly prevalent disease.

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Capítulo VI

BASAL CELL CAVEOLAE: PROGRESSIVE INCREASE THROUGHOUT THE STAGES OF INDUCED CANINE PROSTATIC HYPERPLASIA

6.1 ABSTRACT

The basal cell compartment contains the transient amplifying sub-population of epithelial cells. It is therefore conceivable that it plays a role in the development of BPH. In a previous ultrastructural study of experimentally induced canine BPH, caveolae were observed in the basal cells. Among other roles, caveolae are considered sites for hormone receptor ligand interactions. The purpose of the present study was to determine, by means of ultrastructural morphometry, if the number of caveolae was related to the stages of hormonally induced BPH.

Eight male beagle dogs were used and were divided into two groups (G I, n = 5, experimental group; and G II, n = 3, control group. G I animals were castrated and injected with 25 mg of 5α-androstane-3α,17β-diol, and 0.25 mg 17β-estradiol. G II dogs received only 1 ml of the triolein vehicle. Surgical prostate biopsies were obtained from alternate quadrants before castration and without treatment, and then at 6-week intervals for 36 weeks, resulting in 7 experimental stages, labeled M0 through M6, the latter again without hormonal treatment. The number of caveolae per 100 micrometers of basal plasma membrane was counted.

An increase in the number of caveolae in basal cells was noted from M0 (17%) and M1 (24.9%) to M2 (44.8%), M3 (41.3%), M4 (33%) and M5 (36.2%), the values in the latter four stages suggesting a plateau. The difference between these four stages and the initial ones was statistically significant ($p<.05$). At M6 stage, after hormonal treatment withdrawal, caveolae diminished dramatically in number (18.4%). These difference was also statistically significant ($p<.05$).

The most remarkable ultrastructural finding in this model of experimental BPH was the significant increase in the number of caveolae in basal cells throughout the stages of hormonal treatment, and the dramatic reduction after hormonal withdrawal. This finding suggests that basal cells are crucial in the development of BPH and that they are either a primary target of or are induced by the hormonal stimuli. The role of caveolae in this disease seems to be very important and merits further research.

6.2 INTRODUCTION

Canine prostatic hyperplasia has been accepted as an appropriate animal model for the study of human benign prostatic hyperplasia, although there are important differences between the two species (Murakoshi *et al.*, 2001a). The dog's prostate gland is dependent on a continuous supply of androgenic steroids. Male dog castration will lead to an apoptosis-related, extensive regression of prostatic glandular tissue (Shabisgh *et al.*, 1999). Several types of epithelial cells are present in the prostate gland ducts and acini. They include basal, secretory, and neuroendocrine cells. Basal cells, usually present as a thin layer around secretory cells, are considered the main epithelial proliferative compartment (Yang *et al.*, 2003).

Caveolae were originally reported as flask-shaped, cell membrane invaginations in the gall bladder epithelial and endothelial cells (Palade, 1953; Yamada, 1955), and are now known to be present in a broad variety of cell types (Anderson, 1998). Caveolae are specialized lipid raft microdomains forming 50–100 nm flask-shaped vesicular invaginations of the plasma membrane, which serve as a scaffold for signaling molecules related to cell adhesion, growth, and survival, and are composed of cholesterol, sphingolipids, and several structural proteins known as caveolins; functionally, these plasma membrane microdomains have been implicated in signal transduction and transmembrane transport (Podar *et al.*, 2003; Podar *et al.*, 2002). Several reports have revealed the presence of cell-surface receptors for estradiol in endothelial cells and various other cell types such as granulose cells, endometrial cells, oocytes, spermatozoa, and pituitary cells (Bression *et al.*, 1986). Moreover, ERα have been documented caveolae (Kim *et al.*, 1999).

Although the ultrastructural changes in the dog prostate gland have been extensively reported in the 1960s and 1970s, (Bartsch and Rohr, 1980; Ichihara *et al.*, 1985; Seaman and Winell, 1962; Timms *et al.*, 1976) the occurrence of caveolae in basal cells and their changes have not been addressed in the literature. In a previous study on a canine experimental model of induced prostatic hyperplasia, we noticed the presence of caveolae in basal cell membranes. The purpose of the present study has been to quantify the number of caveolae in basal cells and to compare the changes in the number of caveolae along the several experimental stages in both treated and control animals.

6.3 MATERIALS AND METHODS

6.3.1. ANIMALS

Eight male beagle dogs were used and were divided into two groups (G I, n = 5, experimental group; and G II, n = 3, control group. They were housed individually, fed a standard commercial diet, and provided with water ad libitum. G I animals were castrated and injected with 25 mg of 5α-androstane-3α,17β-diol, and 0.25 mg 17β-estradiol (Steraloids Inc. USA). G II dogs received only 1 ml of the triolein vehicle. The intramuscular injections were given alternately in the hind legs. All animals were treated and cared for in accordance with the University Autonomous of Barcelona School of Veterinary guidelines pertaining to the treatment of experimental animals.

6.3.2. TISSUE SAMPLES

Surgical prostate biopsies were obtained from alternate quadrants before castration and without treatment, and then at 6-week intervals for 36 weeks, resulting in 7 experimental stages, labeled M0 through M6, the latter taken six weeks after withdrawing the hormonal treatment. The biopsies were obtained via a caudal midline celiotomy and were excised from alternating quadrants of the prostate at each interval. The tissues were immediately fixed in 2 % glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for 24 hours. After washing in 0.2 M sodium cacodylate buffer, the tissues were postfixed in a 2% osmium tetroxide solution for 1 hour, with subsequent washings in 0.2 M sodium cacodylate buffer two times for 10 minutes each. The postfixed tissue samples were washed in distilled water, dehydrated in increasingly concentrated ethanol solutions and embedded in Epon 812 resin (Tousimis Research Corporation, USA). Thick, 1 μm control sections stained with toluidine blue were used to select areas with adequate glandular representation. Thin, 90 nm sections were then cut, counterstained with uranyl acetate and lead citrate, and examined with a calibrated CM100 Philips transmission electron microscope (The Netherlands)

6.3.3. QUANTITATIVE ASSESSMENT

Electron micrographs were obtained from the adequately cut basal cells in every section. Magnification ranged from 5,800 to 10,500, and these different magnifications were selected to be able to include the most of a given cell in a single image.From 4 to

10 basal cells were obtained in each sample. The images were printed in a thermal printer, at two images per DinA4 page, resulting in an amplification factor of 2x. The measurements were performed on a digitizing tablet with a pen cursor, using the SigmaScan software package (Jandel Scientific, U.S.A.). The cell membrane length was measured along its basal aspect in all basal cells, and the respective number of caveolae was counted. Thus, the number of caveolae per 100 micrometers of basal plasma membrane was obtained.

6.3.4. STATISTICAL ANALYSIS

Normality of variables was assessed looking at normal probability plots. Analysis was performed by a General Linear Mixed Model. In this model the number of caveolae per 100 μm as the dependent variable and phase of experimental treatment, experimental group, and their interaction were included as explanatory fixed effects. Subject number was included as random effect. Statistical significance was defined as $P<0.05$ for a two-sided test. Analyses were performed using the SAS System for Windows release 9.1 (SAS Institute Inc, U.S.A.).

6.4 RESULTS

6.4.1. COMPARISONS BETWEEN GROUPS 1 AND 2.

The stages M0, M1, M3 and M6 were compared between both groups. The number of basal cell caveolae per 100 μm of cell membrane was significantly different between group 1 and 2 in phases M1 and M3. Thus, the initial (M0) phase values were not significantly different, then the increase in caveolae was significantly higher in the experimental group in both phases M1 and M3. Finally, upon withdrawal of hormonal treatment in the experimental animals, the values in both groups approached again (Table 1, figure 1)

TABLE 1. COMPARISONS BETWEEN DIFFERENT GROUPS AND EXPERIMENTAL PHASES

Comparisons	Mean	Standard error	Upper C.I.	Lower C.I.	Significance
group 1 vs 2 in phase M0	-0,65	0,99	-2,67	1,38	0,519
group 1 vs 2 in phase M1	3,04	0,93	1,40	5,22	0,002
group 1 vs 2 in phase M3	5,62	0,86	3,81	7,42	< 0,001
group 1 vs 2 in phase M6	1,54	0,99	-0,50	3,58	0,132
group 1 in phase M0	2,76	0,82	1,10	4,42	
group 1 in phase M1	2,36	0,77	0,78	3,93	
group 1 in phase M3	4,06	0,67	2,65	5,48	
group 1 in phase M6	1,74	0,67	0,33	3,14	
group 2 in phase M0	2,11	0,56	0,96	3,27	
group 2 in phase M1	5,66	0,51	4,58	6,74	
group 2 in phase M3	9,68	0,54	8,56	10,80	
group 2 in phase M6	3,28	0,74	1,79	4,77	

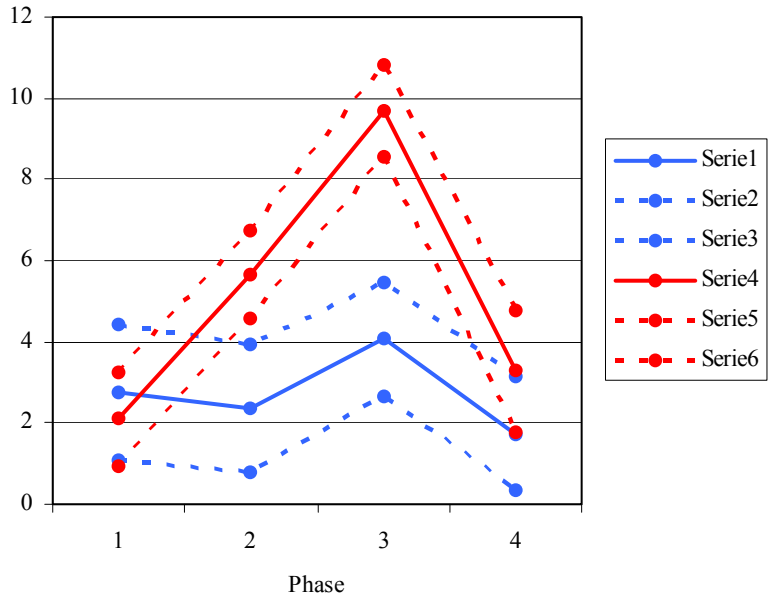

Figure 1. Comparison of caveolae percentages between experimental and control groups. Upper lines represent the experimental group (discontinuous lines for the upper and lower confidence intervals). Bottom lines represent the control group (discontinuous lines for the upper and lower confidence intervals). There is a significant difference between both groups in the two intermediate phases

6.4.2. COMPARISONS BETWEEN PHASES IN GROUP 1

All possible comparisons among experimental phases were statistically tested and most

of them were statistically significant. Essentially, the increase in caveolae was progressive from M0 to M1, to M2, M3, and M4, with a peak reached in M4. Then a significative decline in the number of caveolae was observed for M5 phase and even more for phase M6, in which the number of caveolae approached basal values (Table 2, figure 2)

TABLE 2. COMPARISONS BETWEEN DIFFERENT EXPERIMENTAL PHASES

Phase comparisons	P value
M0 phase vs M1 phase	< 0,001
M1 phase vs M2 phase	0,152
M2 phase vs M3 phase	0,365
M3 phase vs M4 phase	0,004
M4 phase vs M5 phase	< 0,001
M5 phase vs M6 phase	0,002
M0 phase vs M2 phase	< 0,001
M1 phase vs M3 phase	0,013
M2 phase vs M4 phase	< 0,001
M3 phase vs M5 phase	0,105
M4 phase vs M6 phase	< 0,001
M0 phase vs M3 phase	< 0,001
M1 phase vs M4 phase	< 0,001
M2 phase vs M5 phase	0,500
M3 phase vs M6 phase	< 0,001
M0 phase vs M4 phase	< 0,001
M1 phase vs M5 phase	0,450
M2 phase vs M6 phase	< 0,001
M0 phase vs M5 phase	< 0,001
M1 phase vs M6 phase	0,010
M0 phase vs M6 phase	0,302

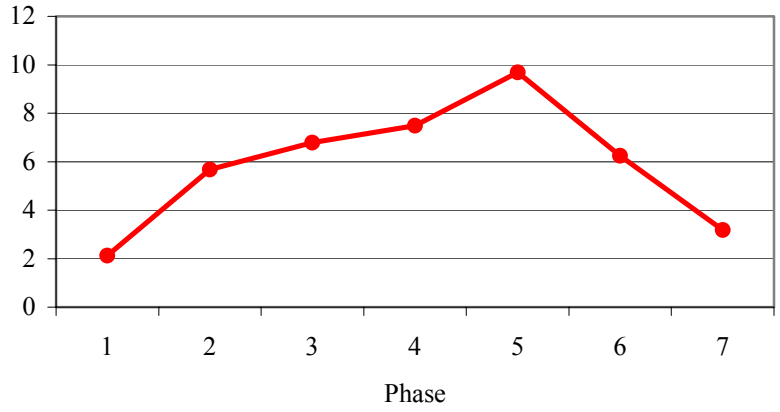

Figure 2. Our results revealed that the joint application of hormones steroids to the experimental dogs induced an increase in the number of caveolae presents in the basal cells of the prostate and we can corroborate it because significant difference ($P < 0.05$).

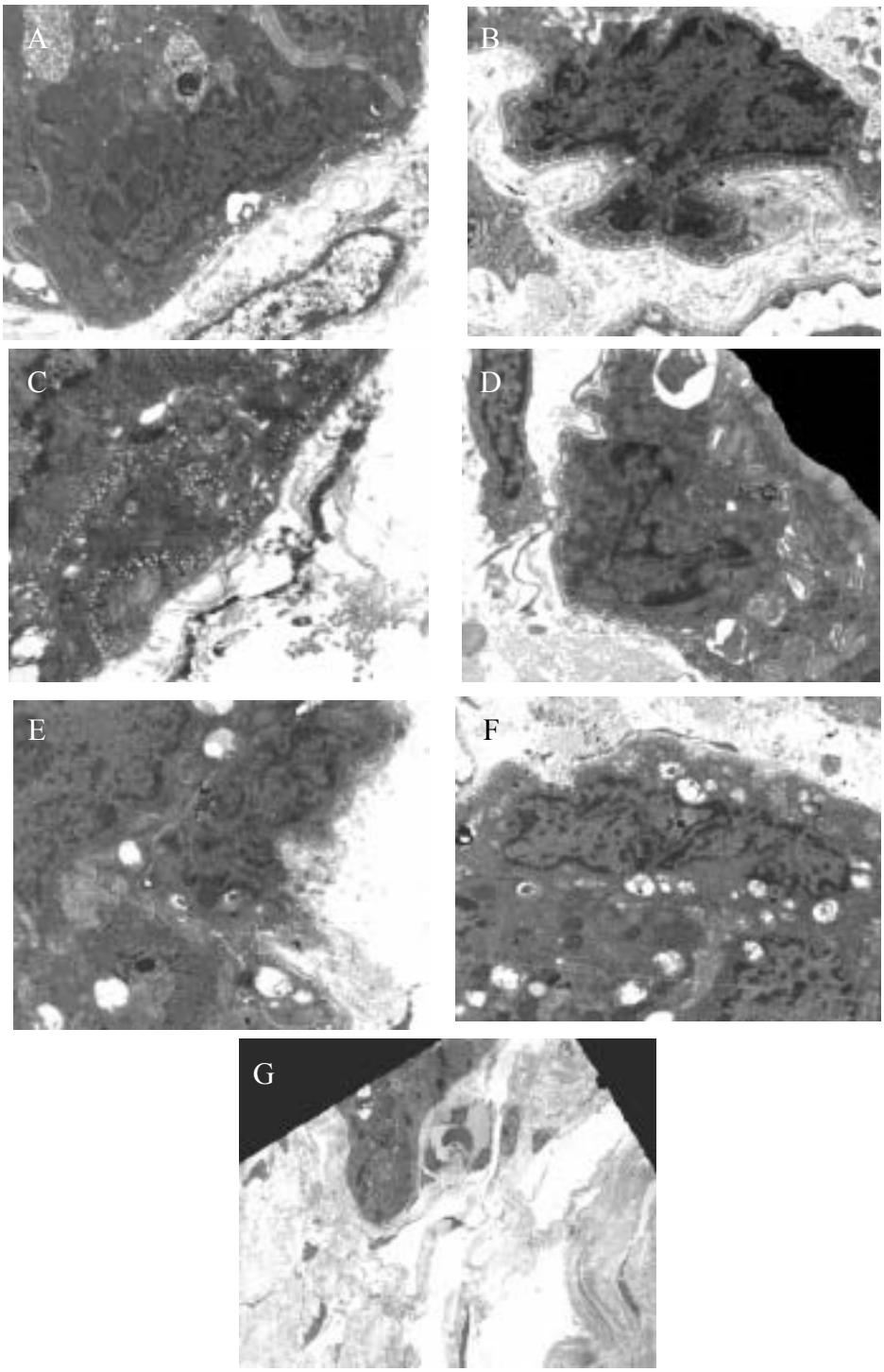

Figure 3. Image in A, shows a basal cell from the first experimental stage (m0), before treatment administration; this cell has scanty caveolae along the cell membrane of the basal domain. Subsequent images, from B through F show stages 2 (M1), 3 (M2), 4 (M3), 5 (M4), and 6 (M5); from the first of these images, a marked increase in the number of caveolae may be noted in response to treatment administration. G shows atrophic prostatic parenchyma after discontinuing the hormonal treatment, perhaps also influenced by the multiple consecutive surgical procedures.

6.5 DISCUSSION

The prostate is a hormonally responsive organ. Androgens are necessary for maintenance of its normal structure and function (Berry *et al.*, 1986b; Leav *et al.*, 2001c; Lowseth *et al.*, 1990), while estrogens act in an almost reciprocal manner, altering normal structure (Brendler *et al.*, 1983; Kwan *et al.*, 1982; Leav *et al.*, 1978). Benign prostatic hyperplasia (BPH) is a very prevalent disease that appears with aging in both dog and men, and is probably related to changes in androgen and estrogen levels in the body (Lowseth *et al.*, 1990; Winter *et al.*, 1995). Young castrated dogs develop prostatic hyperplasia when treated with steroids.

The purpose of the present investigation was to characterize the dynamics of the increase in basal cell caveolae that occurs upon treatment with steroid hormones. This finding is particularly prominent in the basal aspect of the basal cells, facing the prostatic stroma. This is the first report in which the progressive increase in the number of caveolae along the development stages of hormonally induced prostatic hyperplasia is quantified by means of ultrastructural morphometry. This finding points towards the relevance of this structure in the development of prostatic hyperplasia. It also stresses the crucial role of basal cells in the develppment of this disease.

Caveolae assume a variety of shapes, including flat, vesicular and tubular. They may be seen ultrastructurally either opening at the cell surface or closed off, to form a unique endocytic/exocytic compartment, thus each caveola is a dynamic piece of membrane that may be open for receiving and releasing material or closed for processing, storage and delivery to the cell. Biochemical and morphologic techniques have identified a number of molecules that appear to be concentrated in caveolae, relative to the surrounding membrane. Caveolae have a specific lipid composition and are enriched in lipid-modified proteins. They are also rich in receptors and signal-transducing molecules (Anderson, 1998). Cells are constantly targeted by mechanical stimuli from the surrounding cells and the extracellular matrix (Anderson, 1993).

Caveolin has been well recognized as a primary scaffolding protein in the caveolar membrane. It has also been implicated in membrane trafficking of non-clathrin-dependent endocytosis and intracellular cholesterol transport (Lu *et al.*, 2001).

Multiple laboratories have confirmed that membrane steroid receptors can be found in caveolae. One line of evidence suggests that interactions with specific caveolin domains drag membrane estrogen receptors to the plasma membrane. Watson and Gametchu suggest the possibility that multiple membrane localization mechanisms and receptor proteins can act simultaneously or sequentially, and in a cell and receptor context-specific manner. Many studies have shown the presence of membrane receptors in cells of immature developmental stage, cancer cells, or cells subject to steroid-induced proliferation. Thus it is possible that membrane steroid receptors diversify by using developmentally controlled expression mechanisms such as different splice variants (Watson and Gametchu, 2003).

In summary, the present ultrastructural quantitative study has provided direct evidence of the involvement of basal cells in the pathogenesis of prostatic hyperplasia. The dramatic increase in caveolae suggests an extremely enhanced activity of basal cells in this process, probably through an increased interaction with signals from the adjacent stroma or blood capillaries. Future studies should further explore the mechanistic consequences and potential therapeutic implications of this finding.

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Capítulo VII

TRANSCRIPTIONAL SIGNATURE ASSOCIATED WITH THE ONSET OF BENIGN PROSTATE HYPERPLASIA IN A CANINE MODEL

7.1 ABSTRACT

Benign prostatic hyperplasia (BPH) represents the most frequent proliferative abnormality of the human prostate. In spite of the well characterized architecture development of BPH, little is known about the cellular and molecular events that contribute to BPH.

We have developed an animal model to evaluate the follow-up of hormonal-induced BPH and the analysis of gene expression associated with BPH.

Canine specific Affymetrix microarray analysis performed on sequential biopsies obtained in a beagle dog dynamic model, characterized a number of genes altered during BPH. In addition to genes involved in calcification, matrix remodelling, detoxification, and mucosa protection (MGP, MMP2, TIMP2, ITIH3, GST, MT2A, SULT1A1 or FKBP1B, MUC1, TFF3), the up-regulation of TGFB3 and CLU indicates a complete adjustment of the transdifferentiation, senescence and apoptosis programs.

Transcriptome analysis performed on a dynamic animal model that accurately mimics the human clinics, permitted us to characterize a gene expression pattern associated with the onset of BPH.

7.2 INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common age related disorders affecting men, representing the most frequent proliferative abnormality of the human prostate. The development of BPH takes place in an area of the prostate gland accounting for about 5% of this composite organ that surrounds the proximal segment of the urethra, termed transition zone (McNeal, 1990). The progressive obstruction of the urethra leading to urinary retention, bladder function impairment and renal failure (Thomas and Abrams, 2000), involves 80% of men by age 80 and implies that one in three of these patients will require treatment to relieve the obstructive symptoms caused by the disease. In spite of the obvious importance of BPH as a major health problem that significantly affects the quality of life in aging men, its etiology remains unclear (De Marzo *et al.*, 1999).

BPH is characterized by multifocal expansive nodules originated by budding and

branching of the epithelial glandular ducts and acini, and proliferation of prostatic stromal elements (McNeal, 1990; Untergasser *et al.*, 2005). In spite of the well characterized architecture development of BPH, little is known about the cellular and molecular events that contribute to BPH. Recent data from various laboratories support a shift in the balance between cellular growth with apoptosis and senescence (Lee and Peehl, 2004), with a higher proliferative-to-apoptotic ratio in BPH than in normal tissue (Kyprianou *et al.*, 1996), or an increased expression of the anti-apoptotic factor bcl-2 in BPH epithelial cells (Baltaci *et al.*, 2000). Concerning the transdifferentiation and cellular senescence processes that could be involved in the remodelling of stromal tissue, TGF-β1 seems to function as a potent promoter of tissue remodelling in the prostate stroma, by genetically reprogramming fibroblasts into reactive myofibroblasts (Untergasser *et al.*, 2005). Other growth factors reported to play a role in the pathogenesis of BPH include the insulin-like growth factor (IGF) axis and the fibroblast growth factors (FGFs), with suggested autocrine and paracrine modes of action depending on the epithelial and/or stromal production (Lee and Peehl, 2004).

Very recently, DNA microarray technology has been employed by various laboratories to identify candidate genes associated with BPH. Comprehensive analysis of gene expression patterns of BPH would facilitate the understanding of its molecular etiology. In addition, the identification of biomarkers capable of providing a reliable distinction between prostate cancer and BPH is a necessity, as the standard PSA levels can also be caused by prostatitis and BPH (Ziada *et al.*, 1999). In this context, gene signatures have been described that differentially characterise BPH against normal prostate tissue (DiLella *et al.*, 2001; Luo *et al.*, 2002), or against high Gleason grade 4/5 prostate cancer (Stamey *et al.*, 2001). Gene expression profiles between symptomatic versus asymptomatic BPH (Prakash *et al.*, 2002), guided to the association of MIC-1 downregulation in asymptomatic BPH with prostatic inflammation (Taoka *et al.*, 2004). Moreover, gene expression changes of the prostate gland during puberty revealed a subset of genes that shared a expression pattern with BPH (Dhanasekaran *et al.*, 2005). Finally, microarray analysis associated a reduced activity of the 5α-reductase enzyme that catalyses the conversion of testosterone to the more potent androgen, DHT, with prostate cancer when compared to BPH (Luo *et al.*, 2003). In terms of microarray screening for new expression markers, delta-catenin or prostate-specific membrane

antigen (PSMA) were identified as potential candidates for the diagnosis and management of prostate cancer (Burger *et al.*, 2002).

In spite of the highly valuable conclusions of these studies, one important concern of gene expression and BPH is the thorny question of sample selection to analyse any pattern associated with the onset of BPH. BPH samples are obtained from radical prostatectomies of patients with extensive and advanced disease, as the ideal comparison of matched samples of early benign growth and normal prostate transitional zone are limited by insufficient quantities. To circumvent this problem, we have developed an animal model that permitted us to evaluate the follow-up of BPH onset and the analysis of gene expression associated with the initial steps of benign hyperplasic growth. The dog is the only animal known to spontaneously develop prostatic hyperplasia with advancing age (Isaacs, 1984), thus being a unique model for study the pathogenesis of BPH. We took advantage of a model in which prostatic hyperplasia can be induced in castrated dogs by the concomitant administration of 5α-androstane-3α,17β-diol, and 17β-estradiol (Mahapokai *et al.*, 2000).

7.3 MATERIALS AND METHODS

7.3.1. EXPERIMENTAL ANIMALS AND TREATMENT PROTOCOLS

Three male beagle dogs (11/2-2 years) were used for this study. Animals were treated and cared for in accordance with the Autonomous University of Barcelona School of Veterinary guidelines for the treatment of experimental animals. They were housed individually, fed a standard commercial diet, and provided with water ad libitum. Animals were castrated and were injected intramuscularly 25 mg of 5α-androstane-3α,17β-diol, and 0.25 mg 17β-estradiol. Treatment was administered 3 times weekly (Monday, Wednesday, and Friday) for 12 weeks. The intramuscular injections were given alternately in the hind legs.

7.3.2. TISSUE SAMPLES AND IMMUNOHISTOCHEMISTRY

Surgical biopsies of the prostate were obtained via a caudal midline celiotomy and were excised from alternate quadrants, the first sample 2 weeks after arrival of the dogs, before castration and without treatment (T0 sample), and then 6 weeks and 12 weeks

during hormonal treatment (T1 and T2 samples, respectively). Half of the tissues was immediately frozen in liquid nitrogen and stocked at -80ºC until RNA was extracted using the RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany). The other half was fixed on buffered formalin and further processed onto paraffin blocks. Hematoxilin/Eosin and immunohistochemistry staining were performed on four micron sections. Heat-induced epitope retrieval in citrate buffer (pH 7.3) and LSAB+HRP visualization system were used for clusterin immunostaining (1:50 dilution of M-18 antibody from Santa Cruz Biotechnology, Santa Cruz, CA). Absence of primary antibodies were used as negative controls.

7.3.3. SAMPLE LABELLING AND HYBRIDIZATION

Extracted total RNA was used to synthesize double stranded cDNA using the One Cycle cDNA Synthesis Kit (Affymetrix, Inc.) which incorporates a T7 RNA polymerase promoter.

Biotin-labeled antisense cRNA was obtained using the same kit starting with 5 ug of total RNAs and the oligo dT primer 5' GGCCAGTGA-ATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$. cRNAs were purified using the columns from the GeneChip® Sample Cleanup Module (Affymetrix, Inc.) and then 20 μg of cRNAs was fragmented at 94ºC for 30 min in 40 μl of 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM $Mg(OAc)_2$. The fragmented samples were checked in an agarose gel to verify their quality and then added to a hybridization cocktail containing Control oligonucleotide B2 (50 pM) and Eukaryotic Hybridization controls (BioB, BioC, BioD, cre) at 1.5, 5, 25 and 100 pM final concentration respectively from the GeneChip Eukaryotic Hybridization Control Kit (Affymetrix, Inc.), herring sperm DNA (0.1 mg/ml) and acetylated BSA (0.5 mg/ml).

Probe array was equilibrated to RT and prehybridized with 1x hybridization buffer (100mM MES, 1M [Na+], 20mM EDTA, 0.01% Tween 20) at 45ºC for 10 min with rotation. The hybridization cocktail was heated to 99ºC for 5 min in a heat block, transferred to 45ºC heat block for 5 min and spun at maximum speed in a microfuge for 5 min. 200 μl of the hybridization mixture were used to fill the Affymetrix cartridge after removal of the hybridization buffer and the arrays were hybridized at 45ºC for 16 hours with 60 rpm rotation in the Affymetrix GeneChip Hyb Oven 640. GeneChips

were washed and marked with streptavivin phycoerythrin in the Fluidics Station (Affymetrix, Inc.) using the protocol EukGE-WS2 provided by the program MAS 5.0 (Affymetrix, Inc.).

Once washed and streptavidin phycoerythrin-marked the GeneChips were scanned in an Agilent G2500A GeneArray Scanner.

7.3.4. DATA ANALYSIS

CEL files were imported into the affy package (Irizarry *et al.*, 2003) in Bioconductor (http://www.bioconductor.org/) and pre-processed using the rma (robust multi-array analysis) algorithm with the default parameters.

Genes were filtered according to the following criteria: Signal >=log (100), Mean AbsFC >= 1.5. Genes complying with these criteria were then processed using the package limma (a linear model for microarray analysis by Gordon Smyth, Natalie Thorne and James Wettenhall at The Walter and Eliza Hall Institute of Medical Research) and fdr (false discovery rate) was used as the method for multitest correction (Benjamini and Hochberg's stepup method).

7.4 RESULTS

7.4.1. ANIMAL MODEL

Prostatic hyperplasia was induced in castrated dogs by the concomitant administration of 5α-androstane-3α,17β-diol, and 17β-estradiol. After castration, and to focused on the initial steps of induced BPH, we obtained prostate biopsies before hormonal manipulation, and six weeks and twelve weeks after receiving the combination of androgen and oestrogen (green arrows in Fig. 1; upper panel). Light microscopic examination of hematoxylin and eosin stained sections, showed normal tubuloalveolar glands lined by a simple cubical or cylindrical epithelial cells with thin strands of loose intervening stroma in baseline (T0) samples obtained before hormonal manipulation (Fig. 1; lower left panel). On the contrary, an extraordinary overgrowth of the epithelial component at experimental stage T1 was readily apparent, with prominent infolding of tightly packed hyperplastic glandular epithelium protruding towards the glandular lumina and filling them (Fig. 1; lower right panel).

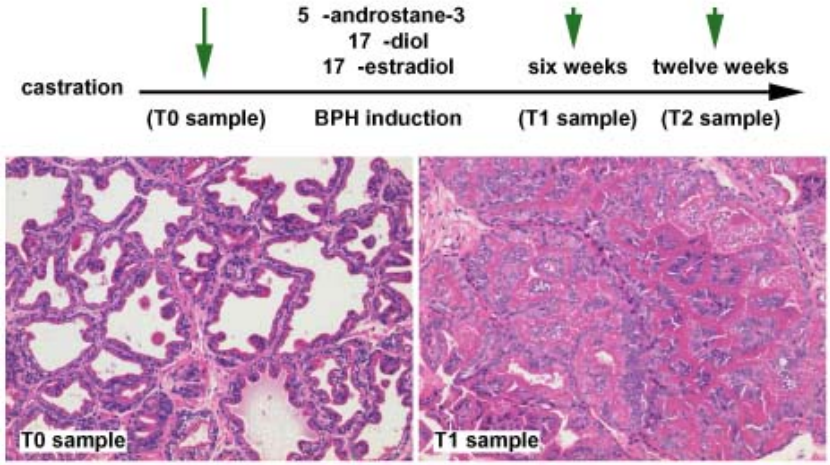

Figure 1 Characterisation of the BPH-induced animal model.

Upper panel, Scheme of BPH induction and biopsy collection. To focused on the initial steps of induced BPH, we obtained prostate biopsies from castrated animals before hormonal manipulation (T0 sample), and six weeks (T1 sample) and twelve weeks (T2 sample) after receiving the combination of androgen and oestrogen. Normal, baseline (T0; lower left panel) and densely packed, experimentally induced (T1; lower right panel) hyperplastic glands (Hematoxylin and Eosin; 400x magnification).

7.4.2. BPH GENE EXPRESSION PROFILING

To analyse gene expression profiles associated with the initial steps of BPH, we performed microarray analysis on prostate samples before and after hyperplasia induction. Three different animals were analysed, and three different biopsies were extracted from each animal all through BPH onset. T0 sample corresponded to normal gene expression profile before BPH induction; T1 sample, as six weeks post-induction gene expression pattern associated with the onset of BPH; and T2 sample, as 12 weeks post-induction gene expression associated with a stably developed BPH (see Fig. 1). Biopsies were subjected to RNA extraction, and further processing to hybridisation with the Affymetrix GeneChip® Canine Genome Array containing over 21,700 transcripts of *Canis familiaris* (see M&M). Individual arrays were used for each of the nine samples analysed, and we then compared global gene expression between samples T1 and T2 versus T0, for each of the three animals. Significant gene expression variations when compared the three gene expression patterns for each animal, were determined after statistical filtering, processing and correction (see M&M). The fact that the first sample

comparison was done on the same animal (T1 versus T0, or T2 versus T0) rendered enough robustness to minimise the number of animal models without compromising the significance of the results. Interestingly, when we analysed the hierarchical clustering for the altered genes found statistically significant associated with BPH, the T1-six weeks samples and the T2-twelve weeks samples clustered together, while T0-pre BPH induction samples clustered in an independent group (Figure 2). This meant that BPH induction occurred as soon as six weeks after hormonal manipulation and characterized by H/E staining (see Figure 1), remained stable at the molecular level during the continuous hormonal treatment. We can conclude from these results that the genes found altered at T1 samples compared to the T0 samples conformed a gene-expression profile associated to the onset of BPH in the beagle model.

Genes found altered during the initial steps of BPH are described in Table I. Among up-regulated genes, we found components of the extracellular matrix, as the endogenous inhibitor of calcification Matrix Gla protein (MGP), that acts as a calcification inhibitor by repressing bone morphogenetic protein 2. Other cell-matrix components found up-regulated in BPH corresponded to protease inhibition processes, on the one hand the Inter-alfa-Trypsin Inhibitor Heavy chain H3 (ITIH3) that functions as serine-type endopeptidase inhibitor activity that prevents or reduces the activity of serine-type endopeptidases. And on the other hand, the Tissue Inhibitor of Metalloproteinase 2 (TIMP2), a natural inhibitor of the matrix metalloproteinase-2 (MMP2) involved in degradation of the extracellular matrix. Concerning detoxification, two genes came up in the microarray comparison up-regulated in BPH, Gluthatione S-Transferase (GST) and Metallothionein-II (MT2A). GST plays an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. MT2A plays a significant role in the detoxification of heavy metals. Two other genes found up-regulated in BPH were the sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 (SULT1A1) and the FK506 binding protein 1B (FKBP1B). Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. FKBP1B is a member of the immunophilin protein family, which play a role in immunoregulation and basic cellular processes involving protein folding and trafficking.

Interestingly, we found mucin-1 (MUC1) and trefoil factor 3 (TFF3) genes up-regulated in post-hormone manipulation samples. MUC1 is a member of the mucin family and encodes a large transmembrane glycosylated phosphoprotein expressed by most normal glandular epithelial cells on the apical surface bordering a lumen. TFF3 is one of the three members of the trefoil factor family of secreted proteins characterized by having at least one copy of the trefoil motif, a 40-amino acid domain that contains three conserved disulfides. It has been hypothesized that TFF peptides and mucins may act in a synergistic manner to protect the mucosa from insults, stabilize the mucus layer and affect healing of the epithelium as mitogenic factors during epithelial restitution after wounding and during inflammation (Kindon *et al.*, 1995; Taupin and Podolsky, 2003). Implications on secretion can also have the up-regulated T-cell differentiation protein MAL, a highly hydrophobic raft-associated integral membrane protein belonging to the MAL family of proteolipids involved in membrane trafficking processes. Finally, two other genes indicated a complete adjustment of the transdifferentiation, senescence and apoptosis programs during BPH onset. These are transforming growth factor, beta 3 (TGFB3), belonging to the beta-type transforming growth factors family of polypeptides that act hormonally to control the proliferation and differentiation of multiple cell types; and the apoptosis-related gene clusterin (CLU), also termed glycoprotein 80, complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, or apolipoprotein J, a secreted heterodimeric glycoprotein expressed in a wide variety of human tissues and fluids.

Table 1. Genes found altered during the initial steps of BPH.

List of genes found statistically altered when T1 samples from hormonal-induced BPHs were compared with their corresponding T0 samples. M values indicate the $\log_2$ of the fold increase, while the statistical significance are determined by the B (log-odds ratio) and p (unadjusted for multiple testing) values.

Function	Gene Title	M	p. value	B
Calcification	MGP	2,4	0,026170142	0,152376069
Matrix remodelling	ITIH3	1,8	0,017490809	0,659259484
	TIMP 2	2,2	0,012446666	1,453799074
Detoxification	GST	2,2	0,005767048	3,03197901
	GST	2,1	0,007483307	2,430670449
	GST-pi	2,1	0,011186252	1,767440713
	MT2A	2,6	0,006796372	2,683526209
Metabolism	SULT1A1	1,8	0,017173077	0,897748357
Immunoregulation/protein folding/trafficking	FKBP1B	1,8	0,017173077	0,784251788
Mucosa protection	TFF3	4,2	0,027452658	0,028578556
	MUC1	5,2	9,35E-05	7,78932636
	MUC1	4,3	0,000284741	6,432677726
	MUC1	2,8	0,001809931	4,401399862
Secretion	MAL	2,3	0,009208285	2,08514438
Transdifferentiation/senescence/apoptosis	TGFB3	2,3	0,017173077	0,74033074
	CLU	5,8	1,79E-05	9,689965299
Unknown	unknown	-1,6	0,016297981	1,088988272
	unknown	1,4	0,017173077	0,761925578
	unknown	-2,5	0,012446666	1,488858444
	unknown	1,6	0,026170142	0,130616013

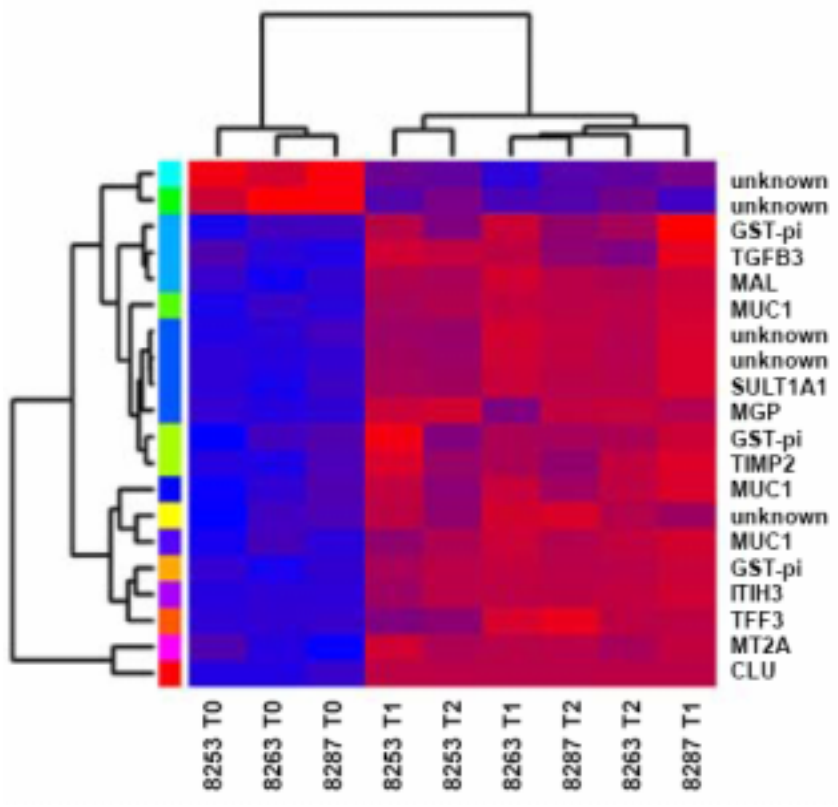

Figure 2. Hierarchical clustering of the nine prostate biopsies obtained from the three different animal models. Cluster analysis was performed with all genes found statistically altered when T1 and T2 BPH samples were compared with the T0 control samples. Note that T0 samples clustered together, in a different group from BPH samples, independently of the animal model. Interestingly, T1 and T2 samples clustered together, indicating that the genes found altered are associated with the onset of BPH.

7.4.3. CLUSTERIN UP-REGULATION ASSOCIATED WITH THE ONSET OF BPH

We next validated the microarray data, and evaluated the accuracy of the animal model at the time of extrapolating the results to the human clinics. For this, we performed immunohistochemistry of the most up-regulated gene, clusterin, on T0 and T1 BPH samples from our beagle hormonal-induced model, and on both animal and human sporadic BPH. As it can be observed in Fig. 3, the baseline samples (T0) were characterized by a negative or extremely weak immunohistochemical expression of clusterin in both the glandular epithelium and the stroma (Fig. 3; upper left panel). On the other hand, experimentally induced hyperplastic glands (T1) showed cytoplasmic, paranuclear, and occasionally membranous staining with clusterin antibody. The stromal cells were mostly negative (Fig. 3; lower left panel). In spontaneous canine and human BPH, weaker staining was observed, but it involved both the epithelial and the

stromal components (Fig. 3; upper and lower right panels, respectively). In addition to validate the data from the microarray analysis, these results extrapolate the overexpression of clusterin to BPH human samples and confirm the accuracy of the animal model to study the molecular alterations associated with BPH.

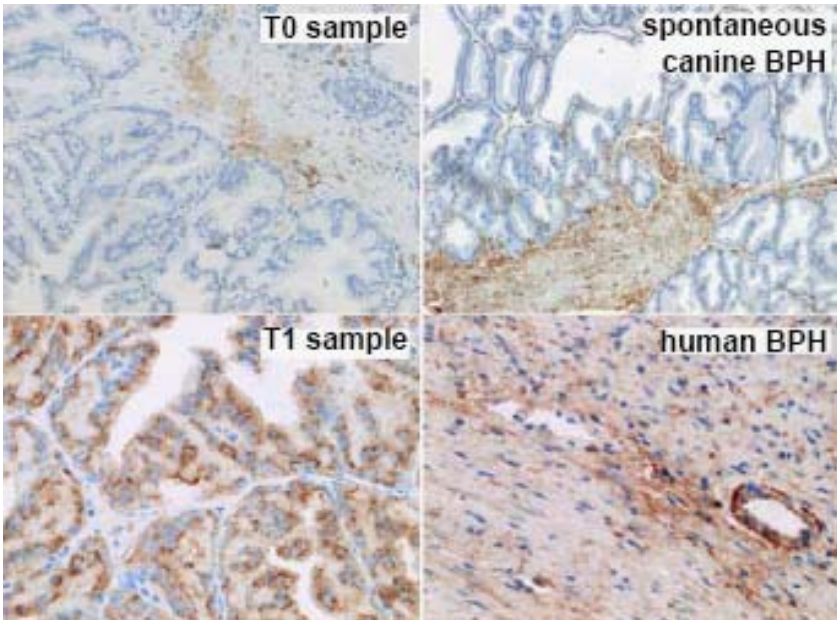

Figure 3 Immunohistochemical detection of clusterin expression.

Upper left panel, Negative baseline sample (T0). Lower left panel, Positive experimental sample (T1), showing positivity restricted mostly to the epithelial cell cytoplasm Upper right panel, Spontaneous canine hyperplasia showing positive stromal and epithelial cells. Lower right panel, Predominantly stromal component in spontaneous human BPH, with positive immunostaining in both the stromal and the epithelial component.

7.5 DISCUSSION

To develop an animal model that could mimic the initial steps of human benign hyperplasic growth and thus be relevant to the study of gene expression associated with the onset of BPH, we focused on the dog in which the bladder muscle is arranged in distinct muscle bundles as in human. Hormonal manipulation to induce an increase in prostate size has been described in the beagle dog as the most appropriate species for investigating abnormal prostate growth comparable to human BPH (Mahapokai *et al.*, 2000).

The integration of the microarray, high-density tissue microarray, and linked clinical

and pathology data represents a powerful approach to molecular profiling of prostate diseases (Dhanasekaran *et al.*, 2005). In this study we tackled the molecular alterations associated with the onset of BPH. The fact that biopsies obtained six weeks after hormonal manipulation clustered together with the ones obtained twelve weeks after BPH induction, indicates that the gene-expression pattern that we characterized is associated with the onset of BPH, remaining stable once the hyperplasic growth has been triggered. This points to a variety of cellular pathways being altered during BPH:

Concerning MGP, soft tissue calcification arises in areas of chronic inflammation, possibly functioning as a barrier limiting the spread of the inflammatory stimulus. Atherosclerotic calcification may be one example of this process, in which oxidized lipids are the inflammatory stimulus. Interestingly, the development of BPH is accompanied by the occurrence of prostatic secretory granules (corpora amylacea), and prostatic calculi generated by calcifications of corpora amylacea or simple precipitation of prostatic secretion, that exacerbate the lower urinary tract symptoms (Geramoutsos *et al.*, 2004). These bodies may also clog prostatic ducts, obstruct prostatic acini, initiate inflammatory reactions and cause abscesses. In prostate carcinomas, MGP has been described highly expressed associated with tumor progression and metastasis (Levedakou *et al.*, 1992). Regarding MMP2 and TIMP2, these genes were found co-expressed in a majority of prostate carcinomas correlating with prognostic variables, TIMP2 expression appearing to have a tumour-promoting role in prostate cancer (Ross *et al.*, 2003). Secretion of both MMP2 and TIMP2 were described by stromal rather than epithelial cells from primary cultures of BPH and prostate carcinomas (Wilson *et al.*, 2002).

In relation to detoxification and GST, benign prostate tissue expresses higher levels of GST-pi, mainly in the basal layer, compared to prostate cancer, suggesting that GST may play a role in the prevention of prostate carcinogenesis through inactivation of reactive electrophiles by conjugation to reduced glutathione (Cookson *et al.*, 1997). MT2A plays a significant role in the detoxification of heavy metals, including the prostate gland (Pannek *et al.*, 2001). In addition, metallothioneins have been associated with protection against DNA damage, oxidative stress and apoptosis. In benign prostatic hypertrophy, MT was mainly localized in the nuclei of epithelial cells while in the

adenocarcinoma MT was mainly localized in the cytoplasm of the epithelial cells (Bataineh and Nusier, 2003).

Relative to SULT1A1, oestrogen sulfotransferase, the enzyme that inactivates estrogen, it has been found selectively expressed in male tissues, thus suggesting a role for this enzyme to protect male-specific tissues against estrogenic activity (Luu-The *et al.*, 2005). Moreover, age-dependent loss of androgen sensitivity has been associated with a marked increase in dehydroepiandrosterone/hydroxysteroid sulfotransferase (rStd) activity, suggesting that intracellular androgen sulfonation can physiologically influence androgen action (Chan *et al.*, 1998). FKBP1B is a cis-trans prolyl isomerase that binds the immunosuppressants FK506 and rapamycin. It is highly similar to the FK506-binding protein 1A, recently described as a potential novel diagnostic marker or target for prostate cancer therapy (Velasco *et al.*, 2004).

MUC1 and TFF3 genes have been found to follow a cell- and tissue-specific pattern in normal tissues, and their expression described in premalignant lesions of the stomach and in gastric carcinomas (Machado *et al.*, 2000). Trefoil peptides are mainly produced by secreting epithelial cells that line the gastrointestinal tract and other epithelial tissues as the respiratory tract, the salivary glands, the uterus and the conjunctiva, being packaged and secreted along with the large-molecular weight glycoproteins mucins (Hoffmann and Jagla, 2002). Aberrant TFF3 peptide expression has been associated with a number of chronic inflammatory diseases including Crohn's disease, ulcerative colitis, and cholecystitis. Concerning the prostate, DNA microarrays have demonstrated overexpression of TFF3 in prostate cancer cells, further confirmed by tissue microarrays, with a staining pattern for TFF3 protein expression increasing with progression from non-tumor to tumorigenic states, that includes 18% of positive BPH staining (Garraway *et al.*, 2004). Similar results were described by the group of De Marzo, in a subset of primary and metastatic prostate cancer specimens as compared to normal epithelium (Faith *et al.*, 2004). Although the contribution of TFF3 expression to prostate carcinogenesis is still unknown, it has been speculated a role reported in cell scattering and tumor invasion, with changes in epithelial cell morphology and motility associated with a modulation of the E-cadherin/catenin cell adhesion complex (Meyer zum Buschenfelde *et al.*, 2004). On the other hand, BPH showed expression of MUC1

on the epithelial cells with no basal or stromal involvement, with alterations in glycosylation postulated to be under the influence of the steroid hormones (Schut *et al.*, 2003). MUC1 expression profiling has been proposed as a molecular prognosis and stratification factor in advanced stage prostate tumours associated with an elevated risk of recurrence (Lapointe *et al.*, 2004). Interestingly, mucinous differentiation has been suggested as an important step in the acquisition of hormone independence phenotype, implying a role for mucins in an unknown pathway of hormonal escape in prostate cancer (Legrier *et al.*, 2004). Also involved on secretion, MAL has been localized to the endoplasmic reticulum of T-cells and is a candidate linker protein in T-cell signal transduction. In addition, this proteolipid is localized in compact myelin of cells in the nervous system and has been implicated in myelin biogenesis and/or function. The protein plays a role in the formation, stabilization and maintenance of glycosphingolipid-enriched membrane microdomains. Together with caveolin-1, MAL has been described located on prostasomes secreted by the prostate cancer PC-3 cell line (Llorente *et al.*, 2004).

Another interesting couple of genes associated with the onset of BPH is conformed by TGFB3 and CLU. TGFb is related to the correct tissue homeostasis and function of the prostate gland, in reference to the interaction stroma/epithelia, becoming increasingly apparent that TGFb intimately regulates the various events associated with the development of BPH (Huang and Lee, 2003). As a regulation factor produced and secreted by the epithelial cells, TGFb acts in a paracrine manner on the fibroblasts of the adjacent stroma, that provides with the growth of the matrix to the functional epithelium affecting its proliferation and differentiation (Untergasser *et al.*, 2005). Likewise, TGFb may induce human prostatic stromal cells to express the smooth muscle phenotype and inhibited their growth (Hisataki *et al.*, 2004). On the other hand, the functional polyvalence of CLU comprises several physiological processes as sperm maturation, tissue remodelling, cell-cell and cell-substrate interactions, stabilization of stress proteins, as well as both induction and inhibition of apoptosis. Interestingly, clusterin is able to respond through its promoter in a cell-type and signal specific manner to a myriad of stress and develop signals, representing a sensible sensor to acute stress (Trougakos and Gonos, 2002). Interestingly, the up-regulation of CLU might mediate the inhibition of spontaneous and androgen-induced prostate growth by a

nonhypercalcemic calcitriol analog (Crescioli *et al.*, 2003). Relative to this and the AP-1 site on the promoter, clusterin is able to respond to TGFb (Jin and Howe, 1997), presumably through its effects on c-Fos protein synthesis and/or stability (Jin and Howe, 1999).

7.6 CONCLUSIONS

In this work, we demonstrate the accuracy of the beagle dog animal model, close to the human clinics, to develop a transcriptome analysis characterizing a gene expression pattern associated with the onset of BPH. Our results point to a number of pathways being altered during the initial steps of the benign prostate hyperplasia, pathways that might be involved both in the promotion and in the tissular response to the hyperplasic growth (Fig. 4). In addition to genes involved in calcification, matrix remodelling, detoxification, and mucosa protection (MGP, MMP2, TIMP2, ITIH3, GST, MT2A, SULT1A1 or FKBP1B, MUC1, TFF3), the up-regulation of TGFB3 and CLU indicates a complete adjustment of the transdifferentiation, senescence and apoptosis programs. In addition to contribute with new clues for the understanding of the hyperplasic growth, this study characterized a number of genes that could represent new potential therapeutic targets. Considering BPH as a major health problem that significantly affects the quality of life in aging men, it is imperative the development of new therapeutic strategies improving the outcome of BPH.

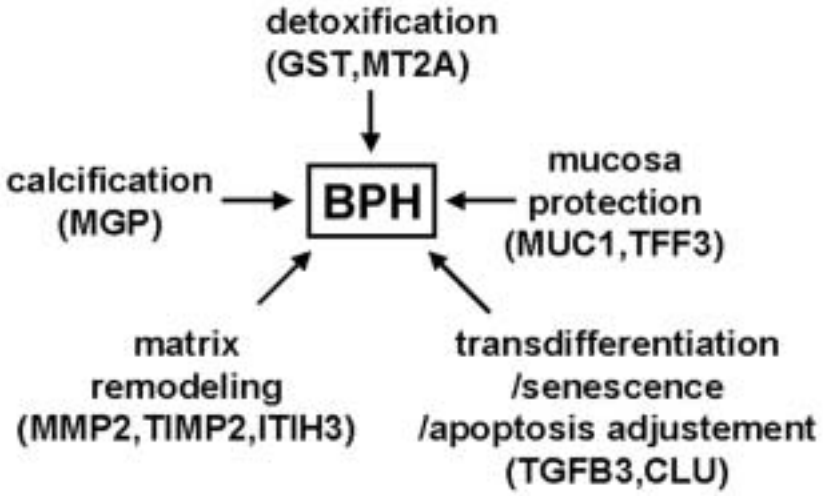

Figure 4. Summary of the different pathways involved on BPH onset, based on the canine transcriptome analysis performed on the hormonal-induced BPH beagle dog animal model. In addition to genes involved in calcification, matrix remodelling, detoxification, and mucosa protection, the up-regulation of TGFB3 and CLU indicates a complete adjustment of the

transdifferentiation, senescence and apoptosis programs.

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Capítulo VIII

DISCUSION GENERAL

Estudios previos realizados hace más de cuatro décadas han demostrado la utilidad del perro como modelo experimental en la HBP humana, ya que a pesar de que todos los mamíferos tienen próstata, sólo el hombre y el perro desarrollan HBP espontáneamente con elevada frecuencia durante la senescencia de la vida, además del hecho de poder inducir experimentalmente dicha patología en perros jóvenes (Berry and Isaacs, 1984; Brendler *et al.*, 1983; DeKlerk *et al.*, 1979; Lowseth *et al.*, 1990; Oesterling *et al.*, 1988; Walsh and Wilson, 1976). A pesar de que existen algunas diferencias entre la HBP humana y canina, podemos considerar que el perro es un buen modelo animal para el estudio de HBP humana (Lowseth *et al.*, 1990; Murakoshi *et al.*, 2000b; Okada *et al.*, 1988).Sin embargo, en los últimos años la gran mayoría de los investigadores han desplazado su interés en la búsqueda de modelos experimentales para dicha patología hacia las ratas y los ratones (Krege *et al.*, 1998; Ma *et al.*, 2004; Prins *et al.*, 2001; Sar and Welsch, 2000; Shappell *et al.*, 2004; Wennbo *et al.*, 1997). Ello se debe probablemente a la mayor facilidad de manejo de estas especies por su reducido tamaño y costo, así como a la posibilidad de producción de transgénicos, unido a las presiones ejercidas por los comités de ética y las instituciones que luchan por evitar el sufrimiento de los animales domésticos.

El primer capítulo experimental de esta tesis doctoral se centró en el estudio de la expresión inmunohistoquímica de receptores de hormonas esteroides en la glándula prostática de perros sanos y con algunas de las diferentes patologías que pueden afectar a dicha glándula espontáneamente, debido a la importancia que tienen estos receptores nucleares para el entendimiento de la biología reproductiva. En este primer estudio, utilizando bloques embebidos en parafina, pudimos comprobar que mediante la utilización de anticuerpos comerciales para AR, ERα, ERβ y PR se observaban variaciones tanto en la intensidad de tinción como en el porcentaje de células que expresan los diversos receptores hormonales en las distintas patologías estudiadas. En resumen, podemos decir que este estudio proporciona la localización celular específica de los mencionados receptores de hormonas esteroideas en la glándula prostática sana, con hiperplasia, prostatitis y cáncer de perros machos adultos.

Es bien conocido que las hormonas esteroides están implicadas en la regulación de muchas funciones corporales, incluyendo la reproducción de machos y hembras. De igual forma se acepta que la acción biológica de estas hormonas es mediada a través de

receptores específicos los cuales se ubican en células específicas en los tejidos diana (Sar and Welsch, 2000).

Nuestro estudio nos corroboró que existe una considerable variación en la expresión de estos receptores entre las diferentes patologías estudiadas. Basándonos en estudios previos realizados en otras especies (Krege *et al.*, 1998; Pelletier *et al.*, 2000; Rosenfeld *et al.*, 1998), consideramos que es necesario ser muy cautelosos a la hora de extrapolar resultados de expresión de receptores de hormonas esteroides entre especies, e incluso entre diferentes individuos de la misma especie, al comparar estudios de biología del tracto reproductivo del macho, ya que son muchos los factores que pueden influir en los resultados obtenidos.

La mayoría de las células epiteliales del tejido prostático incluido en nuestro estudio expresaron los cuatro receptores de esteroides estudiados, mientras que las células estromales solo expresaron AR y ERα. La interacción entre el epitelio y el estroma ha sido estudiada en diversos tejidos (Cunha *et al.*, 1985; Gabison *et al.*, 2005; Slayden and Brenner, 2004), y existe una clara evidencia de que el estroma desempeña un importante papel en la determinación de la diferenciación y función epitelial a través de mecanismos paracrinos (Cooke *et al.*, 1997; Prins *et al.*, 2001). Además, es bien conocido que los andrógenos y los estrógenos modulan la proliferación y diferenciación de la glándula prostática en conjunto con algunos factores de crecimiento y la matriz extracelular (Coffey and Walsh, 1990; Cunha, 1994; Marcelli and Cunningham, 1999; Monti et al., 2001). En dicha glándula, el epitelio es mantenido por los andrógenos y el estroma es un tejido diana tanto para andrógenos como para estrógenos (McConnell, 1995).

La acción de los andrógenos, igual que la de otros esteroides, tales como los estrógenos y la progesterona, es mediada a través de receptores específicos en las células diana, los cuales al unirse a los ligandos provocan un cambio conformacional que resultan en la dimerización y subsiguiente asociación con regiones específicas dentro de las regiones reguladoras de los genes *blanco* (Carson-Jurica *et al.*, 1990). Estudios realizados in vitro, han demostrado que los ERα y ERβ pueden formar homo y heterodímeros, los cuales pueden tener diferentes afinidades o especificidades para algunos ligandos y esto a su vez podría causar diferente activación de genes (Hall and McDonnell, 1999; Sun *et*

al., 1999).

En este estudio, la expresión de AR fue muy similar en las próstatas sanas y en las hiperplasias, disminuyendo en el resto de patologías estudiadas, lo que concuerda con los resultados publicados por otros autores (Murakoshi *et al.*, 2000a; Murakoshi *et al.*, 2000b). En relación a la expresión de ERα, es importante señalar que el porcentaje de células epiteliales positivas fue elevado en las próstatas normales disminuyendo a menos de la mitad del valor en todas las patologías estudiadas y la expresión en células estromales mantuvo un patrón similar. En un estudio publicado por Schulze y Barrack (1987) se describe positividad a ER confinada al núcleo de las células estromales y al epitelio de los ductos prostáticos, pero no en el epitelio de los acinos glandulares. Las diferencias con respecto a nuestros resultados podrían ser ocasionadas por la especificidad de los anticuerpos utilizados, diferencias en el procesamiento de las muestras o de la técnica de revelado empleada, la no utilización de métodos de recuperación antigénica e incluso al hecho de que en esa época sólo se conocía la existencia de una isoforma de ER. En relación a la expresión de la isoforma ERβ, sólo se expresó en células epiteliales, siendo su expresión más elevada en las muestras de tejido sano y disminuyendo en las diferentes patologías. A nuestro conocimiento, este estudio es el primero que reporta expresión de ERβ y de PR en la próstata canina y por tanto no tenemos ningún patrón de comparación. Sin embargo, nuestros resultados fueron similares a los obtenidos por Enmark y colaboradores (1997) en próstata humana y por Pelletier y colaboradores (1999) en próstatas de monos. Igual que en el caso de los ERβ, los PR sólo se expresaron en células epiteliales, pero al contrario que en el resto de receptores estudiados, los PR tienen una expresión muy baja en glándulas normales, que se incrementa en las neoplasias.

El segundo capítulo se basó en la inducción experimental de hiperplasia benigna de la próstata en perros jóvenes de raza Beagle, bajo condiciones controladas. Entre las diversas patologías que pueden presentarse en la glándula prostática del perro, la aparición de HBP es la más común y, aunque es una alteración causada por el envejecimiento en machos sexualmente intactos, puede lograrse su aparición en etapas muy tempranas de la vida del animal mediante la manipulación hormonal (DeKlerk *et al.*, 1979; Leav *et al.*, 2001b; Mahapokai *et al.*, 2000; Walsh and Wilson, 1976; Winter *et al.*, 1995). Por tanto, nos planteamos el seguimiento de las variaciones histológicas e

inmunohistoquímicas que se presentan desde la instauración y desarrollo de la hiperplasia hasta su posterior involución una vez retirado el tratamiento hormonal. En todos los animales del grupo experimental se observó la aparición de hiperplasia glandular caracterizada por un incremento tanto del número de células como del tamaño de las mismas en el epitelio del lumen de los acinos, además de estimular el crecimiento del tejido estromal, especialmente en los septos entre glándulas individuales. Por el contrario, no observamos hiperplasia valorable en los animales del grupo control. Además, observamos áreas de prostatitis en todos los animales de ambos grupos. Nuestros resultados están en concordancia con los obtenidos por otros autores (DeKlerk *et al.*, 1979; Leav *et al.*, 2001b; Mahapokai *et al.*, 2000).

En este estudio, el 100% de las células epiteliales y un elevado porcentaje de células estromales mostraron una uniforme e intensa tinción nuclear para AR y ERα en todas las biopsias tomadas durante el transcurso de la fase experimental, menos en la última, en la que ya se había retirado la aplicación de hormonas. También observamos una ligera tinción citoplasmática en las células epiteliales para este receptor, lo cual era de esperar, ya que ha sido previamente descrito que la administración de andrógenos incrementa tanto la cantidad como la vida media de los AR (Smith *et al.*, 1984; Syms *et al.*, 1985; Syms *et al.*, 1983; Syms *et al.*, 1984). El porcentaje de expresión de los ERβ fue más elevado en las próstatas sanas en comparación a las diferentes patologías estudiadas y sólo se observó inmunoreacción en el núcleo de las células epiteliales. Existe evidencia de que los estrógenos inducen la aparición de hiperplasia y tumores en diferentes tejidos, incluida la próstata en diferentes animales (Fernandez *et al.*, 2004; Leav *et al.*, 1978; Newbold *et al.*, 1990; Scarano *et al.*, 2004) todo lo cual nos sugiere que los estrógenos podrían jugar un importante papel en la inducción de la pérdida del control tanto del crecimiento celular como de la inhibición de la apoptosis. Además existen estudios que demuestran que la aplicación de estrógenos produce metaplasia escamosa en la próstata de perros castrados o hipofisectomizados (Leav *et al.*, 1978; Merk *et al.*, 1986). Partiendo del hecho de que los estrógenos son moduladores de las funciones reproductivas normales de los mamíferos y que su acción en los tejidos es modulada a través de receptores específicos, es lógico pensar que el aumento o disminución de cualquiera de las dos isoformas de ER provocará alteraciones en la histología prostática (Curtis and Korach, 2000). Según el estudio de Kuiper y

colaboradores (1998), ERβ fue el receptor predominante en la próstata de rata y aunque ambos ER tienen una alta homología en el dominio de unión al ADN (95%), sólo muestran un 60% de homología en el dominio de unión al ligando, pudiendo activar diferentes elementos de respuesta a los estrógenos. Por tanto, aunque la función biológica de las dos isoformas de ER en la próstata aún no ha sido definida, en un estudio con ratones a los cuales faltaba el gen para ERβ fue reportado el desarrollo de hiperplasia prostática, lo cual sugiere que este receptor podría actuar como factor inhibidor del desarrollo de crecimiento anormal de la glándula (Leav *et al.*, 2001a).

La presencia de ambos receptores, ERα y ERβ en el epitelio y sólo de ERα en el estroma del tejido prostático nos permite confirmar que los estrógenos tienen una acción directa sobre los dos compartimientos prostáticos en el perro, tal como lo sugieren Royuela y colaboradores (2001) en su estudio con próstatas humanas. De igual forma, el incremento de inmunotinción para ambos ERs en nuestro estudio de BHP inducida en perros jóvenes sugiere que la implicación de los receptores de estrógeno en la hiperplasia prostática concierne principalmente al epitelio.

La evidencia de PR fue muy escasa y de mínima intensidad en todas las muestras tanto del grupo experimental como del grupo control.

El tercer y el cuarto capítulo experimental se relacionan con la descripción detallada de los cambios observados durante el inicio, desarrollo e involución de la hiperplasia prostática a través del microscopio electrónico de transmisión.

Descripciones de los hallazgos de microscopía electrónica de HBP canina han sido reportados previamente (Bartsch and Rohr, 1980; Leav *et al.*, 1978; Merk *et al.*, 1982; Merk *et al.*, 1986; Timms *et al.*, 1976) pero en ninguno de esos estudios ha habido un seguimiento secuencial del proceso de instauración de la hiperplasia con los correspondientes cambios que van produciéndose a medida que el proceso avanza. En nuestro estudio todas las muestras fueron procesadas de la misma forma para garantizar la preservación de la ultraestructura. Los hallazgos de mayor relevancia consistieron en la identificación de abundantes gránulos de secreción en las células luminales que parecen fragmentarse al llegar a la región apical, justo antes de liberar su contenido. Además observamos un notable incremento en el número de vesículas de micropinocitosis (caveolas) de la membrana de células basales en la superficie en

contacto con la lámina basal. Tal y como ha sido descrito previamente, pudimos identificar la presencia de desmosomas en la unión entre células basales y células secretorias o luminales. Las células basales se ubican entre la región basal y las células secretorias, formando una capa discontinua, ya que lo más común fue observar una célula basal interpuesta cada siete u ocho células luminales.

Las caveolas son invaginaciones de la membrana plasmática, de forma aplanada, que están presentes en la pared de una gran cantidad de células epiteliales y también endoteliales (Anderson, 1998). A nivel ultraestructural, se pueden observar caveolas morfológicamente identificables en la membrana plasmática de numerosos tejidos y tipos de células. Según diversos estudios, más del 70% del área de la membrana plasmática alveolar del pulmón puede estar ocupada por caveolas, mientras que otros tipos de células, como las neuronas, carecen totalmente de caveolas (Razani *et al.*, 2002). Por otra parte las caveolas pueden existir, en muchas formas alternas a la tradicional variedad de invaginación de la membrana. Las caveolas estan formadas por oligomerización de las caveolinas, las cuales junto con las flotilinas y las cavatelinas son las proteínas integrantes de la membrana. Todas estas son familias de proteínas que han sido recientemente reportadas como proteínas modificadoras de los "*raft lipídicos*". La función inicial de las caveolas se creía que estaba limitada a los procesos de pinocitosis, pero con el advenimiento de nuevas herramientas para la investigación de su funcionamiento, su rol como simples transportadores vesiculares se ha expandido hasta incluir la transcitosis, endocitosis, homeostasis del colesterol y transducción de señales. Así, las caveolas pueden servir como punto de embarque para numerosos receptores de superficie celular, los cuales son activados por la unión a los ligandos al ser reclutados por las caveolas (Cohen *et al.*, 2004). Se suma a ello el hecho de que se han realizado varios estudios experimentales en diversos sistemas celulares que sugieren la existencia de otros efectos de los esteroides aparte de sus efectos genómicos, ya que esos efectos no pueden ser explicados por el clásico modelo de interacción esteroide-célula diana. Por tanto, estos efectos podrían, tal vez, ser explicados por la existencia de señales generadas por receptores de esteroides que estén ubicados sobre la superficie de la célula, dentro de las caveolas, y a los que llamaríamos efectos "no genómicos" de los esteroides. Estos efectos no genómicos de los esteroides se caracterizan por: 1) Ocurrir muy rápidamente, 2) Ser compatibles con cambios en la síntesis de proteínas y el ARN

mensajero; 3) Tener lugar en células clonadas que carecen de receptores nucleares de esteroides o en células altamente especializadas como los espermatozoides; 4) No poder ser bloqueados por inhibidores del ARNm ni por antagonistas de los receptores genómicos clásicos de los esteroides y5) Se altamente específicos (Revelli *et al.*, 1998; Simoncini and Genazzani, 2003). Estos receptores de superficie (no genómicos) representan una parte de un complejo sistema de regulación celular en el cual los esteroides y péptidos no sólo actúan sobre la misma célula diana, sino que también están envueltos los mismos mensajeros intracelulares, modulando las mismas vías de señalización (Revelli *et al.*, 1998).

El quinto capítulo experimental de esta tesis doctoral consistió en el estudio de la expresión de genes asociados con el inicio, desarrollo y la involución de la HBP en el modelo canino mediante la realización de microarrays de cDNA como herramienta que nos indica el patrón de expresión génica desde el inicio de la patología. El diseño experimental y los criterios bioinformáticas utilizados en este estudio nos permitieron identificar más de 10 genes sobreexpresados, algunos de los cuales han sido relacionados con patologías prostáticas en humanos (Faith *et al.*, 2004; Garraway *et al.*, 2004; Kirschenbaum *et al.*, 1999). Los hallazgos descritos en este capítulo nos permiten demostrar la aplicabilidad de la utilización de los análisis de expresión génica obtenidos de microarrays como una herramienta muy útil para el estudio de las complejas vías moleculares implicadas en el desarrollo de la hiperplasia de próstata. Son necesarios futuros estudios para asignar un rol a los diferentes genes identificados en este estudio.

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Capítulo IX

CONCLUSIONES

1. Los receptores de andrógenos y estrógenos alfa se expresan tanto en el núcleo como en el citoplasma de las células epiteliales de la próstata de perro. También se observa expresión immunohistoquímica de ambos receptores en el núcleo de las células estromales.
2. Los receptores de estrógenos beta y progesterona se expresan en el núcleo de las células epiteliales de la próstata canina pero no en el de las células estromales.
3. Los receptores de andrógenos se expresan en elevado porcentaje de células epiteliales de animales sanos e hiperplásicos y disminuyen su expresión en las muestras tumorales. El porcentaje de positividad a nivel de células estromales es menor al de las células epiteliales y no presenta variaciones en las diferentes patologías caninas estudiadas.
4. Los receptores de estrógenos alfa se expresan en un mayor porcentaje en los animales sanos mientras que los receptores de estrógenos beta mostraron un porcentaje de positividad similar en las diferentes patologías estudiadas.
5. Mientras que el porcentaje de células positivas a los receptores de andrógenos y estrógenos alfa se mantiene elevado durante la inducción hormonal de la HPB, este porcentaje disminuye significativamente después de la retirada del tratamiento hormonal.
6. El porcentaje de células que expresan marcaje para los receptores de estrógenos beta se mantiene alto tanto durante el tratamiento inductivo de HPB como después de su retirada.
7. La inducción hormonal de HPB resulta en una baja expresión immunohistoquímica para los receptores de progesterona, expresión que disminuye significativamente después de la supresión hormonal.
8. El presente estudio describe la presencia de caveolas en la superficie basal de las células basales de la próstata de perros a los que se les ha inducido HPB. El número de estas caveolas incrementa de forma significativa durante la aplicación del tratamiento hormonal para disminuir significativamente después de su retirada.
9. En función de los genes alterados (MGP, MMP2, TIMP2, ITIH3, GST, MT2A,

SULT1A1, FKBP1B, MUC1, TFF3), se han caracterizado una serie de mecanismos implicados en la inducción y respuesta al crecimiento hiperplásico, como calcificación, remodelación de la matriz, detoxificación y protección de la mucosa.

10. La activación de la expresión de TGFB3 y CLU indica un reajuste de los programas de transdiferenciación, senescencia y apoptosis, en relación con los inicios de la hiperplasia benigna de próstata.