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Effects of Age on Growth and ERβ mRNA Expression of Canine Prostatic Cells

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Abstract. *Background: Benign prostatic hyperplasia (BPH) is an age-dependent prostatic disease in human males and dogs. The prostatic stromal estrogen level of health control and BPH patients increases significantly with age, while the dihydrotestosterone (DHT) level is not connected with age. Moreover, experimentally estrogens have induced BPH in the presence of androgens. Our aim was to investigate the effects of age on the proliferation and estrogen receptor beta (ERβ) mRNA of canine prostatic epithelial and stromal cells. Materials and Methods: Epithelial and stromal cells were isolated from canine prostatic tissues. The proliferation of these cell types from dogs of different ages was assessed by thymidine incorporation assay, while the expression and identification of ERβ mRNA were performed by RT-PCR and DNA sequence. Results: Prostatic epithelial cells isolated from 1-year-old dogs exhibited a greater proliferative activity than those of 4-year-old dogs. In contrast, the prostatic stromal cells from 4-year-old dogs proliferated more rapidly than the cells from 1-year-old dogs. ERβ mRNA expression was detected in the canine prostatic epithelial and stromal cells, decreasing with age. The partial DNA sequence showed that the canine ERβ sequence shares 90.0%, 87.0% and 83.0% of its nucleotide homology with human, rat and mouse ERβ, respectively. Conclusion: The decrease in the expression of ERβ in prostatic cells with age reduces its negative control over the androgen receptor, associated with the overgrowth of canine prostatic stromal cells, which further induces the development of canine BPH.*

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The development of benign prostatic hyperplasia (BPH) is mainly associated with androgens, with estrogens only playing a minor role. However, recent clinical data has shown that estrogens play an important role in regulating the growth of normal prostate cells and are associated with prostatic pathology (1-4). In elderly men, the ratio of free estradiol (E_2) to free testosterone (T) was observed to increase by up to 40% (5, 6). Symptomatic BPH patients compared to healthy controls revealed a 33% increase in stromal volume (7). The prostatic stromal estrogen levels of both normal males and BPH patients have been demonstrated to increase significantly with age, while the stromal dihydrotestosterone (DHT) levels in both groups are not correlated with age (8, 9). Moreover, the E_2/DHT ratio in the transitional zone of human BPH patients was observed to be positively correlated with prostate volume, proportion of stroma and age (10). The proliferation of prostatic stromal cells of BPH patients was also found to be associated with higher plasma estrogen and urinary estrogen secretion, but not correlated to the testosterone level (11). In addition, estrogens were experimentally demonstrated to cause a marked dose-dependent stimulation of prostate growth in castrated dogs (12) and induced BPH of dogs in the presence of DHT (13). Because BPH is a stromal disease arising in the periurethral transition zone, which is the most estrogen-responsive part of the prostate (14, 15), the evidence cited above, which includes elevation of the estrogen-to-androgen ratio in aging men, the experimental evidence of a role for estrogen in the regulation of prostatic cell growth, the stromal response to estrogens and the stromal nature of BPH, suggests that estrogens may be of importance in the induction of BPH.

The identification of estrogen receptor beta (ERβ) (16) provided further evidence that estrogens may play an important role in regulating the proliferation of prostatic cells. Estrogens have two receptors; estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (16, 17). Although

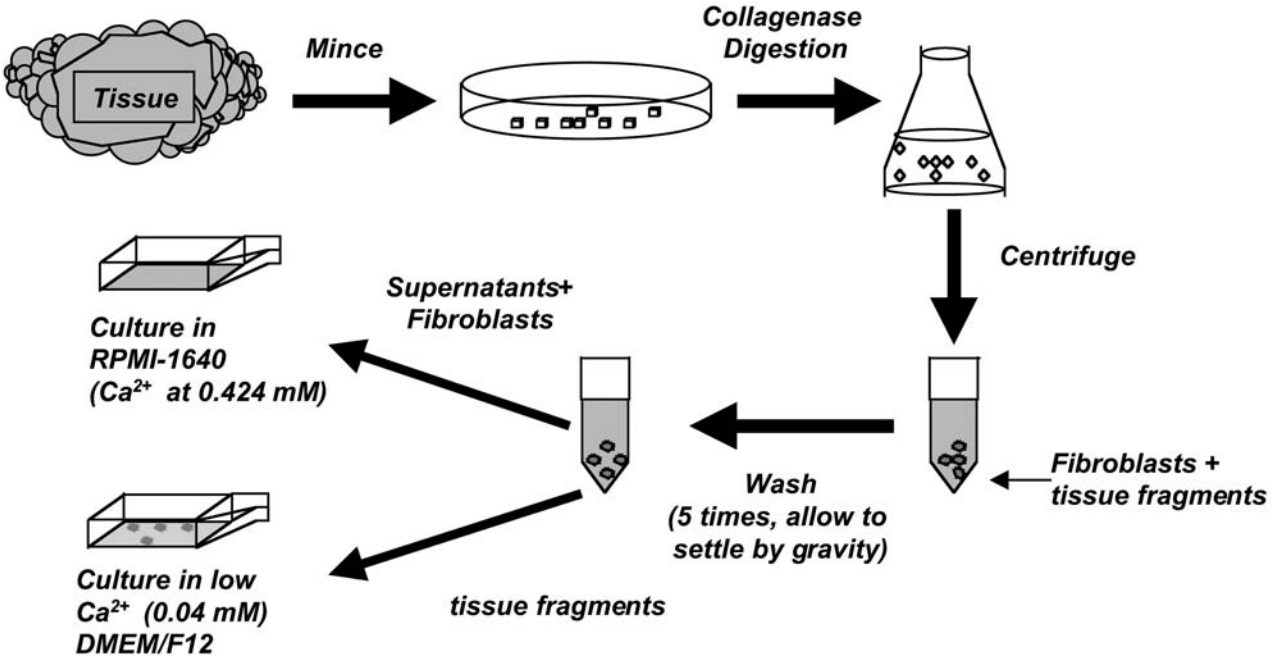

Figure 1. *Isolation of specific cell types from canine prostate tissues.*

the precise biological functions of ERα and ERβ in the prostate are currently undefined, it is known that ERα is predominantly localized in the stromal cells of the prostate (2, 17, 18), while ERβ is localized in the epithelial cells (2). After estrogens bind to ERα and ERβ, ERα stimulates transcription and cellular proliferation, while with ERβ, estrogens inhibit the transcription of ERα and the abnormal growth of the prostate gland (19-22). Both receptors mediate different responses in the target genes and the process of cellular proliferation. This suggests that ERβ may indeed modulate the proliferative effects of ERα-estrogen by blocking pro-proliferative target genes.

In recent years, the occurrence of compounds possessing estrogenic activity in the environment and in food products has received increasing attention because of speculation concerning their ability to adversely affect human and animal endocrinology and their possible etiological role in the diseases of estrogen-sensitive tissues (23, 24). Furthermore, because estrogen-related prostatic hyperplasia is seen to relate to an enhanced estrogenic status in elderly men with lower urinary tract symptoms (25), this age-related factor arouses interest concerning its involvement in the development of BPH. To date, the effect of age on the specific cell types of the prostate is unknown. Recent data from several groups, however, suggest that age and estrogen receptors may be involved in the development of human BPH (12, 13, 20), but there is no such report for the canine prostate. It is also unclear whether ERα and ERβ are present in specific prostatic cell types and whether there are differences in their mRNA expression levels between such specific cell types. Thus, primary cultures of prostatic epithelial and stromal cells derived from dogs of different ages were used to investigate the effects of age on the growth and estrogen receptor mRNA expression of these cells.

Materials and Methods

Isolation of canine prostatic epithelial and stromal cells. The procedure for isolating the canine prostatic epithelial and stromal cells is summarized in Figure 1. Epithelial cells and stromal cells from canine prostatic tissues were separated as previously described, by Canatan's method with modification (26). Prostatic glands from male dogs were soaked in 70% ethanol for 30 sec, then rinsed three times in RPMI-1640 medium (GibcoBRL, Grand Island, NY, USA) containing 5% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA, USA). After the removal of the capsule and urethral segments, the tissues were minced (approximately 2-mm fragments) and then digested in 0.1% collagenase (GibcoBRL) in RPMI-1640 medium supplemented with 5% FCS and an antibiotic-antimycotic mixture (GibcoBRL) for 18 h at 37˚C. After the sedimentation of the remaining undigested tissue fragments, the supernatant containing the epithelial-stromal cell mixture was centrifuged at 200 xg for 5 min at room termerature to collect the cells. After the resuspension of the cell pellet in fresh medium, the cells were permitted to separate by gravity sedimentation for 20 min. The supernatant containing stromal cells and the pellet containing epithelial cells were collected into separate tubes. This process was repeated 5 times with epithelial and stromal cells being pooled into respective fractions.

Cell culture. Canine prostatic epithelial and stromal cells were selected by different concentrations of calcium. Prostatic epithelial

cells were cultured in low-calcium DMEM/F12 medium (Ca^{2+} at 0.04 mM) (GibcoBRL) supplemented with Chelex-100 (BioRad Lab., Richmond, CA, USA) -treated 5% FCS, 10.0 μg/ml insulin (Sigma Chemical Co., St. Louis, MO, USA), 0.02 μg/ml epidermal growth factor (GibcoBRL), 0.5 μg/ml hydrocortisone (Sigma Chemical Co.), 0.1 μg/ml cholera toxin (GibcoBRL) and antibiotic-antimycotic. The canine prostatic epithelial cells were maintained as monolayers at 37˚C, 95% air and 5% CO_2. The canine prostatic stromal cells were cultured in RPMI-1640 (Ca^{2+} at 0.424 mM) with 5% FCS and antibiotic-antimycotic and maintained as monolayers at 37˚C, 95% air and 5% CO_2.

Immunocytochemical staining of primary cultured canine prostatic epithelial and stromal cells. When the canine prostatic epithelial and stromal cells reached about 80% confluence, they were cultured in multichamber slides (Nunc Inc., Naperville, IL, USA). After 24 h, the cells were fixed in –20˚C methanol for 15 min and then were processed for immunocytochemical staining. Briefly, the cells were incubated with prediluted blocking serum [phosphate-buffered saline (PBS) containing 1.5% normal horse serum] for 10 min at room temperature. The cells were incubated with primary antibodies (multi-cytokeratin or vimentin mouse monoclonal antibody) (Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min at 37˚C, then incubated in the presence of a prediluted biotinylated universal secondary antibody (VECTASTAIN Universal Quick Kit, Vector Laboratories, Inc.) for 10 min at room temperature. They were incubated in peroxidase substrate solution [3,3'-diaminobenzidine (DAB) peroxidase substrate kit, Vector Laboratories, Inc.] until the desired stain intensity developed. Each step in the staining procedure was followed by a 5-min wash in PBS.

Thymidine incorporation assay. For the measurement of cell proliferation, the DNA synthesis of canine prostatic cells was determined by thymidine incorporation assay, as previously described (27). Briefly, prostatic epithelial and stromal cells were cultured in 24-well culture plates (Falcon, Lincoln Park, NJ, USA) at $2x10^4$ cells/well, respectively. After 24 h, the cells were then pulsed with 5.0 μCi/ml ^{3}H-thymidine (NEN Corp., Boston, MA, USA) for 3 h. At the end of this period, the cells were washed twice with PBS and fixed with methanol/acetic acid (3:1). Next, the cells were washed with 1 ml of 0.75 M trichloroacetic acid for 30 sec and then lysed with 0.5 ml of 0.2 N NaOH for 1 h. The cell lysates were then neutralized with an equivalent volume of 0.2 N HCl and transferred to scintillation vials. After the addition of 5 ml of scintillation cocktail (Fisher Scientific, Fair Lawn, NJ, USA), the radioactivities were counted on a β-counter. The amounts of (^{3}H) thymidine incorporated into DNA are presented as dpm/well.

RT-PCR analysis. RT-PCR was used to determine the expression levels of ERα and ERβ mRNA in canine prostatic epithelial and stromal cells. The total RNA from these cells was isolated by TRIzol (Invitrogen Co., Carlsbad, CA, USA), according to the manufacturer's instruction. Reverse transcription was performed as previously described (27). Primers for canine ERα and ERβ were designed from the human ERα and ERβ sequence, respectively (28, 29). For the measurements of ERα, ERβ and 36B4 (as internal standard) mRNAs, PCR was performed by mixing 2 μl of RT product with 1.25 μl of $MgCl_2$ (50 mM), 2.5 μl of 10x PCR buffer, 0.2 μl of Taq polymerase (5 U/μl) and 0.3 μl of ERα/ERβ and 36B4 primers in a total 25 μl. For ERα, the 5'-primer was 5'-TAC TGC ATC AGA TCC AAG GG-3' and the 3'-primer was 5'-ATC AAT GGT GCA CTG GTT GG-3'; for ERβ, the 5'-primer was 5'-CAC CTG GGC ACC TTT CTC CTT TAG-3' and the 3'-primer was 5'-CAG CTC TTG CGC CGG TTT TTA TC-3'; for 36B4, the 5'-primer was 5'-AAA CTG CTG CCT CAT ATC CG-3' and the 3'-primer was 5'-TTG ATG ATA GAA TGG GGT ACT GAT G-3'. The PCR for ERα was run for 35 cycles of denaturation at 95˚C for 45 sec, annealing at 60˚C for 45 sec and extension at 72˚C for 1 min. The PCR for ERβ and 36B4 was run for 30 cycles of denaturation at 95˚C for 45 sec, annealing at 54˚C for 45 sec and extension at 72˚C for 1 min. The final RT-PCR products (10 μl) were separated on a 1.2% agarose gel containing ethidium bromide. The specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results are presented as the ratio of ERα/36B4 or ERβ/36B4.

The purification of ERβ PCR products and DNA sequencing. The PCR products were purified using the QIAEX II kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturer's instructions. Briefly, the ERβ PCR product was excised from the agarose gel and the gel slice was weighed. Three volumes of Buffer QX1 were added to 1 volume of gel containing the ERβ DNA fragment. QIAEX II was added, mixed with the Buffer QX1-gel mixture and incubated at 50˚C for 10 min. The mixture was then centrifuged for 30 sec, the supernatant carefully removed, and the pellet washed with 500 μl of Buffer QX1. After this step, the pellet was washed twice with 500 μl of Buffer PE, air-dried for 30 min and then suspended by the addition of 20 μl 10 mM Tris-HCl (pH 8.5). DNA fragments were incubated at room temperature for 5 min and centrifuged for 30 sec. The supernatant containing the purified ERβ DNA was then collected. The purified ERβ DNA at a concentration of 10 ng/μl, was sent to the Plant-Microbe Genomics Facility (PMGF) of The Ohio State University, U.S.A., for DNA sequencing.

Statistical analysis. The data were expressed as the mean±standard deviation (SD) for 4 culture wells. Minitab statistical software for Windows (Minitab Inc., State College, PA, USA) was used for the statistical analysis. Statistical differences between means were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. $P<0.05$ was considered significant.

Results

Morphological characteristics of primary cultured canine prostatic epithelial and stromal cells. The canine prostatic epithelial cells were enriched and grown to nearly 100% confluence and were initially distinguished from the stromal cells by morphological examination. Epithelial cells isolated from the prostatic tissues of 1- and 4-year-old dogs grew in clusters, were polygonal in shape and exhibited prominent round nuclei (Figures 2a and 2b), while stromal cells isolated from the prostatic tissues of 1- and 4-year-old dogs were characterized by a spindle-shaped appearance (Figures 2c and 2d). The morphological identification of the two types of cells was confirmed by immunocytochemical staining for cytokeratin and vimentin, respectively. For the cells morphologically identified as epithelial in nature,

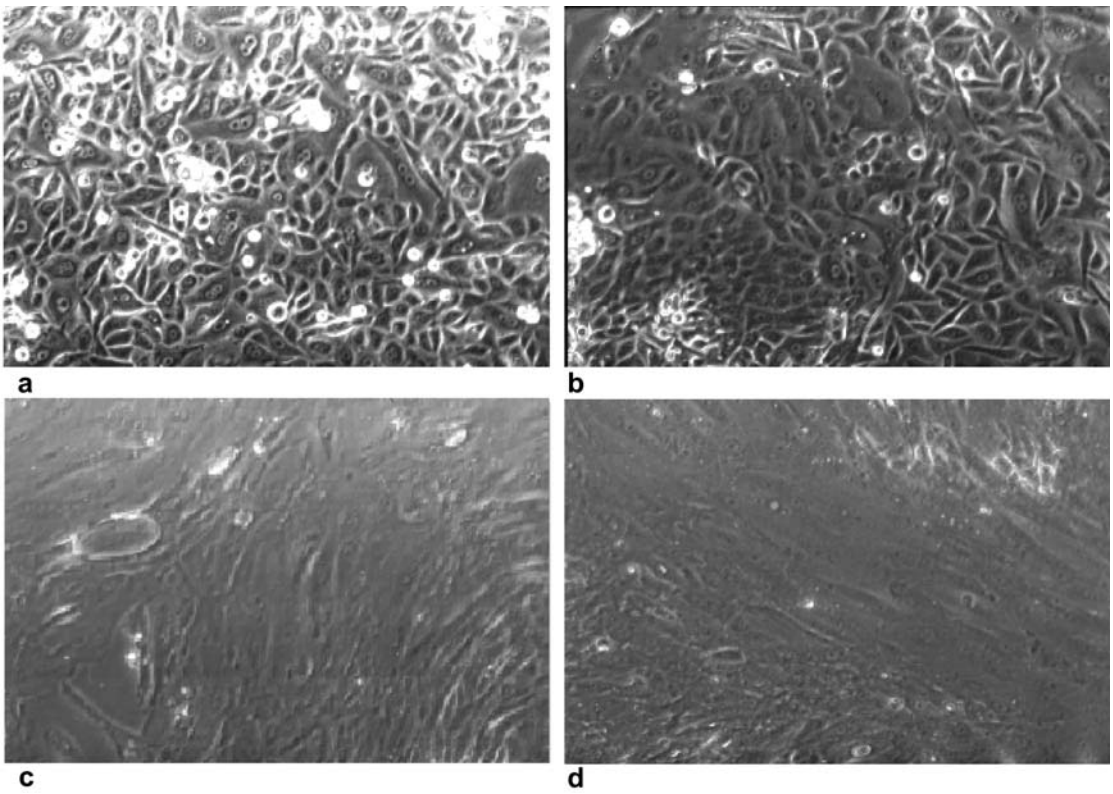

Figure 2. *Phase contrast photomicrograph of cultured prostatic cells from dogs of different ages. Prostatic epithelial cells from prostate specimens of the 1-year dog (a) and 4-year dog (b). Prostatic stromal cells from prostate specimens of the 1-year dog (c) and 4-year dog (d). Epithelial cells grow in clusters and have prominent round nuclei. Stromal cells grow slower in culture than epithelial cells and are characterized by a spindle-shaped appearance (400x magnification).*

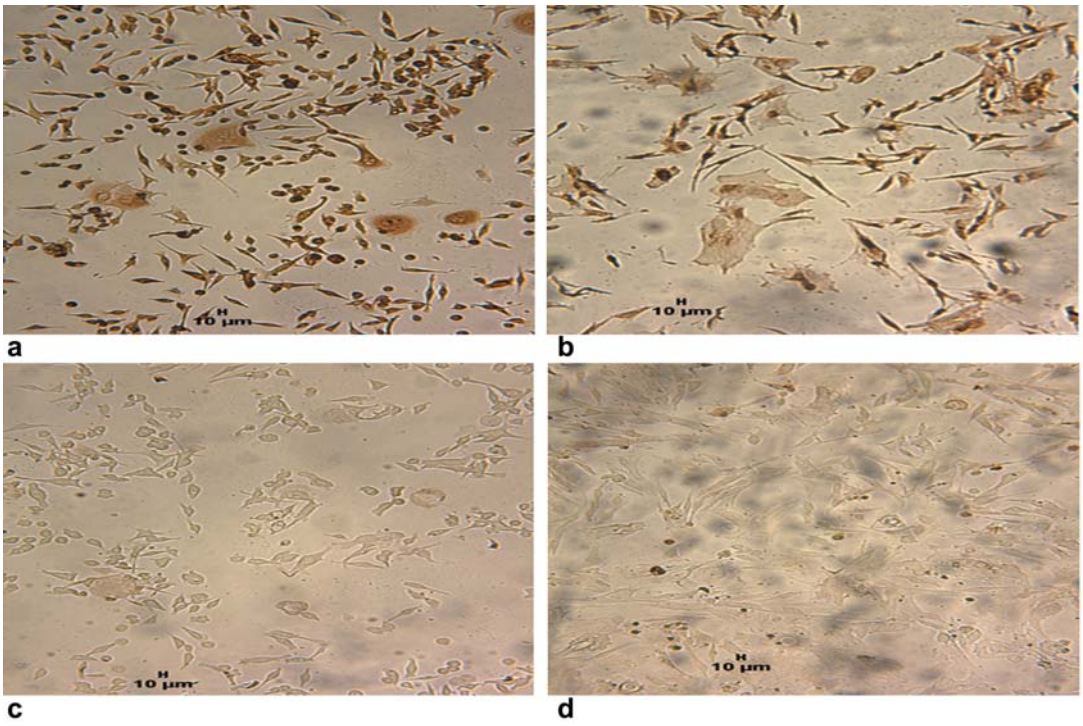

Figure 3. *Immunocytochemistry of cultured prostatic cells from canine prostate tissues. Prostatic epithelial cells were positively stained for cytokeratin (a), but were negative for vimentin (b). Prostatic stromal cells were positively stained for vimentin (c), but were negative for cytokeratin (d) (100x magnification).*

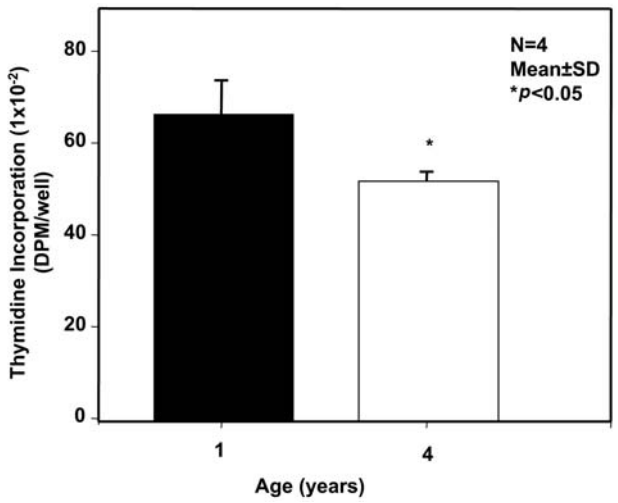

Figure 4. *The proliferation of cultured prostatic epithelial cells from prostate tissues of the 1-year and 4-year old dogs. Each bar represents the Mean±SD of 4 wells. Bar with * represents means that are significantly different from the control group. P<0.05 was considered statistically significant.*

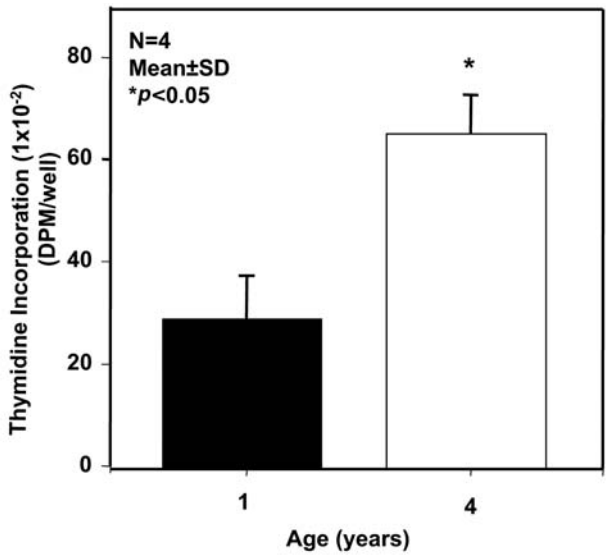

Figure 5. *The proliferation of cultured prostatic stromal cells from prostate tissues of the 1-year and 4-year old dogs. Each bar represents the Mean±SD of 4 wells. Bar with * represents means that are significantly different from the control group. P<0.05 was considered statistically significant.*

immunocytochemical staining revealed that more than 95% of these cells were positively stained for cytokeratin (Figure 3a), but none stained for vimentin (Figure 3b), thus confirming their identity. In contrast, more than 95% of the stromal cells were positively stained for vimentin (Figure 3c), while no staining for cytokeratin was observed (Figure 3d), thereby confirming the stromal nature of this cell population. Together, these immunocytochemical findings validated the isolation and culture methods used for this study. Morphologically, stromal cells could not be distinguished based on the age of the dog from which the cells were isolated. However, the epithelial cells isolated from the 1-year-old canine prostates appeared to exhibit a greater number of binucleate cells than those from the 4-year-old canine prostate (Figures 2a *vs.* 2b). This finding would suggest a greater rate of DNA synthesis in the prostatic epithelial cells isolated from the younger prostate, which was confirmed by a quantitative assay of DNA synthesis.

Comparison of the proliferation of primary cultured canine prostatic epithelial and stromal cells from dogs of different ages. To understand the growth characteristics of primary cultured canine prostatic cells, the proliferation of canine prostatic epithelial and stromal cells was assessed by the thymidine incorporation assay. The thymidine incorporation assay results showed that prostatic epithelial cells from 1-year-old dogs had more DNA synthesis than those from 4-year-old dogs (Figure 4), suggesting that the former cells grow faster than the prostatic epithelial cells from 4-year old canine prostates. In contrast, the prostatic stromal cells from 4-year old dogs exhibited more DNA synthesis than those from 1-year old dogs (Figure 5). The ratio of DNA synthesis (DPM/well) of prostatic epithelial cells to prostatic stromal cells from 1-year-old dogs was 2.28 and from 4-year-old dogs was 0.80. These ratios represent the proliferative rates between prostatic epithelial cells and prostatic stromal cells in this experiment. The observed increase in the proliferation of prostatic stromal cells in older dogs may be related to the etiology of the development of BPH.

Different expression of estrogen receptor beta (ERβ) mRNA in canine prostatic epithelial and prostatic stromal cells from dogs of different ages. To determine whether the mRNA expressions of ERα and ERβ in specific canine prostatic cell types changes with age, they were determined by RT-PCR analysis. RT-PCR detected the expression of ERβ mRNA in canine prostatic epithelial and prostatic stromal cells from 1-year-old dogs (Figure 6), but not in the prostatic stromal cells from 4-year-old dogs, while no expression of ERα mRNA could be detected in our experimental samples. A higher ERβ mRNA expression was observed in prostatic epithelial cells from the 1-year-old dogs than in the epithelial cells from 4-year-old dogs and stromal cells from 1-year-old dogs. Regardless of age, canine prostatic epithelial cells displayed higher ERβ mRNA levels than prostatic stromal cells. Thus, ERβ expression in the canine prostate exhibited cell-specific and age-dependent differences. Moreover, the results showed that the ERβ

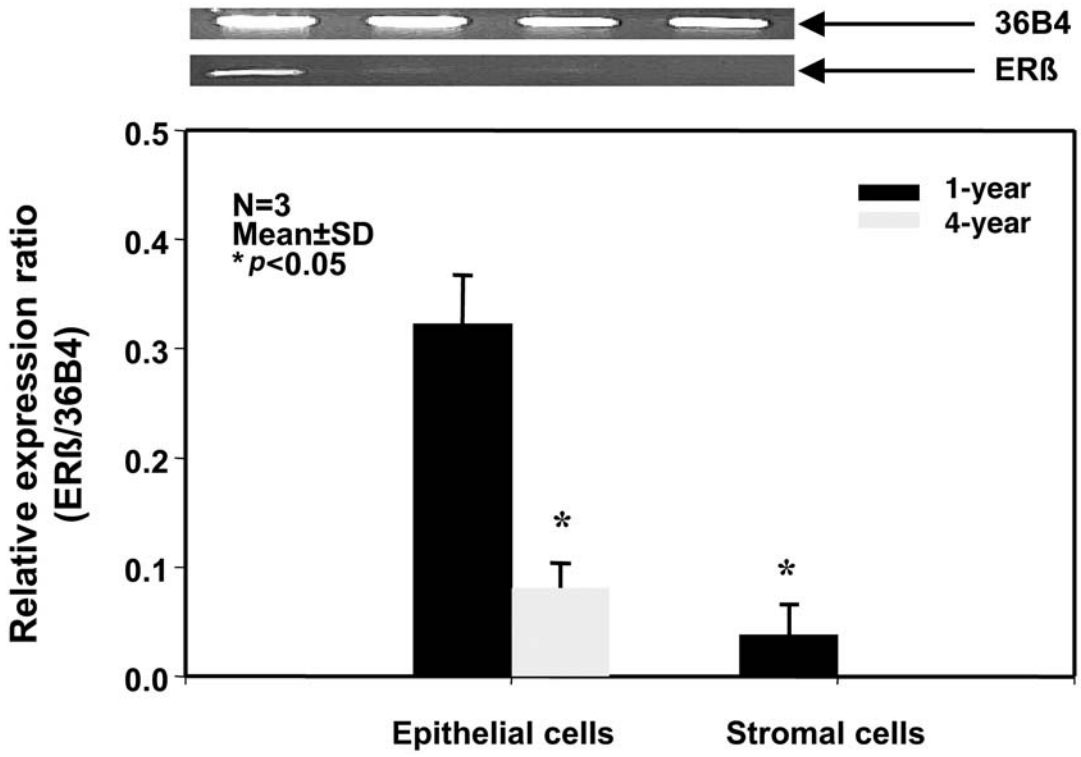

Figure 6. *Expression of estrogen receptor β (ERβ) mRNA in cultured prostatic epithelial and stromal cells from dogs of different ages. ERβ mRNA expression was detected in canine prostatic epithelial and prostatic stromal cells by RT-PCR analysis. Each bar represents the Mean±SD of 3 experiments. Bar with * represents means that are significantly different from the control group. P<0.05 was considered statistically significant.*

mRNA levels in both prostatic epithelial cells and prostatic stromal cells decreased with age.

Partial sequence of canine ERβ. A partial sequence of the canine ERβ cDNA is shown in Figure 7. The canine ERβ cDNA was generated by RT-PCR and was shown to be similar to the human ERβ cDNA. Canine ERβ cDNA is 355 bp long. The canine cDNA nucleotides correspond to nucleotides 663 through 1020 of the human full-length cDNA sequence (29). The canine cDNA sequence shares 90.0%, 87.0% and 83.0% nucleotide homology with the human (29), rat (16) and mouse (30) ERβ cDNAs, respectively.

Discussion

More than 60 years ago, Huggins and Hodges (31) first introduced the concept that estrogen inhibits prostatic growth and observed that the synthetic estrogen diethylstilbestrol (DES) caused a marked decrease in prostate cancer progression. More recently, estrogens were observed to be implicated in the initiation, development and progression of prostatic hyperplasia and carcinogenesis (2, 32-34). High dose T in combination with 17β-estradiol (E_2) stimulates prostatic carcinogenesis in male adult rats (35, 36). Near-physiological doses of either E_2 or DES given to pregnant mice have been reported to imprint on the glands of the adult progeny, resulting in increased prostatic weight, cell number and androgen receptor (AR) content (35). High doses of E_2 given to pregnant rats have also been reported to cause subsequent development of dysplasia in the prostates of the progeny (2, 34). It has become increasingly evident that estrogens may influence both normal and abnormal growth processes in the fetal and adult prostate (2, 34, 35, 37, 38). Furthermore, the aged prostates from normal men and BPH patients have been demonstrated to have an increase in the estrogen/androgen ratio due to a decline in androgen levels (39). These findings indicate that estrogens and estrogen receptors are involved in the development of prostate hyperplasia.

In order to determine the effect of age on the development of human BPH, we measured the proliferation of specific prostatic cell types and the mRNA expression of estrogen receptors in specific prostatic cell types from dogs of different ages, because the growth and development of canine prostate parallels that of human prostate. Our results showed that the ratio of DNA synthesis of the prostatic epithelial cells to prostatic stromal cells from the 1-year-old

	10	20	30	40	50	60
Canine	CAGCT**C**TT**G**C	GCCGGTT**T**TT	**A**TC**T**AT**T**GTA	CACTGATT**T**G	T**AT**CTGG**G**CA	**T**ATG**T**AA**T**CA
Human	CAGCT**C**TT**G**C	GCCGGTT**T**TT	**A**TC**G**AT**T**GTA	CACTGATT**T**G	T**AG**CTGG**G**CA	**A**ATA**T**AA**T**CA
Rat	CAGCT**C**TT**A**C	GCCGGTT**C**TT	**G**TC**T**AT**G**GTA	CACTGATT**C**G	T**GG**CTGG**A**CA	**G**ATA**T**AA**T**CA
Mouse	CAGCT**T**TT**C**C	GCCGGTT**C**TT	**G**TC**T**AT**T**GTA	CACTGATT**C**G	T**GG**CTGG**A**CA	**G**ATA**T**AG**T**CA

	70	80	90	100	110	120
Canine	TTATGTCCTT	GAATGCTTCT	TTTAAAAAAG	GCCTTACATC	CTTCACA**C**GA	CCA**G**AC**T**CC**A**
Human	TTATGTCCTT	GAATGCTTCT	TTTAAAAAAG	GCCTTACATC	CTTCACA**C**GA	CCA**G**AC**T**CC**A**
Rat	TTATGTCCTT	GAATGCTTCT	TTTAAAAAAG	GCCTTACATC	CTTCACA**T**GA	CCA**A**AC**G**CC**A**
Mouse	TTATGTCCTT	GAATGCTTCT	TTTAAAAAAG	GCCTTACATC	CTTCACA**G**GA	CCA**G**AC**A**CC**G**

	130	140	150	160	170	180
Canine	TA**G**TGATA**T**C	C**A**GATGC**G**TA	ATC**G**CTGCAG	AC**C**GC**A**CAGA	AGTG**G**GCATC	CCTCTTT**G**A**A**
Human	TA**G**TGATA**T**C	C**C**GATGC**G**TA	ATC**G**CTGCAG	AC**A**GC**G**CAGA	AGTG**A**GCATC	CCTCTTT**G**A**A**
Rat	TA**A**TGATA**C**C	C**A**GATGC**A**TA	ATC**G**CTGCAG	AC**G**GC**G**CAGA	AGTG**A**GCATC	CCTCTTT**G**C**G**
Mouse	TA**A**TGATA**C**C	C**A**GATGC**A**TA	ATC**A**CTGCAG	AC**G**GC**G**CAGA	AGTG**A**GCATC	CCTCTT**G**G**C**G

	190	200	210	220	230	240
Canine	**C**TTGGAC**T**AG	TAA**T**G**G**GGCT	GGC**A**CAAC**T**G	**T**T-CC**A**C**T**AA	-CTTCCT**T**TT	CAGTGTCTCT
Human	**C**TTGGAC**C**AG	TAA**CA**GGGCT	GGC**G**CAAC**G**G	**T**TCCC**A**C**T**AA	**C**CTTCCT**T**TT	CAGTGTCTCT
Rat	**T**TTGGAC**T**AG	TAA**CA**GGGCT	GGC**A**CAAC**T**G	**C**TCCC**A**C**T**AA	**G**CTTCCT**T**TT	CAGTGTCTCT
Mouse	**C**TTGGAC**T**AG	TAA**CA**GGGCT	GGC**A**CAAC**C**G	**C**TCCC**GC**CAA	**G**CTTCCT**C**TT	CAGTGTCTCT

	250	260	270	280	290	300
Canine	CTGTTTACAG	G**T**AAGG-G**G**G	**T**TCTAG**T**GAT	CTTGCTTCAC	ACCA**A**GGACT	CTTTTGAGGT
Human	CTGTTTACAG	G**T**AAGG**T**G**T**G	**T**TCTAG**C**GAT	CTTGCTTCAC	ACCA**G**GGACT	CTTTTGAGGT
Rat	CTGTTTACAG	G**T**AAGG**T**G**T**G	**C**TCTAG**T**GAT	CTTGCTTCAC	ACCA**A**GGACT	CTTTTGAGGT
Mouse	CTGTTTACAG	G**C**AAGG**T**G**T**G	**T**TCTAG**T**GAT	CTTGCTTCAC	ACCA**A**GGACT	CTTTTGAGGT

	310	320	330	340	350		
Canine	TC**T**GCATA**C**A	G**AA**G**T**GA**C**AA	**C**TGGC**AA**TGG	**ATGG**CTAAAG	GA**N**A**A**AGGTG	**C**CCAGGTG	355
Human	TC**C**GCATA**C**A	G**AT**G**T**GA**TA**A	**C**TGGC**GA**TGG	**ACCA**CTAAAG	GA**GAA**AGGTG	**C**CCAGGTG	358
Rat	TC**T**GCATA**GA**	G**GA**G**C**GA**TG**A	**T**TGGC**AA**TGG	**GTCG**CTAAAG	GA**GAC**AGGTG	**C**CCAG	355
Mouse	TC**T**GCATA**GA**	G**AA**G**C**GA**TG**A	**T**TGGC**AG**TGG	**GTCG**CTAAAG	GA**GAG**AGGTG	**T**CCAG	355

Figure 7. *Partial sequence of canine estrogen receptor β (ERβ). Sequence analysis of the canine ERβ cDNA revealed that it is similar to the human ERβ cDNA. Canine ERβ cDNA is 355 nucleotides long. The sequence shares 90.0%, 87.0% and 83.0% of its homology with the human, rat and mouse cDNAs, respectively. The nucleotide sequence corresponds to the 3' untranslated region of the human ERβ cDNA. Nucleotides in bold are different among species.*

dog is higher, while the ratio of DNA synthesis of the prostatic epithelial cells to prostatic stromal cells from the 4-year dog is lower. A high ratio (>1) means that the proliferative rate of prostatic epithelial cells is higher than that of prostatic stromal cells, while a low ratio (<1) indicates the opposite. Our result suggests that changes in the prostatic epithelial cells/stromal cells ratio in males occur with age. When dogs are young, prostatic epithelial cells proliferate more than stromal cells (ratio is high), but the opposite is true for older dogs, where the ratio is low. The decrease of this age-related ratio implied that more proliferation in the prostatic stromal cells with age might be related to etiology of the development of BPH. Our result is consistent with an other report in which prostatic epithelial cells underwent programmed cell death (apoptosis), but no apoptotic cells could be detected in the prostatic stromal cells (40). Our results indicate that the deregulation of this balance results in pathology such as hyperplasia or neoplasias in prostates.

Estrogen, another major class of circulating steroids, was shown to act synergistically with androgens to induce BPH in dogs by enhancing sensitivity to androgens through increasing AR levels (41). Moreover, estrogenic activity is mediated by the interaction between estrogen receptors and the hormone with subsequent activation of the receptors (2, 15, 16). Thus, it can be inferred that this age-dependent decrease in prostatic ERβ results in increased ERα-mediated or AR-mediated stimulatory effects on prostatic stromal cell function. It can be speculated that such a process may be involved in the induction of canine BPH.

Although estrogens have long been considered to play a minor role in male reproductive organs, the identification of

ERβ implied that estrogens might also play an important role in the growth of the prostate. Several reports have shown that ERα and ERβ play different roles in prostatic cell proliferation. ERα stimulates cell proliferation, while ERβ restrains the stimulatory effects of ERα in the rodent uterus (21) and inhibits epithelial cell proliferation in the rodent prostate (22). ERβ activation may decrease the AR content in prostates of wild-type mice but a higher AR level and age-related prostatic hyperplasia appears in ERβ knockout mice (22). These findings indicate that ERα and ERβ may have different roles in the pathogenesis of BPH. Thus, we examined the ERα and ERβ mRNA expressions in prostatic specific cells so that we could determine if the increased proliferation of canine prostatic stromal cells with age was related to the change of ERα and ERβ mRNA expressions. Our results showed that ERβ mRNA expression was found in both prostatic epithelial and prostatic stromal cells, except for stromal cells from the prostates of 4-year-old dogs. The findings of the present study clearly indicated that there is a progressive reduction in ERβ mRNA levels in both prostatic epithelial and prostatic stromal cells with age. Although most publications have reported the expression of ERβ solely in prostatic epithelial cells (42-44), our data showed that there is an expression of ERβ in prostatic stromal cells from young dogs, which is rapidly lost with age. These results implied that the greater proliferation of canine prostatic stromal cells with age is associated with the reduction in age-dependent ERβ mRNA. No ERα mRNA expression was detected in any prostatic primary cultured cells. Our data are in agreement with the results of Lau *et al.* who did not detect ERα in human prostate primary cultures (4). Linja *et al.* also found that the level of the expression of ERα mRNA in BPH tumor samples was very low and was similar to that of ERα-negative breast cancer samples (44). These results indicate that estrogens are involved in the growth and differentiation of prostatic cells *via* ERβ, not ERα. Recently, various studies have shown that the change of ERβ mRNA and protein is associated with the development of BPH and prostate cancer (45-48). For example, Horvath *et al.*, using IHC, found that the ERβ protein was progressively lost in prostate hyperplasia and neoplastic lesions (45). Pasquali *et al.* also obtained similar results, showing that malignancy of prostate is associated with the loss of ERβ expression (46). However, Royulela *et al.* claimed that the increased expressions of ERα and ERβ might be involved in hyperplasia and prostate cancer (47). Our data supported the findings of Horvath *et al.* and Pasquali *et al.*, in which the loss of ERβ expression is related to prostate tumor progression. Our results suggest that the reduction in age-dependent ERβ mRNA expression might play an important role in the etiology of the development of canine BPH, by leading to an increase of the AR content of prostate glands associated with the enhanced proliferation of canine prostatic epithelial and prostatic stromal cells. Our above conclusion is consistent with other reports (4, 18, 19, 22, 45, 49, 50, 51, 52). A publication, showing that E_2 directly mediated AR transcriptional activity *via* a new E_2-AR-ARA_{70} pathway in human prostate cancer cells (53), further confirmed our hypothesis. Thus, it is possible that anti-estrogens will play an important role in the treatment of benign prostatic hypertrophy. The development of a new estrogen antagonist with specific affinity for ERβ, may be our strategy against human BPH in the future.

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