Effect of Castration on Extracellular Matrix Remodeling and Angiogenesis of the Prostate Gland

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Abstract. This study was conducted to evaluate the long term effect of castration on the prostate gland proliferation, extracellular matrix remodeling and angiogenesis. Prostate gland proliferation was assessed by immunolocalization of proliferating cell nuclear antigen (PCNA). The expression level of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and metalloproteinase-13 (MMP-13) by the prostate gland were assessed by immunohistochemistry and quantitative real-time PCR. The expression of the above mentioned parameters by the prostate gland of mature intact dogs were compared to that of castrated dogs six months post-castration. The results showed that castration induced a remarkable atrophy of the prostate gland which was associated with a highly significant decrease in the PCNA proliferation index. Although TGF-β protein was immunolocalized to the epithelial and stroma cells of the prostate gland from both intact and castrated dogs, castration induced a significant up-regulation of TGF-β mRNA expression. VEGF mRNA expression and its encoded protein immunolocalization were decreased significantly by the prostate gland from castrated dogs as compared to that of intact dogs. Castration, on the other hand, resulted in no significant change in MMP-13 mRNA expression despite an effect on its cellular immunolocalization which appeared to be localized to the epithelial and stromal cells of the prostate gland from castrated dogs as compared to epithelial cells of the prostate gland from intact dogs. These results indicated that castration-induced prostate gland regression continued to exert a potent suppressive effect on prostate gland proliferation which might be mediated by the elevated level of TGF-β. Moreover, the low expression level of VEGF might reflect a reduced blood flow demand by the regressed and growth-dormant prostate after castration.

Key words: Prostate, Castration, Proliferation, VEGF, MMP-13, TGF-β

THE prostate gland development from fetal to pubertal and later maintenance of its structural and functional integrity during adulthood is dependent on adequate supply of androgen. Therefore, castration at any time during the male lifespan leads to a rapid involution of the gland which appeared to be due largely to a massive death of secretory epithelial cells. However, all other cellular components of the prostate gland also suffer different changes in response to androgen ablation which ranged from the cellular death to alteration in their proliferation and differentiation capacities [1, 2]. These cellular components include basal cells, smooth muscles, and endothelial cells. In deed, it has been shown that androgen ablation effect on the prostate gland vascular system preceded its effect on glandular secretory epithelial cells and, therefore, it has been suggested that it is the triggering step that leads to prostate gland involution [3]. The response of the vascular system was characterized by a rapid reduction of the blood flow to the prostate gland and was associated with apoptosis of the vascular endothelial cells [3, 4]. The effect of androgen on the endothelial cells appeared to be indirect and might be mediated by vascular endothelial growth factor (VEGF) [5]. Evidence
supporting this mediator role, indicated that androgen stimulates [6, 7] and castration inhibits VEGF expression [7–9].

The effect of castration on prostate gland growth has been shown to be associated with modulation of transforming growth factor β (TGF-β) expression. TGF-β is a growth factor which exerted a wide range effects on different prostatic cell proliferation, differentiation and death [10]. Its elevated level of expression by the prostate gland after castration suggested that it acts as a mediator of castration-induced epithelial [11] and stromal cell death [12]. In addition, in vitro studies indicated that TGF-β acts to inhibit prostate stromal cells proliferation and induced their myodifferentiation [13–15]. Similarly, TGF-β seems also to mediate castration-induced prostate cancer cell death [16]. However, despite the association of TGF-β expression with prostate cancer cell death, alteration of TGF-β expression has been implicated in reactive stroma and extracellular matrix remodeling (ECM), which are associated with prostate cancer [17, 18]. In support for the later, it has been shown that TGF-β acts to stimulate the expression level of MMP-13 which belongs to a family of structurally related endopeptidases that are involved in degradation of various components of ECM macromolecules [19]. MMP-13, also known as collagenase-3, is an active member of this family which acts to degrade fibrillar collagen II of the ECM [20]. It has been detected to be expressed by different prostate cancer cell lines, prostate cancer tissue and benign prostate hyperplasia [21–23]. Its expression pattern by prostate cells appeared to be varied according to the malignancy of prostatic cells and, therefore, it has been suggested to be a diagnostic marker for prostate cancer [24].

Although the early events of castration induced regression of the prostate gland is well characterized, the later effects as the time progress after castration is not well defined. Evidence for this derived from the time-dependent response of the prostate cancer to castration. While castration induced an initial regression of the prostate cancer, the regressed gland eventually escapes the castration remedy and resumes its growth. Furthermore, castration of dogs has been shown to increase the risk of prostate cancer progression later on life [25]. Recent evidence from our laboratory indicated that castration of dogs for one month resulted in a remarkable increase in vimentin expression [1] which has been shown to be associated with prostate cancer progression [18]. As these data underlined the importance of analyzing a long term effect of castration on the prostate gland, this study was conducted to examine the expression level of factors that are involved in cell proliferation, ECM remodeling and angiogenesis after six months of castration. The parameters which have been chosen to be investigated include PCNA labeling index, TGF-β, VEGF and MMP13.

Materials and Methods

Eight adult dogs aged between 2 to 4 years were divided into two groups. While the dogs of the first group were left intact, the dogs of the second group were castrated according to the method of Carne [26] under general anesthesia. After six months of castration, dogs of both groups were euthanized by intravenous injection of over dose of 10% thiopentone sodium. The protocol of animal handling, castration, and euthanasia were approved by the Jordan University of Science and Technology Animal Care and Use Committee (JUST-ACUC).

Preparation of tissues

Prostate glands of intact and castrated dogs were divided into two symmetrical parts. The first part was stored in liquid nitrogen for mRNA isolation and further PCR analysis. The second part was washed with normal saline, fixed in 4% buffered formaldehyde for 4 hours, and then routinely processed and embedded in paraffin.

Immunohistochemistry

The immunohistochemistry was performed as previously described [2]. Five µM sections were picked on vecta bond (Vector laboratories, Buringame, CA, USA) coated slides and were then deparaffinized in xylene and rehydrated in descending series of alcohol concentrations. In order to expose the masked antigenic sites, sections were subjected to autoclave at 121°C in citrate buffer (10 mM sodium citrate pH 6.0) for 10 minutes and allowed to cool at room temperature for 20 minutes. To quench endogenous peroxidase activity, sections were incubated in 1% methanolic H₂O₂ for 20 minutes. Non-specific binding sites were blocked with DAKO protein block for 30 minutes (DAKO® Protein Block Serum-Free) (DAKO Corp., Carpentaria,
Sections were then incubated with specific monoclonal primary antibodies of PCNA (DAKO Corp., CA), TGF-β (Santa Cruz Biotechnology, Inc), VEGF (Santa Cruz Biotechnology, Inc) and MMP-13 (Serotec, Oxford, UK) at a dilution of 1:30, 1:75, 1:100 and 1:150 respectively. The primary antibodies were applied for 1 hour at room temperature in a humidified chamber. At the end of each primary antibody incubation, the sections were washed three times with phosphate buffer saline (PBS) and sections were then incubated with universal biotinylated immunoglobulin (L.V.DAKO LSAB® + Kit, HPR) (DAKO Corp., CA) for 30 minutes at room temperature in a humidified chamber. The sections were then washed three times with PBS and incubated with streptavidin (L.V.DAKO LSAB® + Kit, HPR) (DAKO Corp., CA) for 30 minutes at room temperature in a humidified chamber. The immunoreactions were developed by 1 mg/ml solution of diaminobenzidine tetrahydrochloride (DAB) (DAKO Corp., CA). The reaction was terminated in distilled water, and the sections were counterstained with Mayers haematoxylin, dehydrated, mounted and examined under light microscope. The negative controls in which the primary antibodies were omitted and substituted with PBS were processed parallel to the examined sections in each assay.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from a frozen prostate using an SV Total RNA isolation kit and performed according to the manufacture protocols (Promega Corporation, USA). The RNA concentration was determined by measuring the absorbance at 260 nm. Samples were then stored at −20°C for subsequent RT-PCR analysis. 0.2 μg of total RNA was used to synthesize a complementary DNA (cDNA) using the reverse transcription kit (Promega Corporation, USA). The RT reaction was carried out at 25°C for 5 min followed by 42°C for 60 min and then at 95°C for 5 min. The samples were then placed on ice for 5 minutes and stored at −20°C for PCR amplification. PCR was performed using a commercial PCR kit containing sybergreen florescent dye (SyberGreen Supermix; Biorad Laboratories, USA) in the presence of 2 μM of specific primers. Real time PCR (Rotor-gene 3000, Corbett research, Australia) was used to quantitatively analyze the mRNA expression level of TGF-β, VEGF and MMP13. The primers were designed to be specific for canine sequence using the web-based QuantiProb design software (www.qiagen.com). The forward primer for TGF-β1 was ATG GCT GTC CTT TGA TGT and reverse primer was GTG TGT TAT CTT TGC TGT. The forward primer for VEGF was TCC AAT CTC TCT CTC CCT and reverse primer was TCT GTA TAT TGA AAC C. The forward primer for MMP-13 was GGG GAC TTC TAT CCA TTT and the reverse primer was TGG GTA AAC TCG. The amplification reactions were started with an initial denaturation at 95°C for 2 minutes, flowed by 45 cycles each composed of denaturation at 95°C for 30 second, annealing at 55°C for 30 second and extension at 72°C for 30 second. At the end of the 45 cycles the melting curve for the reactions was performed at temperature range from 72°C to 95°C. Relative mRNA expression for TGF-β, VEGF, and MMP-13 was determined using the 2−ΔΔC_T method and normalized to GAPDH expression [27].

PCNA labeling index

PCNA labeling index was calculated as described previously [28]. The percentage of PCNA positive cells were counted among 500 cells in randomly chosen fields at magnification of ×400 from each dog in all groups.

Statistical analysis

Statistical analysis of PCNA labeling index and mRNA expression was performed using one-way analysis of variance (ANOVA) on Minitab software. Results were presented as the mean ± SEM. A probability of less than 0.05 (P<0.05) was considered statistically significant.

Results

As expected castration for six months induced a pronounced regression of the prostate gland which was characterized by a remarkable shrinkage of its size. Histological evaluation revealed a presence of small cells with a scant cytoplasm located within atrophied...
acini and ducts. The collapsed glandular structures are devoid completely of tall columnar epithelial cells. While proliferating cells of the prostate gland of intact and castrated dogs were confined to the glandular epithelial cells, very few proliferating cells were detected by the stoma cells. The gland of intact dogs expressed PCNA which was localized to the nucleus of the glandular epithelium (Fig. 1A) and this immunolocalization was decreased dramatically after castration (Fig. 1B). The PCNA proliferation index was calculated as a percentage of positively stained epithelial cells within 500 cells of the glandular compartment. The results revealed a highly significant reduction in the prostate gland proliferation index of castrated dogs as compared to that of intact dogs (Fig. 1C).

The TGF-β immunolocalization and its mRNA expression were compared in the prostate gland from intact and castrated dogs. The results showed that TGF-β was mainly localized to the stromal cells of the prostate glands of intact dogs with very faint immuostaining localized to the epithelial cells (Fig. 2A). Castration induced a slight shift in TGF-β immunolocalization from the stromal cells to the remaining glandular cells of the atrophied acini (Fig. 2B). These changes in the immunolocalization of TGF-β were associated with a two fold increase in its mRNA expression (Fig. 2C).

Vascular endothelial growth factor is an angiogenic factor and therefore, it was used as a marker to assess angiogenesis of the prostate gland of intact and castrated dogs. The results showed that VEGF was expressed predominantly by the epithelial cells of the prostate gland of intact dogs (Fig. 3A). After 6 months of castration there were no detectable immuostaining of VEGF by the prostate gland of castrated dogs (Fig. 3B). The reduction in VEGF protein immuostaining by the prostatic cells of castrated dogs was supported by its mRNA expression. As shown in Fig. 3C there is a highly significant reduction of mRNA encoding for
VEGF by the prostate gland from castrated dogs as compared to that of intact dogs.

The effect of castration on extracellular matrix remodeling was assessed by the immunolocalization of MMP-13 which is known to be involved in the turnover of connective tissue matrix components. As shown in Fig. 4A, the results showed that MMP-13 was immunolocalized to the cytoplasm of the glandular cells of the prostate gland of intact dogs. However, the immunostaining was more intense in the epithelial cells which were localized immediately close to the basement membrane. Although, MMP-13 was continued to be immunolocalized to the epithelial cells of the prostate gland after 6 months of castration, the stromal cells were induced to express MMP-13 (Fig. 4B). Quantitative MMP-13 mRNA analysis revealed that there is a slight (1.5 fold) but not significant increase in its expression (Fig. 4C).

Discussion

The results of this study illuminated some aspects of long term effect of castration on the expression levels of factors that are involved in prostate gland proliferation, blood flow and extracellular matrix turnover. Previously we have demonstrated that castration resulted in no changes in the proliferation pattern of basal cells and induced stromal cell proliferation [2]. However, the results of this study extended our previous investigation by showing that as the time progressed after castration there is a significant cessation of basal and stromal cell proliferation. These changes in prostatic cell proliferation were associated with an increase in TGF-β mRNA expression. Previous reports have shown that castration-induced normal and prostate cancer regression was paralleled by an increase in TGF-β expression and therefore, TGF-β was implicat-
ed in prostatic cell apoptosis [11, 12, 16]. However, the results of this study indicated that the TGF-β mRNA expression remained elevated after six months of castration. The presence of high level of TGF-β for this extended period of time might be involved in suppressing the proliferation of the remaining cells after castration. In fact TGF-β is a known negative regulator of prostatic cell growth where it acts to inhibit epithelial and stromal cell proliferation [12, 29]. Taking together, it seems likely that TGF-β might act to suppress prostatic cell proliferation and to maintain a low cellular turnover of the atrophied gland after castration.

MMP-13 is a protease enzyme that acts to degrade collagen II of the extracellular matrix and it is implicated in the progression of different tumors [30]. However, the expression of MMP-13 by prostate cancer, benign prostate hyperplasia and prostate cancer cell lines remained uncertain and controversial. While some studies showed that MMP-13 cellular expression and its plasma concentration levels from normal and patient with prostate cancer and benign prostate hyperplasia was associated with the stage of prostate gland malignancy [24], other studies failed to establish such kind of correlation [22, 23]. Recent report indicated that androgen acts to stimulate the expression level of MMP-13 by LNCaP prostate cancer cell line [23]. The results of this study extended the existing knowledge by showing that normal canine prostate gland expressed MMP-13 and its encoded protein. Although these results revealed no significant effect of androgen ablation on MMP-13 mRNA expression, the immunolocalization of its protein to the glandular epithelial cells of the prostate gland from intact dogs and its localization to epithelial and stromal cells of the pros-

![Image](image_url)

**Fig. 3.** Effect of castration on the immunolocalization of VEGF and its mRNA expression. VEGF was localized to the epithelial cells of the prostate gland of intact dogs (A) and it was disappeared after six months of castration (B). Relative mRNA expression of VEGF was decreased significantly (P<0.05) by the prostate gland from castrated dogs as compared to that of intact dogs (C).
tate gland from castrated dogs, seem to suggest a role of androgen in the regulation of MMP-13. As the functional role of MMP-13 is directed towered the preferential degradation of ECM constituent [20], the spatial immunolocalization of MMP-13 by the epithelial cells which are in close proximity to the basement membrane of the prostate gland of intact dogs and its localization to the stromal and remaining epithelial cells after castration appeared to suggest a biological role of MMP-13 that is remained to be determined.

Androgen ability to maintain normal structural integrity of the prostate gland has been attributed to its ability to maintain adequate blood supply to the gland where vascular endothelial growth factor has been recognized as androgen-mediator that plays a central role in the regulation of blood flow [5]. Castration-induced normal, benign and prostate cancer has been shown to be associated with a suppression of VEGF expression [7, 8]. Although androgen ablation has been shown to induce apoptosis of both vascular endothelial cells and prostatic epithelial cells, it appeared that its apoptotic effect on vascular endothelial cells preceded that on prostatic epithelial cells [3]. Hence, the early events of prostate gland regression after castration can be attributed to a rapid reduction in blood flow and induction of vascular endothelial cell apoptosis [3]. The results of this study indicated that castration for long time exerted an extended suppressive effect on VEGF. Therefore, the low level expression of VEGF might reflect a low blood flow requirements by the regressed prostate gland. This conclusion appeared to be consistent with the observations that a progressive growth of the prostate gland required a paralleled increase in VEGF expression. These observations showed that androgen supplementation which drives prostate gland re-growth was associated with an increase in VEGF ex-

Fig. 4. Effect of castration on the immunolocalization of MMP-13 and its mRNA expression. While MMP-13 was localized to the glandular epithelial cells of the prostate gland of intact dogs (A), it was localized to epithelial and stroma cells of the prostate gland of castrated dogs (B). There was no significant difference in the relative mRNA expression of MMP-13 by the prostate gland from intact and castrated dogs (C).
pression [31]. Similarly, re-growth of regressed tumor after castration is associated with an increase in VEGF [9] and if androgen ablation failed to induce tumor apoptosis there is no reduction in VEGF expression [8].

Castration has been suggested as a risk factor that might contribute to increase the incidence of prostate cancer in dogs. The results of this study indicated that there is an increase in TGF-β expression which has been shown to be associated with prostate cancer and implicated in prostate cancer angiogenesis [10, 17]. However, TGF-β has been shown to behave differently in normal and tumor tissue. Therefore, the association between the elevated level of TGF-β and the reduced cell proliferation rate after six months of castration seems to support its role as a negative regulator of prostatic cell proliferation. TGF-β, in addition, has been shown to stimulate MMP-13 expression [19] which was implicated in cancer progression [30]. The results of this study indicated that castration exerts no significant effect on MMP-13 expression despite its stimulatory effect on TGF-β expression. Since androgen supplementation has been shown to stimulate MMP-13 expression [23], the expected effect of TGF-β on MMP-13 might be counter balanced by androgen ablation. Moreover, the very low level of VEGF expression after six months of castration also reflected a low blood flow demand by the growth-dormant prostate. Although this conclusion argued against a role of castration in prostate cancer, it should not be overlooked that castration might require a longer time to stimulate the production of factors that are associated with prostate cancer.

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References


