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Smooth Muscle Cell Behavior in the Ventral Prostate of Castrated Rats

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ABSTRACT: Smooth muscle cells (SMC) play roles in prostatic development and function. The cells also respond to tissue injury and hormonal variations, alternating between a fully differentiated and contractile phenotype and a dedifferentiated synthetic or secretory phenotype. However, the phenotypic changes in SMC after androgen deprivation have not yet been described. The ventral prostate of control and castrated rats was processed for routine histology, immunocytochemistry, reverse transcriptase polymerase chain reaction (RT-PCR), and scanning electron microscopy (SEM). The maintenance of SMC phenotype was confirmed by immunocytochemistry and by RT-PCR. Stereological analyses were done to define the relative and absolute volume of the SMC. SMC were elongated and flattened against the epithelium. After castration, the cells shortened concomitantly with pleating of the cell surface, leading to a spinous aspect. SEM showed that the smooth surface of SMC became progressively folded. Immunocytochemistry demonstrated both smooth muscle myosin heavy chain and smooth muscle α-actin in the prostatic SMC 21 days after castration, whereas RT-PCR amplified the message for smoothelin. Stereological analysis showed an increase in the relative volume of SMC in relation to the whole gland and the stroma. A decrease in the absolute volume of SMC occurred only within the first 7 days after castration and remained unchanged thereafter. The prostatic SMC are affected by the absence of androgens and there is a critical transition point during the first week in which the total volume occupied by SMC diminished. The remaining SMC showed a marked phenotypical change. These findings indicate that ventral prostate SMC maintain their differentiated phenotype after castration. The alterations in SMC behavior correlate with general stromal modifications taking place after castration.

Key words: Androgen, castration, SEM, stereological analysis, smoothelin, smooth muscle myosin.

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The prostate gland consists of a secretory epithelium and a stroma, the organization and functional status of which are under androgen control. The stroma is dynamic and directly influences the behavior of the epithelium and is also activated in response to injuries (Cunha et al, 1996; Thomson, 2001).

The main cell types found in the stroma are smooth muscle cells (SMC) and fibroblasts (Shapiro et al, 1992). These cells synthesize regulatory and structural components of the extracellular matrix to create a microenvironment that regulates the growth and functional differentiation of other cell types. These activities are regulated by androgens, which maintain the organ homeostasis (Farnsworth, 1999; Tuxhorn et al, 2001; Thomson et al, 2002). Prostatic SMC express androgen receptors (Prins et al, 1991), but the effects of androgens on the phenotype and behavior of these cells are poorly known, with often contradictory findings.

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Androgen deprivation by surgical or chemical castration leads to a marked involution of the prostate. After surgical removal of the testis, there is a residual 5% of circulating testosterone that is produced peripherally by the adrenal gland. This residual testosterone is insufficient to maintain organ homeostasis and marked alterations occur in the prostatic epithelial and stromal compartments.

The epithelial alterations correspond mostly to a decrease in secretory activity and the loss of epithelial cells by apoptosis. The stroma shows an increase in cell and extracellular matrix density. The extracellular matrix is extensively remodeled, with changes in the basement membrane (Carvalho and Line, 1996) and in collagen fibers (Vilamaior et al, 2000). Although the modifications in the epithelial basement membrane are attributed to the remaining epithelial cells (Carvalho and Line, 1996), those involving collagen fibers were associated with SMC (Vilamaior et al, 2000).

The SMC phenotype changes in response to physiological and pathological conditions regardless of the tissue of origin of the cells, including the prostate (Zhao et al, 1992; Worth et al, 2001). This process corresponds to a shift from a differentiated contractile phenotype to a secretory dedifferentiated phenotype. The dedifferentiation of SMC is characterized by the downregulation of smooth

muscle-specific markers (Aikawa et al, 1993; Owens, 1995; Boesch et al, 1999; Doevendans and van Eys, 2002) such as smoothelin, which is an actin-associated protein (Rensen et al, 2002).

In this work we examined SMC behavior in the prostate of castrated rats using light and scanning electron microscopy (SEM), stereology, immunocytochemistry, and reverse transcriptase polymerase chain reaction (RT-PCR). Marked morphological modifications were observed, with the cells adopting a spinous aspect. There was a reduction in absolute cell volume but the expression of some SMC markers was maintained. These results indicate that SMC retained their differentiated state after castration.

Materials and Methods

Animals and Histological Processing

Three-month-old Wistar rats were used. Orchiectomy was done via a scrotal incision under anesthesia with chloral hydrate (300 mg/kg intraperitoneally). Ventral prostates were removed 7, 14, and 21 days after surgery. Age-matched rats were used as controls. At least 3 rats were used for each experimental point.

The ventral prostate was removed and immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline for 24 hours. The tissues were then washed, dehydrated, cleared in xylene, and embedded in historesin for stereological analysis or in Paraplast Plus for immunocytochemistry.

Stereological Analysis

Sections 2 μm thick were cut and stained with hematoxylin and eosin (H&E). Six microscopical fields were photographed for each group and the stereological analysis was done using Weibel's system, including the 168-point grid (Huttnen et al, 1981) and the alleatory sampling instructions. The relative volume of the stroma and SMC was determined by calculating the total volume of each of these compartments, on the basis of the mean prostatic weight. To verify the modification within the stroma, the ratio between the relative volume of the SMC and the relative volume of the stroma was calculated. The results were compared using the Student's two-sample *t*-test.

SEM

SEM was used to examine the surface morphology of SMC after sample preparation by the KOH-collagenase digestion method described by Murakumo et al (1993), with slight modifications. The ventral prostate of noncastrated rats and rats castrated 21 days before were removed and fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 week at room temperature. The prostates were placed in 30% KOH for about 8 minutes at 62°C and washed in 0.1 M phosphate buffer solution overnight. They were then immersed in a solution of collagenase type D 1 mg/mL (Sigma Chemical Company, St Louis, Mo) in 0.1 M phosphate buffer, pH 7.4, for 6 hours at 37°C, and subsequently washed in the same buffer for 1 h. The tissues were postfixed in 2% tannic acid solution for 2 hours, rinsed in distilled water for more than 1 hour, and treated with 1% osmium tetroxide for 2 hours at room temperature. After dehydratation in a graded ethanol series, the tissues were critical point-dried in liquid CO_2. Some tissue pieces were freeze-fractured in liquid nitrogen before drying. The specimens were mounted on aluminum stubs with double-sided adhesive tape, sputtered with gold, and examined in a Jeol 5800 scanning electron microscope.

Immunocytochemistry

Sections 5 μm were collected on silanized glass slides, dewaxed with xylene, and rehydrated in a descending ethanol series. Endogenous peroxidase activity was blocked by treating the tissue sections with 3% hydrogen peroxide in water for 30 min. Nonspecific protein–protein interactions were blocked by incubating sections with 3% bovine serum albumin (BSA) (Sigma) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h. For the smooth muscle myosin heavy chain antibody reaction, sections were pretreated with 0.4% pepsin in 0.01 N HCl at 37°C for 30 minutes before blocking the endogenous peroxidase activity.

Monoclonal antibodies against smooth muscle α-actin (diluted 1:100; Sigma) and smooth muscle myosin heavy chain (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, Calif) were used. The primary antibodies were diluted in 1% BSA in TBS-T, and applied to the sections for 1 hour at room temperature. After three 5-minute washes with TBS-T, the sections were incubated with a secondary peroxidase-conjugated antibody against mouse polyvalent immunoglobulins (Sigma) diluted 1:100 in 1% BSA in TBS-T for 1 hour. Sections were washed again and the peroxidase activity detected with 3,3′-diaminobenzidine. Negative controls were obtained by omitting the incubation with the primary antibody. The slides were counterstained with methyl green, air dried, and mounted in Entellan (Merck, Darmstadt, Germany). Observations were made using a Zeiss Axioskop microscope and photographs were taken using Kodak 100 Proimage film.

RT-PCR

Total RNA was isolated by the guanidinium thiocyanate extraction method, using TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, Calif) according to the instructions provided by the manufacturer. The amount of RNA was determined by measuring the absorbance at 260 nm and using a correction factor of 40. For cDNA synthesis, 5 μg of RNA in a final volume of 20 μL were used. The reverse transcription reaction was done using AMV reverse transcriptase (USB Corporation, Cleveland, Ohio) for 60 minutes at 42°C and 30 minutes at 52°C. PCR was done in a final volume of 25 μL with 150 ng of cDNA, 1.5 U of Taq polimerase (Promega), 3 mM of $MgCl_2$, and 12 pmol of the following set of primers (Invitrogen, São Paulo, Brasil) for smoothelin (Rensen et al, 2002): forward 5′-GTCGACATCCAGAACTTCCTCC-3′; reverse 5′-CGCAGGTGGTTGTACAGCGA-3′. The conditions were 2 minutes of initial denaturation at 94°C, 30 seconds at 94°C, 30 seconds at 54°C, and 30 seconds at 72°C (35 cycles) with a final extension of 5 minutes at 72°C. The PCR products were analyzed on 2% agarose gels and vi-

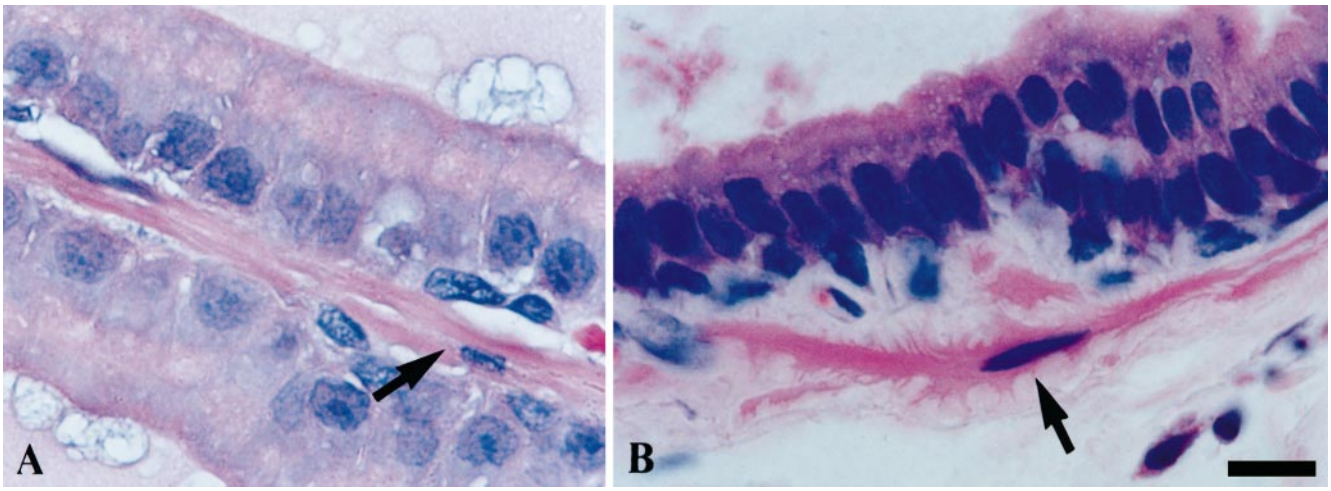

Figure 1. Hematoxylin and eosin-stained sections of the ventral prostate of a rat, showing elongated smooth muscle cells (SMC) (arrows), with the smooth appearance in control rats in which they usually occured as a single layer below the epithelium **(A)**. This arrangement contrasted with appearance of the SMC 21 days after castration (arrows): relaxed, showing a spinous aspect **(B)**. Bar = 10 μm.

sualized by ethidium bromide staining. The PCR markers used were BenchTop PCR Markers (Promega). β-Actin mRNA expression was used as an internal control for all groups. Positive and negative controls for smoothelin expression were rat lung and PC3 human prostatic cancer cell, respectively.

Results

Morphology and SMC Volumes

H&E staining showed that control SMC were elongated and flattened against the epithelial basement membrane (Figure 1A). After castration, the cells shortened and developed a pleated cell surface, leading to a spinous appearance (Figure 1B). The SMC lost contact with each other and showed wide extracellular spaces between cells. Usually, more than one layer of SMC was found below the epithelial structures after castration.

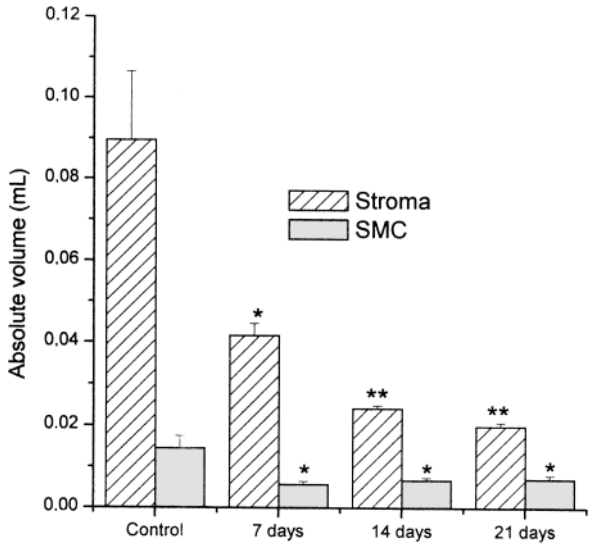

Figure 2. Relative contribution of smooth muscle cells (SMC) to the stroma and to the whole prostate in control and castrated rats. There was a progressive increase in the volume fraction occupied by SMC in the prostate after castration. In contrast, the increase in the contribution of SMC to the stromal volume was only seen 14 days after castration. (*P = .1; **P = .01).

As determined by stereology, the absolute volume of the stroma decreased progressively after castration, whereas the absolute volume of the SMC decreased only within the first 7 days after castration and remained unchanged thereafter (Figure 2). Stereology also showed that SMC occupied 5% of the prostate volume and 14% of the stromal volume. The relative volume of SMC increased after castration for whole gland and the stroma (Figure 3). The SMC are asymmetrically distributed in the ventral prostate with respect to the proximal–distal axis of the acini (Nemeth and Lee, 1996) and this contributes to a large variation in the absolute volume of the SMC, which is reflected by a larger standard deviation for the control (Figure 3) as compared to the castrated prostate, in which there is a proximalization on the pattern of SMC distribution.

The treatment with KOH–collagenase allowed visualization of the changes in the smooth muscle cells by SEM. The SMC of control rats were flat and elongated, with the usual smooth surface (Figure 4A). The SMC of castrated rats (21 days after castration) were relaxed and had an irregular, rough surface (Figure 4B).

Immunocytochemical Identification of SM-MHC and SM α-A and Smoothelin mRNA Expression

The SMC were identified using 2 specific antibodies for components of cytoskeleton present in differentiated SMC. Immunocytochemistry showed SM-MHC (Figure

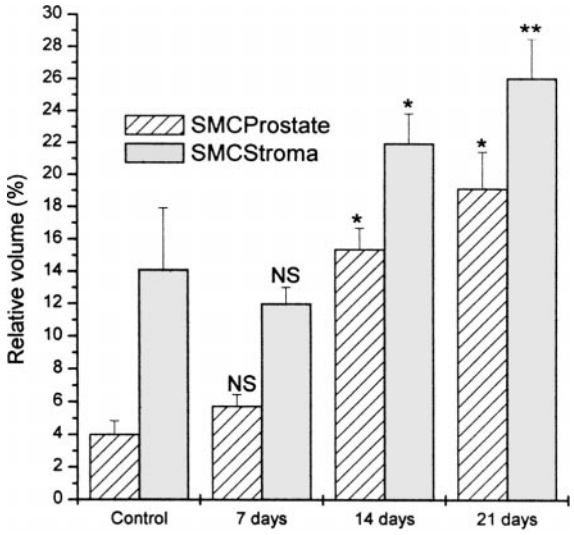

Figure 3. Absolute volume of the stroma and of the smooth muscle cells (SMC) compartments in the ventral prostate of noncastrated rats (control) and 7, 14, and 21 days after castration. There was a progressive reduction in the absolute volume of the stroma. The absolute volume of the SMC decreased within the first 7 days and remained unchanged thereafter. (*P = .1; **P = .01).

5A through D) and SM α-A (Figure 5E through H) in both prostatic SMC of noncastrated rats and in rats deprived of androgen for 21 days.

Presence of mRNA for smoothelin was determined by RT-PCR from the prostate of noncastrated rats and in rats castrated 7, 14, and 21 days before sacrifice (Figure 6A). Two bands with ~440 base pairs (bp) and ~300 bp, corresponding to smoothelin were found. β-Actin expression was used as an internal control (Figure 6B).

Discussion

Prostatic SMC have functional androgen receptors and modulate prostatic function by producing paracrine effectors for epithelial function under androgen stimulation (Hayward et al, 1996). It is therefore conceivable that SMC respond to androgen withdrawal. The importance of SMC in the morphogenesis and maintenance of the prostate has been stressed. These cells may act on the epithelium through paracrine mechanisms after proper stimulation by androgens and other regulatory signals (Farnsworth, 1999; Thomson et al, 2002). Castration caused a transition from the elongated, smooth profile to a retracted cell with a highly folded surface, and a spinous morphology, as shown by histology and SEM. The membrane folding observed may reflect the excess membrane present after the loss of cytoskeletal components.

Stereology showed that SMC occupied 5% of the rat ventral prostate, in contrast to the 22% occupied by these cells in the human prostate (Shapiro et al, 1992). Thus, the rat ventral is less fibromuscular than the human prostate. The relative volume of the SMC increased after castration, demonstrating that these cells contributed to a larger fraction of the stroma, which in its turn represented a larger fraction of the prostatic tissue, as previously re-

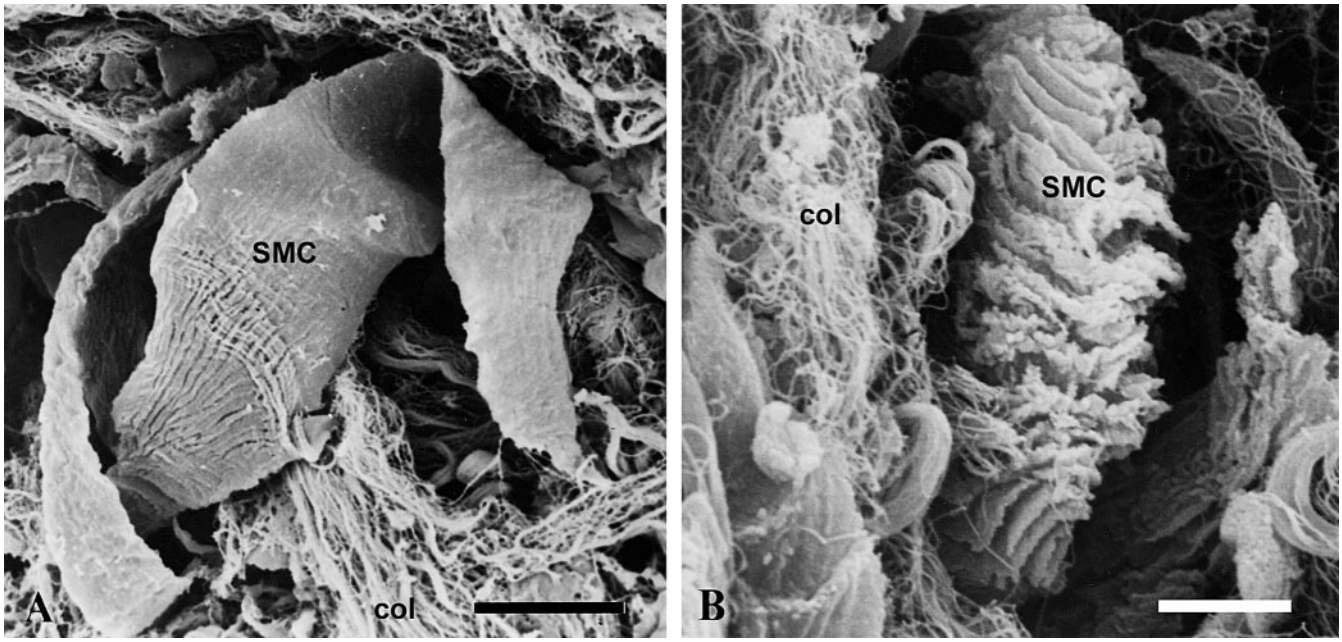

Figure 4. Scanning electron microscopy of the rat ventral prostate after treatment with KOH–collagenase. In noncastrated rats, smooth muscle cells (SMC) were flat and elongated, with the usual smooth surface **(A)**. In castrated rats (21days after orchiectomy), the SMC were relaxed and had a highly folded surface **(B)**. **(A)**, bar = 20 μm. **(B).** COL indicates collagen fibers. Bar = 10 μm.

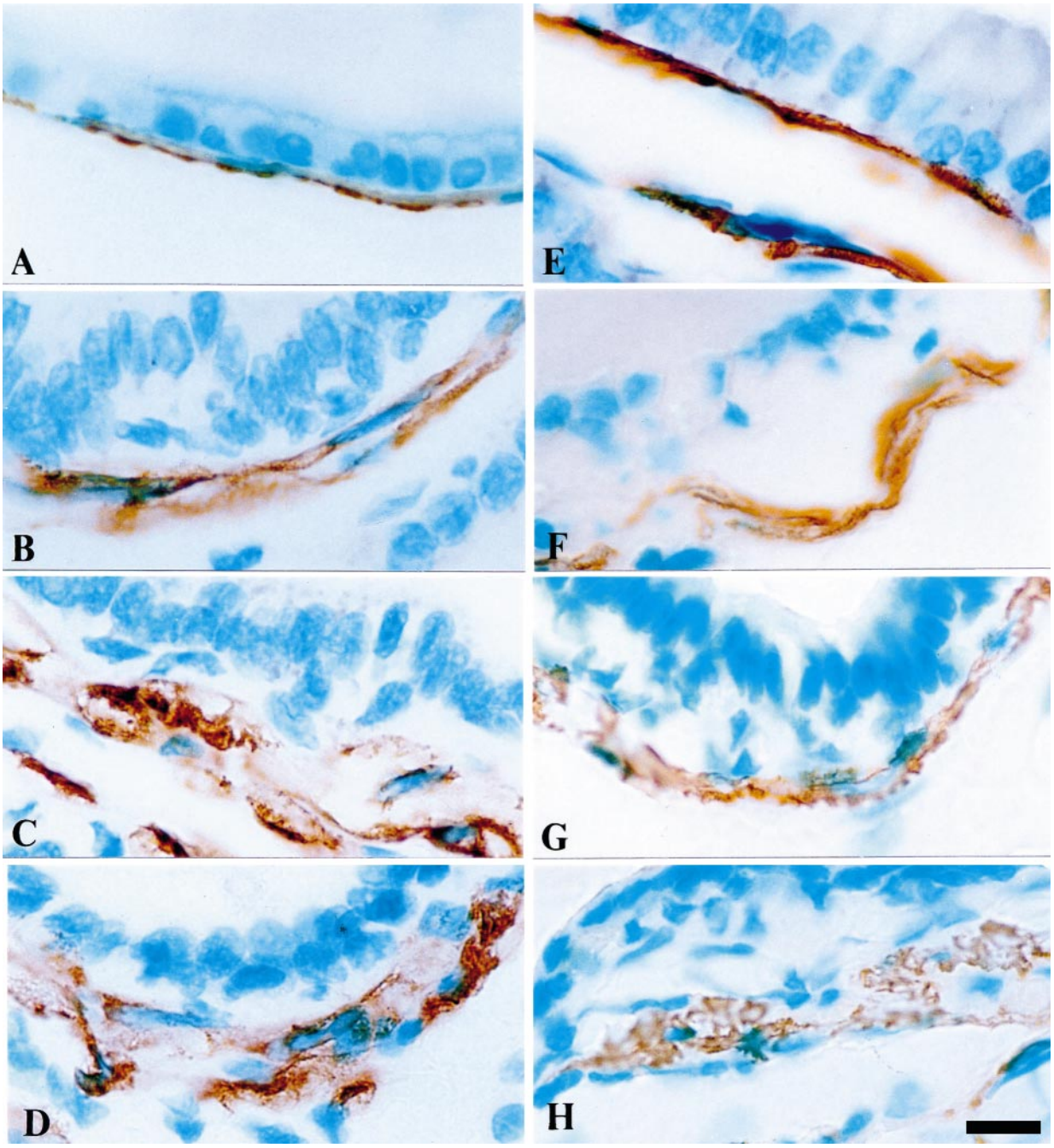

Figure 5. Immunocytochemical localization of smooth muscle myosin heavy chain **(A–D)** and α-actin **(E–H)** in the ventral prostate of a control **(A, E)** and 7 **(B, F)**, 14 **(C, G)**, and 21 days **(D, H)** after castration. Immunoreactivity was observed in all cases. In contrast to the reaction for SM α-actin, the reaction for SM-MHC was not uniform. The reaction follows the general morphology of SM cells (SMC) in each group, as seen in Figure 1. Bar = 10 μm.

ported (Zhao et al, 1992; Holterhus et al, 1993; Vilamaior et al, 2000).

The new SMC morphology and the contribution of these cells to the stromal volume did not mean that the cells were inactive. Instead, the SMC changes correlated with the general stromal modifications and appeared to be responsible for some of them, including the reorganization of collagen fibers, with which they were intimately associated after castration (Carvalho and Line, 1996).

The analysis revealed a critical transition phase in the absolute volume of the SMC within the first week after castration. This effect may be attributed not only to the reduction in the volume of individual cells, but also to cell deletion by apoptosis, since SMC may be among the

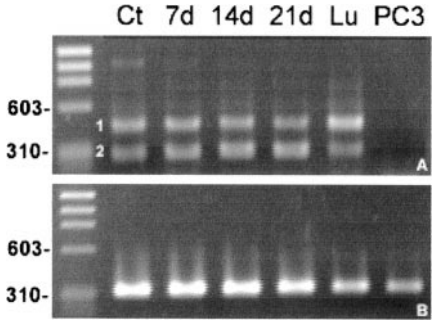

Figure 6. **(A)** Reverse transcription polymerase chain reaction (RT-PCR) using mRNA isolated from the prostate of control and castrated rats at different stages after castration. Smoothelin mRNA was found even 21 days after castration. **(B)** Amplification of the β-actin mRNA. Ct indicates control; 7d, 14d, and 21d, 7, 14, and 21 days after castration, respectively; Lu, lung; PC3, PC3 prostatic epithelial cancer cells; 1 and 2, two mRNA for smoothelin corresponding to the visceral and vascular isoforms, respectively. Key molecular markers are indicated on the right.

stromal cells undergoing apoptosis in the early period after castration, coincident with a peak of the apoptosis in prostatic epithelium after deprivation androgen (Shabisgh et al, 1998, 1999; Staack et al, 2003).

After this period, the total volume occupied by SMC up to 21 days was maintained, despite the overall reduction in the stromal absolute volume. A reduction of the absolute volume occupied by SMC in the first week after castration contributes to the reduction in their volume, as shown by the difference between the control and castrated rats 7 days after castration. The difference between the relative and absolute volumes reported here can be understood by recalling that the prostatic weight (and respective volume) is greatly reduced after androgen withdrawal.

Some of the SMC surface changes seen in castrated rats were similar to those observed in hypertrophic SMC (Gabella, 1990). However, the cell volume was clearly reduced in the former. This phenomenon has not been properly investigated but may indicate that SMC in castrated rats undergo progressive atrophy (Zhao et al, 1992; Niu et al, 2001, 2003).

The atrophy of SMC may be caused by the decreasing expression of some critical components of the contractile machinery. Cellular atrophy is characterized by the accumulation of large lipofuscin granules in the cytoplasm and a decreased cell volume. Atrophic cells were noted at the 14th day after castration and were more evident thereafter (Niu et al, 2001, 2003). In contrast, our results showed a decreased absolute volume of the SMC after the 7th day after castration and this stayed unalterable until 21 days after castration.

The SMC markers SM α-A, SM-MHC, and smoothelin were detected, even 21 days after castration. The RT-PCR for smoothelin transcripts resulted in 2 bands. These 2 bands correspond to alternatively spliced isoforms of smoothelin, as described by Rensen et al (2002), who attributes one of them to smoothelin A, found in visceral and urogenital tissues, and the other to smoothelin B, observed in vascular tissues. The results are then discriminating between the contribution of both prostatic and vascular SMC.

These observations suggest that, within the time scale of our experiments, the SMC of the ventral prostate maintain their differentiated phenotype after androgen withdrawal. In contrast to our observations, Hayward et al (1996) reported a loss of bundles of prostatic smooth muscle after castration, and showed that this was accompanied by the ordered and sequential disappearance of some markers of smooth muscle differentiation. However, these authors' protocol followed the alterations for up to 100 days. Since a series of SMC-specific markers (Doevendans and van Eys, 2002) was recently suggested for the characterization of the fully differentiated phenotype of these cells, the differentiated state of SMC in the ventral prostate of castrated rats awaits further investigation.

SMC have been associated with the stromal reorganization that occurs in prostatic epithelial metaplasias (Tuxhorn et al, 2001). However, more recently, these same authors proposed that SMC are displaced by the reactive stroma (consisting mostly of fibroblast-derived myofibloblasts) and invading epithelial cells (Tuxhorn et al, 2002).

The stromal reorganization in the prostate gland of castrated rats shares some similarities with the reactive stroma during epithelial cell invasion, including increases in the levels of type I collagen (Tuxhorn et al, 2002), tenascin (Ibrahim et al, 1993; Xue et al, 1998), and hyaluronan (Lokeshwar et al, 2001). However, these processes are distinct with respect to the cell types responsible for these extracellular matrix alterations. SMC (and likely fibroblasts) are important in the stromal reorganization after castration, whereas myofibroblasts participate in the stromal reaction in metaplasias.

The SMC changes reported here agree with the proposal of Cunha et al (1996), who suggested that prostatic function was dependent on the balance between epithelial and SMC factors acting on each other. Our results indicate that after androgen deprivation and epithelial regression, SMC partly assume the role of adapting the stromal compartment to the reduced activity of the epithelial structures.

This function is secondary to a primary role in eliciting epithelial death by preventing the production of survival factors or by releasing death signals by stromal cells (Kurita et al, 2001). Although these primary functions are attributed to the stroma, we cannot rule out the participation of SMC, especially because, in contrast to fibroblasts, they possess active androgen receptors (Prins et al, 1991).

Acknowledgments

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