Transforming Growth Factor β1 Transduced Mouse Prostate Reconstitutions: II. Induction of Apoptosis by Doxazosin

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BACKGROUND. To study the possible relationship between adrenergic activities and the pathogenesis of benign prostatic hyperplasia (BPH), we tested the effect of doxazosin, an α1-adrenoceptor antagonist, on prostatic growth in vivo using a mouse model for BPH.
METHODS. The mouse prostate reconstitution (MPR) model system with retroviral (BabeTGF-β1Neo) transduction of transforming growth factor beta 1 (TGF-β1) was used to induce focally hyperplastic BPH-like lesions and increase the number of catecholaminergic neurons. The mice were treated with daily intraperitoneal injections of doxazosin (3 mg/kg).
RESULTS. Doxazosin caused a significant reduction in the wet weight of BabeTGF-β1-infected MPRs. The percent of PCNA-positive epithelial cells was similar in the doxazosin-treated and water only, control groups. There was a significant increase in the number of epithelial cells undergoing programmed cell death, apoptosis, in the doxazosin group (apoptotic index = 4.7 for doxazosin group vs. 3.1 for control group, $P < 0.05$). The doxazosin-induced apoptosis was more apparent in TGF-β1 transduced MPRs than BAGα control MPRs, and was not seen in the prostates of the adult male mice into which the MPRs were engrafted.
CONCLUSIONS. Our data demonstrate a novel and potentially important biological activity of doxazosin in vivo in this mouse model of BPH. *Prostate 33:157–163, 1997.*
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KEY WORDS: **α-adrenoceptor; catecholamines; benign prostatic hyperplasia; doxazosin; mouse prostate reconstitution**

INTRODUCTION

Although many physiological responses of specific α-adrenoreceptor antagonists have been attributed to smooth muscle relaxation, the biological activities of these agents in prostatic cells have not been well documented. More specifically, a role for α-adrenoreceptor-mediated activities in the pathogenesis of benign prostatic hyperplasia (BPH) or in the regulation of prostate growth has not been clearly established. Multiple factors such as age, abnormal hormone and growth factor levels, or genetic alterations have been suggested for BPH pathogenesis [1,2]. The clinical and pathologic complexity of BPH has led to the development of diverse therapeutic approaches. Recently, clinical studies have demonstrated the therapeutic activity of pharmacological agents that are α-adrenoreceptor antagonists [3,4] for BPH. In other organ systems, adrenergic agonists exert growth-promoting influences during development [5] and stimulate growth and DNA synthesis in a variety of target cells through α-adrenoceptor mediated pathways [6–8].

We have developed a mouse model which produces phenotypic changes that mimic, in part, aspects of BPH [9]. Since we had observed increased accumu-

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lation of TGF-β1 in the stroma associated with human BPH foci [10], we modified the mouse prostate reconstitution (MPR) model system [11] by infecting mouse urogenital sinus cells with a retrovirus, BabeTGF-β1Neo, which transduces the TGF-β1 gene [9]. These TGF-β1 transduced MPRs develop into fully differentiated prostatic tissues with focal lesions composed of both stromal and epithelial hyperplasia, with an increased density of neuronal cells including catecholaminergic neurons and their fibers [12]. We used this model to test the effects of doxazosin, an α-adrenoreceptor antagonist, on the growth of normal and TGF-β1 transduced mouse prostate in vivo. When doxazosin was systemically administered to mice engrafted with BabeTGF-β1-infected MPRs, a significant reduction in the wet weight and increase in the apoptotic index of the MPRs was observed, compared to controls treated with water only. These results provide experimental evidence that doxazosin, an α-adrenoreceptor antagonist with documented activity for BPH, alters prostate growth through the induction of apoptosis, in vivo, in the MPR model.

MATERIALS AND METHODS

Mouse Prostate Reconstitution

MPRs were prepared as described previously [11]. Briefly, the urogenital sinus (UGS) was removed from day 17 embryos of C57BL/6 mice. The UGS tissues were digested with trypsin and collagenase, and the dissociated cells were infected with the retrovirus BabeTGF-β1Neo or BAGα in the presence of polybrene (8 μg/ml), as previously described [9]. The cells were then centrifuged and mixed with rat tail collagen with 1.5×10^6 cells/ml of collagen solution. Aliquots were placed in DMEM with 10% fetal calf serum overnight at 37°C. The next morning, the cells treated with the retroviruses were implanted separately under the renal capsule of adult (4-month-old) male C57BL/6 mice.

Administration of Doxazosin

Animals were caged separately on a 12 hr light:dark schedule, and fed a standard diet with water ad libitum. They were randomly assigned to either control (sterile water) (n = 11; 4 BAGα and 7 TGF-β1 MPRs) or experimental (doxazosin) groups (n = 21; 11 BAGα and 10 TGF-β1 MPRs). On the fifth day after the implantation of MPR, doxazosin-masylate (UK-33274-27; Pfizer Pharmaceuticals, New York, NY), dissolved in sterile water, was administered intraperitoneally (i.p.) at a dose of 3 mg/kg body weight daily for 24 days. Previous data has demonstrated that a complete blockade of α1-adrenoceptor of rodent cells can be achieved at this dosage [13,14], since the effective dose in rodents is about 0.3 mg/kg. Toxic effects are not anticipated from the dose we used, since the lethal dose is in excess of 200 mg/kg. All control mice received similar volumes of sterile water i.p. on the same schedule.

Tissue Preparation

Four weeks after the implantation, each MPR was cleanly dissected from under the kidney capsule, weighed, fixed in 10% buffered formalin and embedded in paraffin for sectioning and immunohistochemistry. Sections were serially cut at 6 μm in thickness. Representative sections were stained with hematoxylin & eosin (H&E) for histological evaluation. The remaining sections were used for immunohistochemical staining. The ventral lobe of the prostate gland from the host animal was also fixed, paraffin embedded, and sectioned.

Antibodies and Immunohistochemistry

Polyclonal antibodies against TGF-β1 (antibody CC, a gift from Drs. Michael Sporn and Kathleen Flanders which identifies extracellular TGF-β1), cytokeratin 14 (antibody RK-14, a gift from Dr. Dennis Roop, Baylor College of Medicine, Houston, TX), and tyrosine hydroxylase (antibody AB152, Chemicon International, Temecula, CA) were used as previously described [9,12]. To determine proliferative activity, sections were stained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Clone PC10, Dako Corp., Carpinteria, CA). After a 10-min microwave treatment (in 10 mM citrate buffer, pH 6.0), the sections were reacted with 1% H_2O_2 for 10 min, blocked with 3% normal horse serum for 20 min, then incubated with the primary antibody diluted 1:200 in phosphate buffered saline for 90 min. This was followed by incubation with biotinylated horse anti-mouse IgG1, then avidin-biotin complex (ABC) (Vector Laboratories, Burlingame, CA) and reaction with 3,3′-diaminobenzidine/H_2O_2. Sections were counterstained with methyl green to facilitate the identification of stomal vs. epithelial cells. The specificity of immunoreactions was verified by incubating some sections with phosphate buffered saline or with mouse IgG_a substituted for the specific primary antibodies.

In Situ Labeling of Apoptotic Bodies

The terminal transferase/uridine nick end labeling (TUNEL) technique developed by Gavrielli et al. [15] was used for labeling the cells undergoing apoptosis.

After deparaffinization with xylene and hydration, with a gradient of ethanol, the sections were treated with 2% H_2O_2 for 10 min to inactivate endogenous peroxidase. The slides were treated with DNase-free Protease K (20 mg/ml, Boehringer-Mannheim, Indianapolis, IN) for 10 min at 25°C. Following a 20 min incubation in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl, pH 7.2, 1 mM cobalt chloride, 140 mM sodium cacodylate), the sections were incubated in hybridization buffer [containing 1 μl of TdT (United States Biochemical, Cleveland, OH) and 2.5 μl of biotinylated dUTP (Boehringer-Mannheim) in 100 μl TdT buffer] for 1 hr at 37°C. The reaction was terminated by rinsing with 0.3 M sodium chloride and 0.03 M citrate chloride. The sections were then blocked with 2% bovine serum albumin, reacted with ABC (Vector laboratories) and 3,3′-diaminobenzidine/H_2O_2. Sections were counterstained with methyl green to enable the identification of stomal vs. epithelial cells. Specificity was confirmed by eliminating the biotinylated dUTP from hybridization buffer. As a positive control, sections were pretreated with DNase [15].

Quantitative Histological Analyses

Computer assisted image analysis of stained sections was performed as previously described [9,12]. The effect of doxazosin on histomorphology was evaluated by determining the volume of the glandular epithelium, stroma, and the lumen in each MPR on H&E-stained sections. The area of each focus of stromal/epithelial hyperplasia was measured, and the number of foci counted and expressed per mm^2 total prostatic area. Neuronal densities in each MPR were measured and expressed as the numbers of neurons per mm^2 stromal area. The apoptotic bodies and PCNA labeled cells were counted after projection on a Trinitron video screen at 200× magnification so that each measuring field had a real area of 300 × 400 μm. From each MPR, 20 to 25 measuring fields from six to eight sections were randomly selected, and the percent of the total number of epithelial cells staining positively PCNA determined. The apoptotic index (AI) was expressed as the number of apoptotic bodies per 1,000 epithelial cells, whereas apoptotic bodies located in the stroma were expressed as the number of apoptotic bodies per mm^2 stromal area.

Statistical Analysis

The significance of the differences in MPR wet weight, volume fraction percentages, and neuronal densities were analyzed by analysis of variance and *P*-values determined by the Bonferroni/Dunn test. A *P*-value less than 0.05 was considered statistically significant. The significance of the differences in apoptotic index and the percent of the total number of epithelial cells staining positively for PCNA between doxazosin and control treatments were determined by Mann-Whitney rank test.

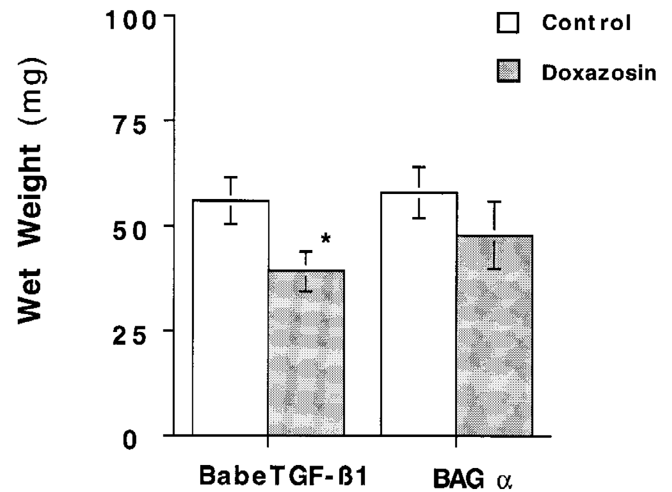

Fig. 1. Effect of doxazosin on wet weight. MPRs infected with either BabeTGF-β1, or BAGα, were harvested 4 weeks after the implantation from the doxazosin-treated or the control (sterile water-injected) mice, and weighed before tissue processing. Control (water) animals are indicated by the clear bars and doxazosin treatment animals by the shaded bars. Error bars represent ± standard error of the mean (*$P < 0.05$).

RESULTS

All mice engrafted with either the BabeTGF-β1- or BAGα-infected MPRs were treated with doxazosin or sterile water, as described above, and remained healthy throughout the course of the treatment. At 4 weeks after MPR implantation under the kidney capsule, the animals were sacrificed and no gross changes in the tissues were apparent. The average wet weight of the BabeTGF-β1-infected MPRs was more than 30% lower ($P < 0.05$) in the mice treated with doxazosin (39.2 ± 4.8 mg) compared to those treated with sterile water (55.6 ± 5.5 mg) (Fig. 1). The wet weight of BAGα-infected MPRs was also lower in the doxazosin-treated group relative to the sterile water controls. However, this difference did not achieve statistical significance. Consistent with previous observations [9], there was no difference in wet weight between the BabeTGF-β1- and BAGα-infected MPRs in the control water groups.

Focal BPH-like lesions composed of epithelial and stromal hyperplasia were associated with the BabeTGF-β1-infected MPRs. Morphological characterization of these BPH-like lesions has been detailed pre-

TABLE I. Morphological Features of Hyperplastic MPR Lesions*

MPR treatment	BabeTGF-β1		BAGα	
	Control	Doxazosin	Control	Doxazosin
Number	11	10	4	7
Focal lesion density lesions/mm^2 (± SEM)	0.122 (0.02)	0.112 (0.01)	0.036 (0.003)	0.042 (0.01)
Focal lesion size mm^2 (± SEM)	0.063 (0.006)	0.052 (0.008)	nd	nd
Neuronal density cells/mm^2 (± SEM)	2.697 (0.52)	3.339 (0.64)	0.749 (0.31)	0.657 (0.12)

*Hyperplastic MPR lesions were defined as focal areas of stromal and epithelial hyperplasia. nd, not determined.

viously [9,12], and similar features were observed in the present set of experiments. In the BabeTGF-β1-infected MPRs, hyperplastic lesions composed of cytokeratin 14-positive cells with extracellular accumulations of TGF-β1 and an increased density of neuronal cells were readily apparent. Quantitatively, the frequency of focally hyperplastic lesions and the density of neuronal cells in the BabeTGF-β1 infected MPRs was significantly higher than that in the BAGα controls (Table I). The frequency of focal lesions and the neuronal density in BabeTGF-β1-infected MPRs in the animals treated with doxazosin were similar to the control BabeTGF-β1-infected MPRs treated with water (Table I).

To determine if the doxazosin-induced reduction in wet weight of the BabeTGF-β1 MPRs was the result of decreased prostatic secretion or from growth inhibition, the volume fractions of lumen, glandular epithelia, and stroma were measured morphometrically using computer-assisted image analysis. There was no significant difference in either the volume percentage of the lumen or the lumen:epithelia:stroma ratios between the doxazosin treated and the sterile water control group (Table II). These results suggest that the doxazosin-caused reduction in wet weight of MPRs cannot be attributed to reduced secretion.

The proliferative activities of the glandular epithelial cells were evaluated with anti-PCNA immunohistochemistry. The PCNA antibody gave strong nuclear staining (Fig. 2A). The PCNA-immunoreactive epithelial cells were counted on MPR sections and expressed as a percent of the total number of epithelial cells. There was no significant difference in the percent of PCNA-positive epithelial cells between control and doxazosin treatments in BabeTGF-β1- or BAGα-infected MPRs, or in the ventral prostate of the engrafted mice (Fig. 2B). Overall, there did seem to be a slightly higher percent of PCNA-positive epithelial cells associated with the BabeTGF-β1-infected MPRs, but this did not achieve statistical significance.

With the TUNEL procedure, apoptotic cells were easily identified in the sections from retrovirus infected MPRs (Fig. 3A) or the ventral prostate of the engrafted mice (not shown). The apoptotic cells localized in the glandular epithelia were counted and expressed as an apoptotic index (AI) or the number of apoptotic cells per 1,000 epithelial cells (Fig. 3B). The AI in water only control BabeTGF-β1-infected MPRs ($n = 11$) was 3.1, but significantly higher at 4.7 ($P < 0.05$) in the doxazosin-treated group ($n = 10$). The AI of the glandular epithelia in the water only control BAGα-infected MPRs was similar to the water only control BabeTGF-β1-infected MPRs, but was increased in the doxazosin-treated animals, however, this increase did not achieve statistical significance. Doxazosin treatment did not lead to significant change in AI in the glandular epithelia of the ventral prostate of the engrafted mice. Doxazosin treatment was associated with a higher density of apoptotic bodies per mm^2 stromal area, as compared to the water-treated controls in both the BabeTGF-β1- and BAGα-infected MPRs, although these differences did not achieve statistical significance (Fig. 3C).

TABLE II. Effect of Doxazosin on the Volume Fractions of Lumen, Epithelium, and Stroma in BabeTGF-β1-Infected MPRs*

Treatment	No.	Volume fraction % (± SEM)		
		Lumen	Epithelium	Stroma
Control	11	44.93 (3.55)	27.43 (3.74)	27.64 (3.85)
Doxazosin	10	42.39 (4.19)	28.24 (2.26)	29.37 (3.35)

*Volume fractions were determined by computer assisted image analysis with measurements of the area of each compartment as a percent of the total area on H&E stained slides from the BabeTGF-β1-infected MPRs.

DISCUSSION

The primary objective of this study was to determine if a therapeutic α1-adrenoceptor antagonist could affect normal or hyperplastic prostatic growth in vivo. We used a TGF-β1 transduced MPR model

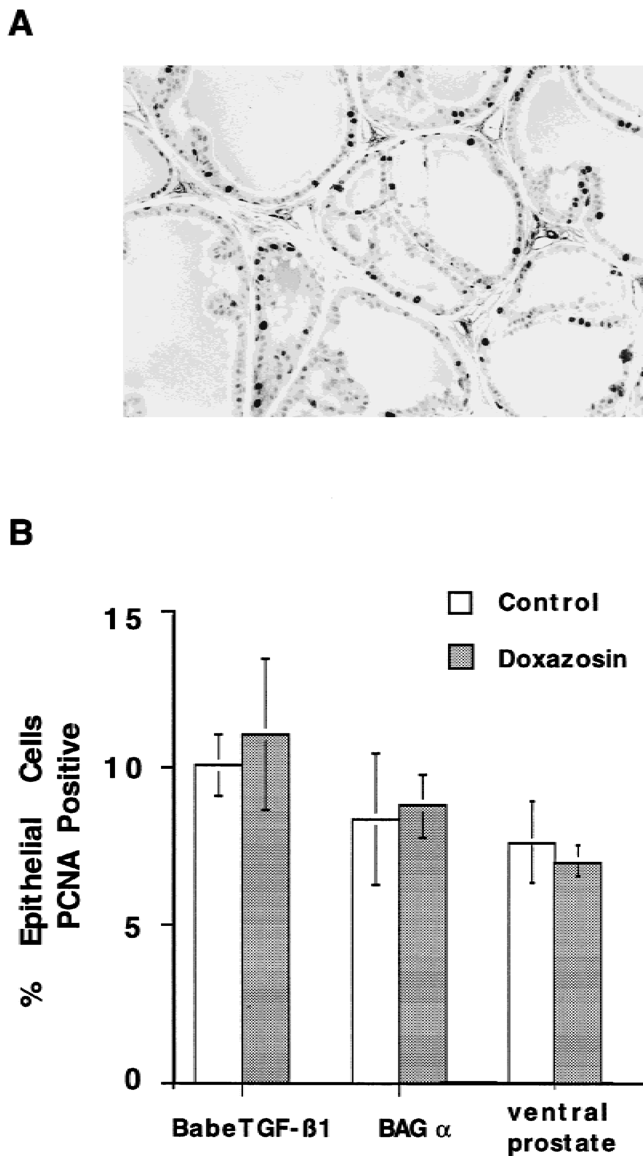

Fig. 2. Effect of doxazosin on proliferative activities of the epithelial cells. **A:** Representative field illustrating PCNA positively staining cells in a BabeTGF-β1-infected MPR (original magnification, ×200). **B:** Comparison of proliferative activity expressed as % PCNA positive epithelial cells in sections from BabeTGF-β1-infected MPRs, BAGα-infected MPRs, and from the ventral prostate of the adult male host animals. Shading and standard error bars as in Figure 1.

system which develops BPH-like focal hyperplastic lesions as well increased catecholaminergic neurons [9,12]. We selected an α1-adrenoceptor antagonist, doxazosin, which has documented therapeutic activity for BPH. The dose chosen, 3 mg/kg daily, was higher than the effective dose determined in other animal studies [13,14], but well below the lethal dose. The effects of doxazosin on MPR growth as well as on the BPH-like lesions were evaluated by histochemical and morphometric assessments.

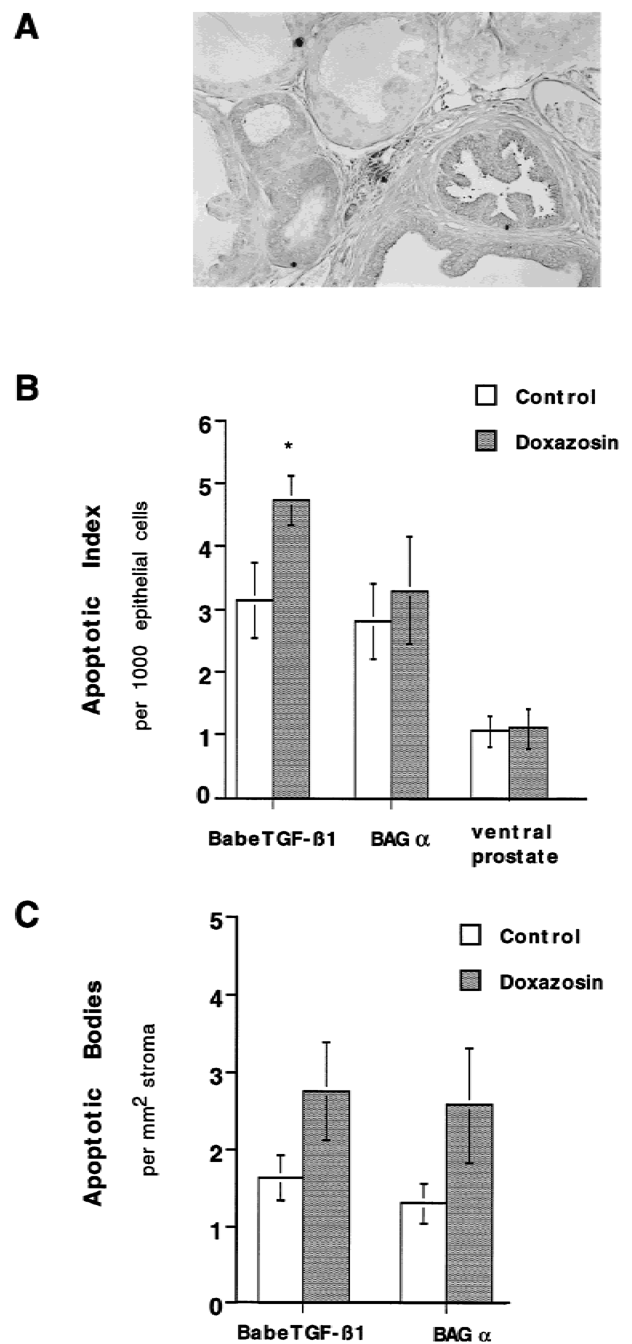

Fig. 3. Effect of doxazosin on apoptosis. **A:** Representative micrograph depicting apoptotic bodies in a BabeTGF-β1-infected MPR (original magnification, ×200). Apoptotic bodies were labeled with the TUNEL technique. **B:** Apoptotic index (the number of apoptotic bodies per 1,000 epithelial cells) in epithelial compartments of the MPRs infected with BabeTGF-β1, or BAGα, and in the ventral prostate of the adult male host animals. **C:** Number of apoptotic bodies per mm^2 stromal area. Shading and standard error bars as in Figure 1 ($^*P < 0.05$).

The results indicate that doxazosin treatment led to inhibition of prostatic growth as indicated by a reduced wet weight. This weight reduction did not correlate with a change in morphometry as the volumes of the lumen, stroma, and epithelia were proportionally similar. We therefore evaluated the growth rate by measuring the percent of epithelial cells positive for PCNA and, surprisingly, the percent of PCNA positive epithelial cells in the doxazosin-treated groups, whether in the BabeTGF-β1- or BAGα-infected MPRs, did not differ significantly from that in the sterile water treated controls, suggesting that the inhibition of prostatic growth associated with doxazosin treatment was not caused by inhibition in proliferative activity.

The growth potential of tissues is determined by a dynamic balance between two processes: proliferation and programmed cell death (apoptosis) [16]. As the inhibition of MPR growth by doxazosin could not be attributed to an inhibition of proliferative activity, apoptosis in the MPRs was investigated with the TUNEL technique (Fig. 3). The apoptotic index in the glandular epithelia of the BabeTGF-β1 infected MPRs from doxazosin treated animals was significantly higher than that in the sterile water treated controls. Thus, an increased apoptotic rate, but not a decreased proliferative rate, may be responsible for the inhibition of prostatic growth resulting from doxazosin treatment. Although sympathetic/catecholamine activities have been suggested in promoting DNA synthesis in a variety of cells through an α1-adrenoceptor mediated mechanism, to our knowledge this is the first report of induction of apoptosis by systemic administration of an α1-adrenoceptor antagonist in vivo.

The effect of doxazosin on the inhibition of prostatic growth did not appear to be BPH-lesion-specific in this TGF-β1 transduced MPR model of BPH. Although the mean density and size of the focal lesions was slightly smaller in the doxazosin-treated MPRs, doxazosin treatment did not significantly decrease these parameters in the BabeTGF-β1 transduced MPRs, when compared with the sterile water treated controls. The apoptotic bodies labeled by TUNEL technique were present among normally appearing glands, as well as in the BPH-like foci, and present at an increased frequency in the epithelium (Fig. 3B) and the stroma (Fig. 3C) of the doxazosin treated animals from both MPR groups. Therefore, the reduction in MPR wet weight was apparently due to an effect of doxazosin on both normal and hyperplastic prostate cells. It is interesting to note that the biological activities of doxazosin demonstrated in this study, which achieved statistical significance, were limited to TGF-β1-transduced prostatic tissues. It is, therefore, possible that there is interaction (either direct or indirect) between doxazosin activities and TGF-β1 expression. We have previously demonstrated that high levels of TGF-β1 accumulation were not only associated with BPH in humans [10], but also with the stroma of the developing mouse prostate [17].

The inhibition of prostatic growth by doxazosin appeared to be most prominent in the MPR tissues, as doxazosin treatment did not affect either the percent epithelial cells PCNA positive or AI of the epithelial cells in the host ventral prostate. Although the MPRs show morphological characteristics of differentiated prostatic tissues by the fourth week after the implantation, they also still have some features of a developing organ. For example, they have a higher percent of PCNA positive epithelial cells, when compared to the ventral prostate of adult mice. The fact that doxazosin treatment induced increased apoptosis in the MPRs, especially in the BabeTGF-β1-transduced ones, but not in the mature host prostate, suggests a mechanism of action for doxazosin within the context of prostatic development. It has been suggested that the stromal and epithelial abnormalities associated with BPH arise from a ''reawakening'' of fetal developmental processes [18]. Thus, it is conceivable that human BPH lesions may be more sensitive to the effects of doxazosin than normal adult prostatic tissue. A potential mediator for both abnormal cellular growth manifest as BPH, and fetal prostatic development, is TGF-β1 [10,17].

The mechanism(s) for the induction of apoptosis by doxazosin remains to be explored. The existence of α1-adrenoceptors in both stromal and epithelial cells in the MPR tissues has been confirmed by a radioreceptor assay with ^{3}H-prazosin (unpublished observations). The dosage of doxazosin used in the present study is pharmacologic and high enough for a complete blockade of α1-adrenoceptors in rodent cells [13,14]. Nevertheless, whether doxazosin induces apoptosis through the α1-adrenoceptor or through other signal transduction pathways remains unresolved.

Although the present study did not address the mechanism for the doxazosin action in the induction of apoptosis, it does establish a relationship among α1-adrenergic activity, TGF-β1, and prostatic growth. In the TGF-β1 transduced MPR system, focal accumulations of extracellular TGF-β1 in stroma are associated with hyperplastic prostatic lesions and increased neuronal densities [9,12], presumably through neurotrophic effects [19–21]. An increase in release of catecholamines resulting from increased neuronal density may affect the growth of prostatic cells in both the stroma and epithelia. It is possible that sympathetic activity in the MPRs might play a role as a trophic (or anti-death) factor during development; therefore, the removal or blockade of this factor may induce increased apoptosis in the MPR tissues. It is also pos-

sible that doxazosin affects the vascular tissues of the MPR and thus produces apoptosis in prostatic cells through an indirect mechanism. Further investigations are necessary to clarify the mechanism of doxazosin induced apoptosis.

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