Effect of Epristeride on the Expression of IGF-1 and TGF-β Receptors in Androgen-Induced Castrated Rat Prostate

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The development of benign prostatic hyperplasia (BPH) is an androgen-dependent process that may be mediated by a number of locally produced growth factors. Among them, insulin-like growth factor 1 (IGF-1) and transforming growth factor β (TGFβ) are thought important in regulating prostate growth and homeostasis, and their expression undergoes changes in proliferative prostatic disease. Epristeride, a 5α-reductase inhibitor, is an effective drug in the treatment of BPH, inducing regressive changes in the prostate. This study was designed to assess the effects of epristeride on expression of these two factors at mRNA and protein levels in castrated rats maintained with exogenous testosterone. Epristeride treatment caused significant reduction in ventral prostate weight in a dose-dependent manner. There was a positive correlation between IGF-1 mRNA expression and ventral prostate weight and an inverse correlation between TGF-β1 mRNA expression and ventral prostate weight. Immunohistochemistry showed strong IGF-1 receptor immunoreactivity in the prostatic epithelial cells of untreated animals. *In situ* hybridization demonstrated high levels of IGF-1 mRNA expression both in the prostatic stromal and epithelial cells of untreated rats. In treated rats, both IGF-1 receptor protein and IGF-1 mRNA levels decreased significantly, and IGF-1 mRNA was mainly expressed in prostatic stromal cells. Weak expression of TGFβ receptors at the protein level and TGFβ at the mRNA level were found in the prostatic hyperplastic epithelial cells of untreated rats. In treated animals, intense TβRII immunoreactivity was observed in epithelial cells, and a higher level of TGFβ mRNA was observed in both epithelial cells and stromal cells compared with control animals. In our opinion, the effect of epristeride on rat prostatic atrophy might be mediated via local growth factor(s).

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Development of benign prostatic hyperplasia (BPH) appears to be dependent on the conversion of testosterone to dehydrotestosterone (DHT), which is enzymatically mediated by a steroid 5α-reductase (1). Epristeride is a potent and specific inhibitor of type II 5α-reductase and has proved to be effective in reducing prostate size and the circulating and intraprostatic levels of DHT (2, 3); however, its molecular mechanism is not very clear.

The prostate gland requires androgens for proliferation and maintenance of its function. BPH development has been associated with aging and hormonal control, but age-related changes in androgen secretion alone do not explain the hyperplastic development of the gland. It could be that estrogens and androgens act synergistically; in castrated dogs, hyperplastic development is induced by their simultaneous administration or by injection of androstenedione (an aromatizable androgen), whereas androgens alone fail to produce this effect (4, 5). In addition to hormones, a whole battery of other regulators is involved in the fine-tuning of prostate growth and differentiation. Among them are many polypeptide growth factors, which are generally locally produced and exert their effects either in the tissue in which they are formed or in the adjacent tissue. Efforts to identify pivotal growth factors and studies on their effects have been prompted by the observation that prostatic cells in culture need substances other than androgens for proliferation (6). These polypeptide growth factors are positive and negative regulators of prostatic growth and function. Insulin-like growth factors (IGFs) are considered to be the most important positive growth factors. Several studies have indicated that IGFs are mitogenic in prostate tumor cells and normal prostate cells. The prostate stroma secretes IGF-1 and the

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epithelial cells respond to IGFs through the interaction of these growth factors with the type 1 IGF receptor (7, 8). The principal IGF produced in the rat prostate is IGF-1, whereas in humans, the predominant species is IGF-2.

TGFβ plays an important role in the regulation of prostatic growth. It inhibits growth of prostatic epithelial cell in culture and mediates programmed cell death after androgen withdrawal. It is the only known negative regulator of prostatic growth.

TGFβs have been detected in normal and diseased human prostate (9, 10). TGFβ seems to be a potent inhibitor of prostatic epithelial cell proliferation and has been shown to mediate apoptotic epithelial cell death *in vitro* (11). Signaling by TGFβ is dependent on binding to cell surface receptors, three of which, designated TβRI, TβRII, and TβRIII have been cloned. Receptors type I and II are serine/threonine kinases (12). In a proposed model, TGFβ binds to the type II receptor, and then the type I receptor is recruited and phosphorylated by the type II receptor. Sex steroids appear to be involved in modulating the TGFβ system, since androgen ablation upregulates both TGFβ and TGFβ receptors in castrated rat (13).

To assess whether epristeride-induced atrophic changes in BPH could be mediated by IGFs and TGFβ, we compared the expression and cellular localization of IGF-1 and TGFβ in androgen-treated castrated rats treated with epristeride and controls.

Materials and Methods

Drug and Agent. Epristeride was kindly donated by Yangzhou Pharmaceutical Factory (People's Republic of China). All reagents used in immunohistochemistry were purchased from Santa Cruz Biotech (Santa Cruz, CA). *In situ* hybridization kits were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Animal Treatment. All animals used in these studies were maintained in compliance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences.

Sprague-Dawley male rats were housed in groups of two per individual microisolator cage under controlled temperature (21°–22°C), humidity (50%), and light conditions (12:12-hr light:dark cycle; lights on at 0700 hr). Animals were fed a commercially available chow, and water was available *ad libitum.* Castration was performed via a scrotal incision under ether anesthesia before starting the experiment. Both the testes and epididymis were removed. After castration, the rats were maintained under standard laboratory conditions for 7 days. Then the animals were randomly divided into four groups, with each group containing eight rats. Epristeride was given orally once a day at 0800 hr via gastric intubation, using methylcellulose as solvent. The volume of an individual gastric feeding was 1.0 ml/100 g body wt. Each group of rats received exogenous androgen via daily subcutaneous injections of testosterone propionate (0.5 mg/rat) in combination with either 0, 3, 10, or 30 mg/kg body wt of epristeride every day for 30 days. Prostates of eight rats in each group were obtained for investigation 24 hr after the last administration. After weighing, each ventral prostate was divided into two parts; one part was paraffin sectioned for detection of the IGF-1 receptor and TGFβ receptor by immunohistochemistry, and the rest was frozen in liquid nitrogen and stored until use.

Immunohistochemistry Staining for IGF-1 Receptor and TGFβ Receptor Expression. Ventral prostatic tissue, after various treatments, was processed for immunohistochemical staining. The primary antibody used was the anti-IGF-1 receptor or anti-TGFβ receptor II rabbit polyclonal antibody (Santa Cruz Biotech). Each prostate was fixed with 4% formalin, embedded in paraffin, and sectioned at 6 μm as preciously described (14). Briefly, the sections from representative paraffin-embedding tissue samples were deparaffinized, rehydrated, and incubated with the primary antibody for 1 hr at 37°C with a 1:150 dilution of IGF-1 receptor and 1:100 dilution of TGFβ receptor II rabbit polyclonal antiserum. The slides were then incubated with goat anti-rabbit ABC regents (Vector Laboratories, Burlingame, CA) according to the instructions of manufacturer. Immunoreactive sites were detected by immersing the slides in 0.05 M Tris-HCl buffer containing 0.05% diaminobenzidine and 0.01% H_2O_2. Negative controls included substitution of the first antibody with normal rabbit serum or omission of the first and/or the second antibodies. Slides were not counterstained. To determine the relative content of IGF-1 receptor and TGFβ receptor, transmittance was quantitated using a microphotometer at 650 nm wavelength (15). The transmittance of blank space without any tissue in the same section was assigned 100%.

***In Situ* Hybridization for IGF-1 and TGFβ1 mRNA Expression.** For *in situ* hybridization, cryostat sections (6 μm) were dried in room temperature, fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS), pH 7.4, for 20 min and then were washed two times in PBS.

IGF-1 and TGFβ1 antisense oligonucleotide probe (2.5 pmol/μl, Oncogene Co., Cambridge, MA) was labeled with digitoxin (DIG)-dd-UTP (1 nmol/μl, mol wt: 1116.7. Roche-Boehringer Mannheim, Germany) by a 3′-end labeling system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Labeled probe was purified by using Microspin G-50 Column (Amersham Pharmacia Biotech). Briefly, tissues were dehydrated by passage of the slides through increasing concentrations of ethanol in distilled water. The slides were put into a freshly prepared protease-K solution (1 μg/ml in 50 mM Tris-HCl and 5 mM EDTA, pH 8.0) at room temperature for 20 min, rinsed in PBS-glycine (0.1 mM), and then acetylated for 10 min in a freshly prepared acetic anhydride solution (1:400 in 0.1 mM triethanolamine, pH 8.0). After dehydration with graded ethanol, sections were briefly air dried before hybridization. The hybridization mixture (10 mM Tris-HCl [pH 7.5], 50%

formamide deionized, 0.6 M NaCl, 1 mM EDTA, 1× Denhardt's solution, 1 μg/ml sonicated salmon sperm DNA, 500 μg/ml transfer RNA, 10% Dextran sulfate, and 10 mM dithiothreitol) containing 5 pmol DIG-labeled oligonucleotide in a final volume of 25 μl was applied to each section and covered with a siliconized coverslip. Hybridization was continued for 18 hr at 42°C in a sealed humid chamber. The slides were then incubated at room temperature with 4× SSC and 2× SSC for 30 min, respectively. After incubation with blocking solution (buffer1 containing 0.1% Triton X-100 and 2% normal sheep serum) for 1 hr, slides were incubated for 3 hr in a humid chamber with buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum, and 1:500 dilution of sheep anti-DIG-alkaline phosphatase (Fab fragments). Then the slides were washed with buffer 1 for 20 min with constant shaking. After incubation with buffer 2 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM $MgCl_2$) for 10 min, slides were immersed in a color solution containing (1:50 dilution of nitro-blue tetrazolium (NBT)/5-Bromo-4-chloro-3-indolylphosphate (BCIP) in buffer 3) for 5 hr. When color development was optimal, the reaction was stopped in buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA). Sections were mounted using an aqueous mounting solution. The specificity of *in situ* hybridization of oligonucleotide probe for IGF-1 and TGFβ mRNA was studied by hybridization with the respective DIG-labeled sense probe.

Relative content of IGF-1 and TGFβ mRNA was assessed by microphotometer at 470 nm wavelength. The transmittance of blank space without any tissue in the same section was assigned 100%.

Statistics. Data were expressed as mean ± SD and were analyzed by analysis of variance (ANOVA) followed by Dunnett test. Relation between prostate weight and IGF-1 mRNA/TGFβ mRNA level was assessed with simple linear regression analysis, and Pearson (r) correlation coefficients were presented. Differences were considered significant with a P value less than 0.05. Statistical analyses were performed using the SPSS for Windows (v. 10.0).

Results

Male rats given various doses of episteride daily for 30 days did not show any statistical difference in body weight (data not shown). Episteride treatment, however, caused significant reduction in ventral prostate weight in a dose-dependent fashion (Table I and Fig. 1A). There is a positive correlation between IGF-1 mRNA expression and ventral prostate weight ($r = 0.5578$, $P < 0.01$; Fig. 1B) and an inverse correlation between ventral prostate weight and TGFβ1 mRNA levels ($r = 0.7417$, $P < 0.01$; Fig. 1C).

Morphology of Prostate Treated with Episteride. Histologically, untreated control prostates were made up of nodules in which tall columnar cells and papillary infoldings were prominent, whereas treated prostates showed moderate to marked atrophic changes in the epithelial compartment. As a consequence of epithelial atrophy, a relative increase of stroma was seen in the prostates treated with episteride (data not shown).

Effect of Episteride on IGF-1R and TβRII Protein Expression. The expression of IGF-1R at the protein level was analyzed by immunohistochemistry in rat prostatic tissue samples. Control samples showed strong IGF-1R immunoreactivity in epithelial cell, whereas in episteride-treated sample, IGF-1R was reduced markedly as compared with the control group in a dose-dependent manner (Table II and Fig. 2, A and B).

Using polyclonal anti-TGFβ receptor II, we detected the expression of TβRII at the protein level. TGFβ receptor protein was mainly localized in epithelial cells. In control rat prostates, this protein expression was weak, whereas in treated groups, TGFβ receptor expression showed intense immunoreactivity, also localized in epithelial cells. In the stroma, no immunostaining for TGFβ receptor was seen in either episteride-treated or control prostate. TβRII protein level of episteride-treated samples was increased significantly in a dose-dependent manner as determined by relative quantitative estimation with a microphotometer (Table II and Fig. 2, C and D). Little or no color reaction was observed in all negative control slides.

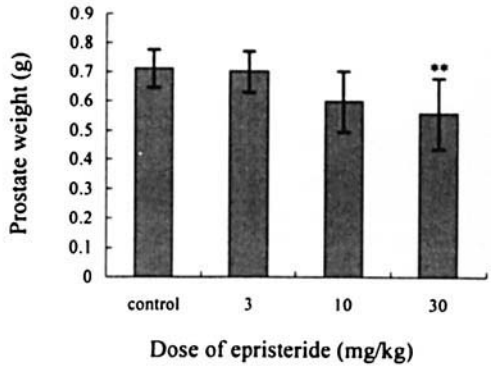
Effect of Episteride on IGF-1 and TGFβ mRNA Expression. To examine the effect of episteride on IGF-1 and TGFβ mRNA expression, we detected all samples by *in situ* hybridization with the DIG-labeled antisense oligonucleotide probe. Hybridization with antisense IGF-1 oligonucleotide probe revealed positive staining in many stromal cells but comparatively less in isolated epithelial cells. IGF-1 mRNA level was decreased significantly in a dose-dependent manner as observed in episteride-

Table I. Effect of Episteride on Ventral Prostatic Weight

Treatment	Ventral prostatic weight (g)
Castrated + testosterone (control)	0.711 ± 0.065
Castrated + testosterone + episteride (3 mg/kg body wt)	0.701 ± 0.122
Castrated + testosterone + episteride (10 mg/kg body wt)	0.600 ± 0.103
Castrated + testosterone + episteride (30 mg/kg body wt)	0.559 ± 0.070[a]

Note. Data were presented as mean ± SD ($n = 8$ in each group).
[a] $P < 0.01$ compared with control group.

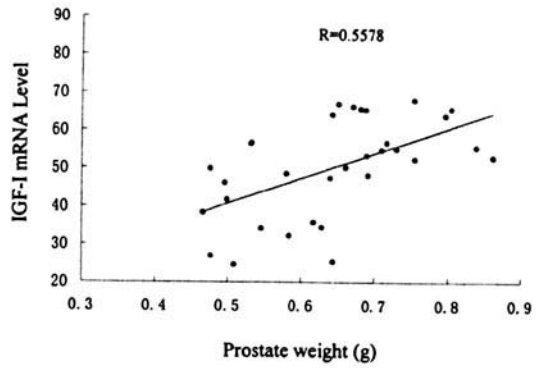
A



*P<0.05 *vs.* control group.

**P<0.01 *vs.* control group

B



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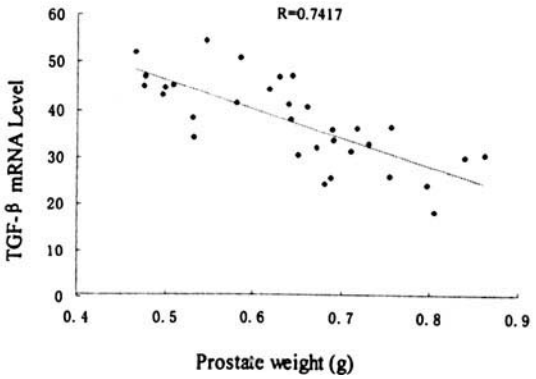

Figure 1. Effect of epristeride on rat prostate weight and correlation between prostate weight and IGF-1 mRNA level and TGFβ1mRNA level. (B) Relationship between prostate weight and IGF-1 mRNA level (data expressed as value of OD_{470}) of prostate in all rats (n = 32), i.e., castrated rats maintained with testosterone and castrated rats maintained with testosterone plus either 3, 10, or 30 mg/kg body wt of epristeride. (C) Relationship between prostate weight and TGFβ mRNA level (data expressed as value of OD_{470}) of prostate in all rats (n = 32), i.e., castrated rats maintained with testosterone and castrated rats maintained with testosterone plus either 3, 10, or 30 mg/kg body wt of epristeride. OD_{470} = –log transmittance. *P < 0.05 vs control group. **P < 0.01 vs control group.

Table II. Relative Contents (Transmittance) of IGF-IR and TβRII Protein

Group	IGF-IR	TβRII
Control	45.47 ± 8.03	75.97 ± 5.57
Epristeride (3 mg/kg)	53.29 ± 5.50[b]	67.98 ± 5.43
Epristeride (10 mg/kg)	57.81 ± 6.80[a]	61.48 ± 7.61[b]
Epristeride (30 mg/kg)	61.38 ± 5.75[a]	56.65 ± 6.64[a]

Note. Data were semiquantitatively assessed by microphotometer at 650 nm. Values were expressed as mean ± SD.
[a] P < 0.01 vs control group.
[b] P < 0.05 vs control group.

treated prostates compared with control prostates (Table III and Fig. 3, A and B). The specificity of hybridization was demonstrated by the abolition of hybridization when the tissue sections were hybridized with DIG-labeled sense IGF-1 probe.

The hybridization signal for TGFβ using specific oligonucleotide was confined to stromal cells, with little positive staining seen in epithelial cells. In the control group, stromal cell staining was weakly positive, whereas in epristeride-treated groups, especially in the 30 mg/kg group, strong positive staining was observed in stromal cells. TGFβ mRNA expression was increased in a dose-dependent manner in treated groups (Table III and Fig. 3, C and D). When the labeled sense probes for TGFβ were used in parallel tissue sections, the hybridization signals were abolished (data not shown).

Discussion

Until recently, surgery was the only effective treatment for BPH. Advances in hormone therapy have changed the urologist's approach to BPH. Epristeride has proved effective in suppressing serum and intraprostatic DHT concentrations to castrated levels, with subsequent reduction in prostate size (2). We demonstrate that epristeride administration was associated with a decrease in ventral prostate weight, a decrease in IGF expression, and an increase in TGFβ expression in ventral prostate. Epristeride treatment caused significant reduction in ventral prostate weight in a dose-dependent fashion. Our study demonstrated both a positive correlation between IGF-1 mRNA expression and prostate weight and a negative correlation between TGFβ mRNA expression and prostate weight. These effects of epristeride on gene expression in the prostate could be expected to result in a less favorable microenvironment for the proliferation of IGF- and TGFβ-responsive cells.

Currently there are several studies that provide evidence that IGFs are mitogenic and that their expression undergoes changes in proliferative prostatic disease effect on the prostate (6, 7). BPH is an extremely common disorder, affecting a large proportion of elderly men (16). The etiology of this condition remains unknown, but it appears to be related to local factors rather than to systemic hormonal changes. Mckeehan *et al.* (17) found that there were direct mitogenic effects of insulin, epidermal growth factor,

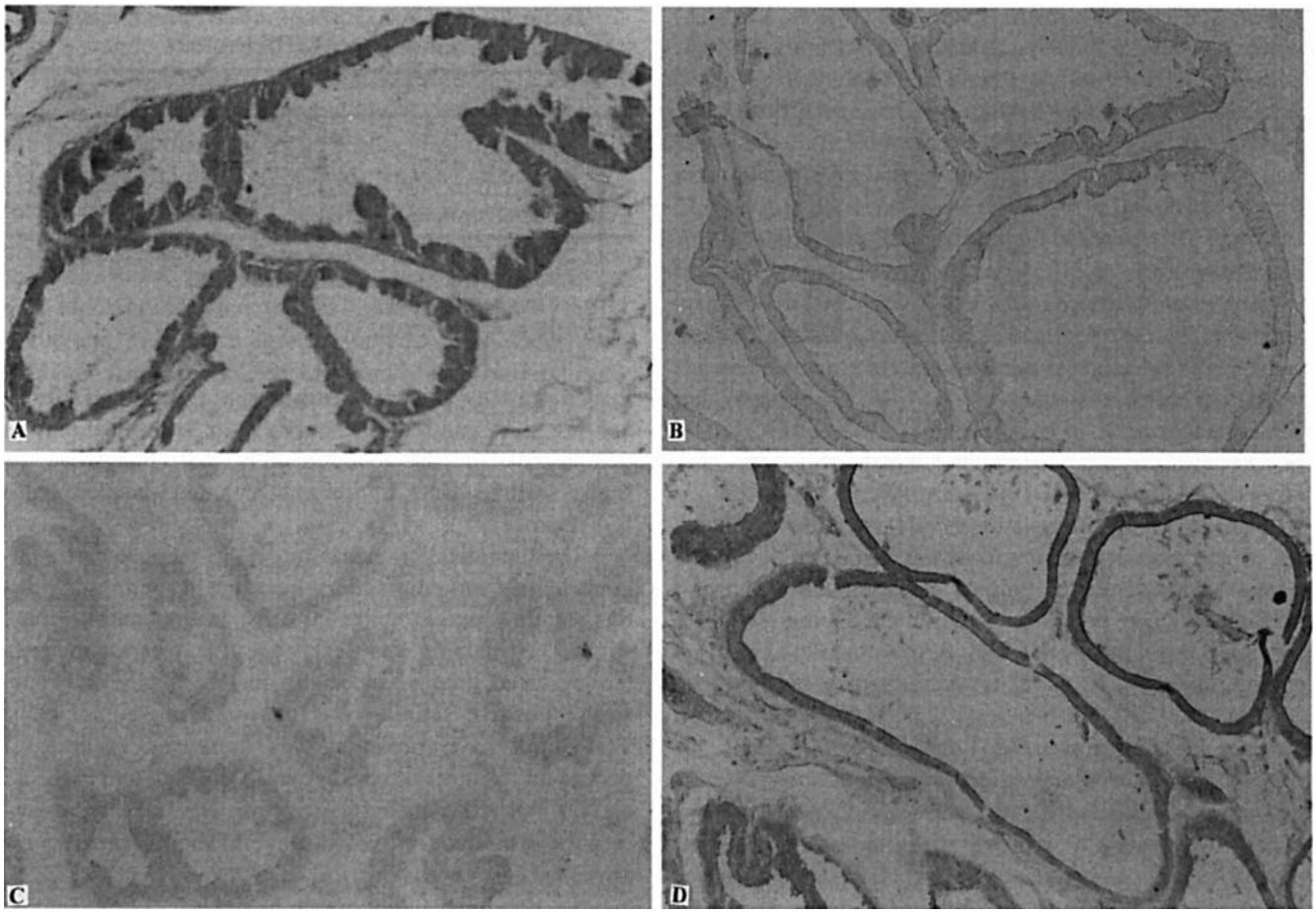

Figure 2. Immunohistochemical staining for IGF-1R and TβRII in sections of the rat prostate. The sections from representative paraffin-embedded tissue samples were deparaffinized, rehydrated, and preincubated with 1% H_2O_2 to abolish endogenous peroxidase activity. The slides were incubated with anti-IGF-1R or anti-TβRII, respectively, followed by ABC staining without counterstaining. Magnification ×33. (A) The prostatic epithelial cells stained for IGF-1R in the control group. (B) The prostatic epithelial cells stained for IGF-1R in 10 mg/kg epristeride-treated group. (C) The prostatic epithelial cells stained for TβRII in control group. (D) The prostatic epithelial cells stained for TβRII in 10 mg/kg epristeride-treated group.

Table III. Relative Contents (Transmittance) of IGF-I and TGFβ mRNA

Group	IGF-I	TGFβ
Control	34.31 ± 3.31	72.91 ± 8.79
Epristeride (3 mg/kg)	44.53 ± 5.26	65.25 ± 6.60
Epristeride (10 mg/kg)	52.06 ± 6.90[a]	59.26 ± 6.27[b]
Epristeride (30 mg/kg)	67.08 ± 7.55[a]	51.34 ± 5.09[a]

Note. Data were semiquantitatively assessed by microphotometer at 470 nm. Values were expressed as mean ± SD.
[a] $P < 0.01$ vs control group.
[b] $P < 0.05$ vs control group.

glucocorticoid, cholera toxin, unknown pituitary factors, and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free primary cell culture. Moreover, it has been reported that IGF-1 is a mitogenic factor for human prostate cells in culture, and its action is mediated through binding to type 1 IGF receptor (18, 7). In the human prostate, increasing information suggests that epithelial cells are the predominant target cells of IGF-1 action. In fact, *in vivo* IGF-1 receptors are localized in the epithelial cell (19). The fact that IGF-1 and IGF-2 could not be detected in conditioned medium from prostatic epithelial cells suggests that IGFs, which are produced in the stroma, act as paracrine growth factors in normal prostatic epithelium. This pattern of IGF expression appears to be unchanged in BPH tissue. Some authors found that in hyperplastic prostates, IGF-1 mRNA was localized exclusively in the stromal cell (20, 21). Epristeride, an inhibitor of type II 5α-reductase, is effective in BPH treatment by reducing prostate size with regressive changes in the glandular component (22). In this study we analyzed the expression levels of IGF-1 and its receptor IGF-1R in control testosterone-treated castrated rat, and we compared these with epristeride plus testosterone-treated castrated rats in an attempt to establish a link between the potential role of IGFs in the pathogenesis of BPH and the mechanism of action of epristeride. Our data demonstrated that IGF-1 mRNA was expressed strongly and to a large extent in castrated rat prostatic epithelium and stroma. In specimens from epristeride-treated rat prostate, this expression was markedly decreased and mainly detected in stromal cells.

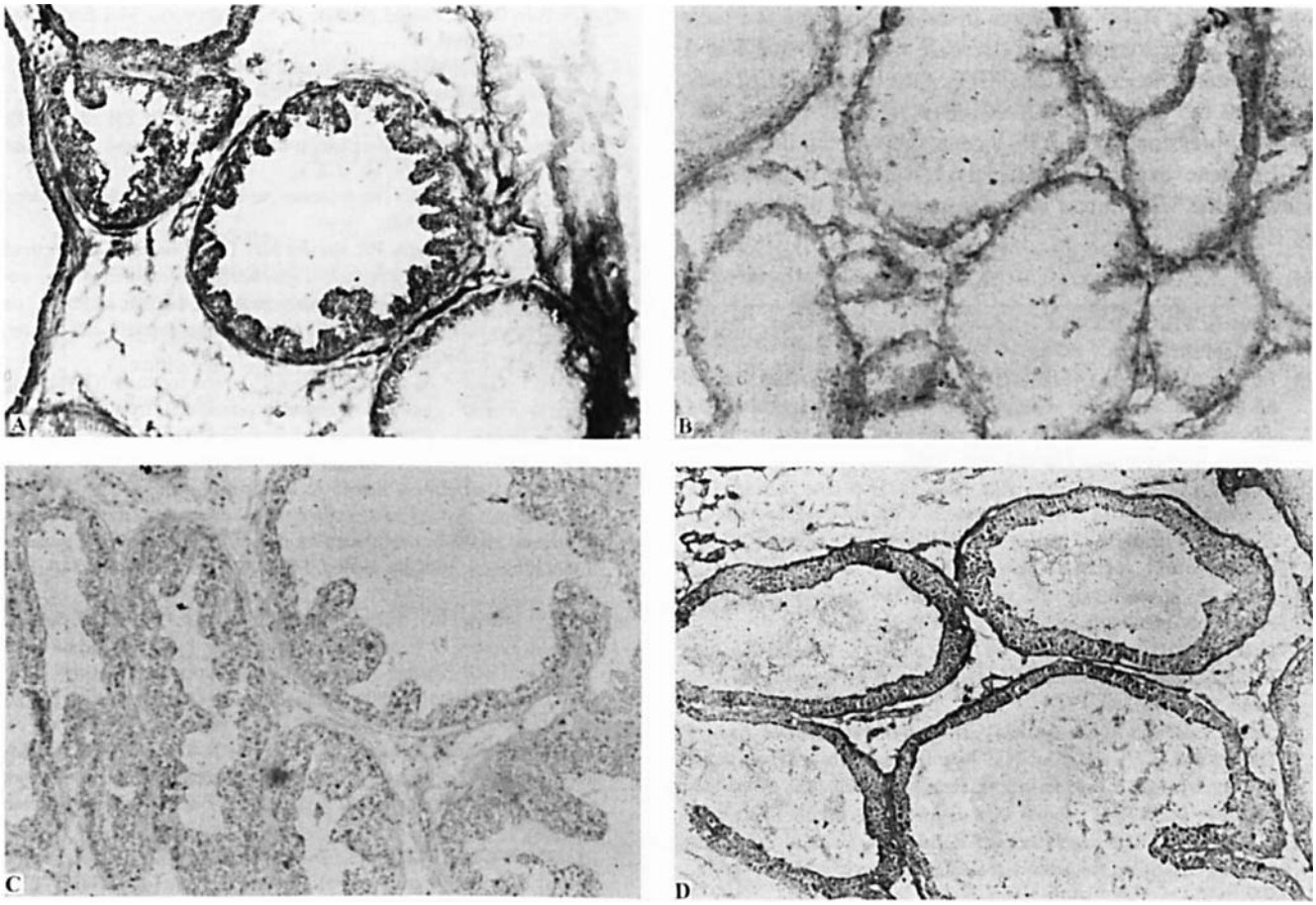

Figure 3. *In situ* hybridization of rat prostate tissue with DIG-labeled IGF-1 and DIG-labeled TGFβ1 antisense oligonucleotide probe. Magnification ×33. (A) Representative prostate tissue of control rats hybridized with the DIG-labeled IGF-1 antisense oligonucleotide probe. (B) Representative prostate tissue of rats treated with 10 mg/kg epristeride hybridized with the DIG-labeled IGF-1 antisense oligonucleotide probe. (C) Representative prostate tissue of control rats hybridized with the DIG-labeled TGFβ antisense oligonucleotide probe. (D) Representative prostate tissue of rats treated with 10 mg/kg epristeride hybridized with the DIG-labeled TGFβ antisense oligonucleotide probe.

Using immunohistochemistry, the IGF-1 receptor was shown to be basically located in the prostatic epithelial cells, with no immunoreactivity present in the stroma. As regards the semiquantitative analysis, significant differences were found between the specimens treated with epristeride and the untreated group. Moreover, IGF-1 mRNA expression was consistent with its receptor protein. These findings suggest that epristeride's therapeutic action in the treatment of BPH might be attributed to the suppression of IGF-1 autocrine/paracrine loops, which in turn reduce ventral prostate weight as described.

Apoptotic cell death has been proposed as a mechanism to explain involution in androgen-deprived prostate. In the rat, castration triggers apoptosis in epithelial and stromal cells throughout the ventral lobe (23, 24). The TGFβ signaling system plays an established role in apoptotic cell death in different transformed and nontransformed epithelial cell types (25, 26). In the prostate, TGF-β1 has been detected only in the secretory cells and in the basal cells by some authors (27), and also in the stroma by others (28). Undoubtedly, the TGFβ system is regulated by androgens. Castration increases the levels of TGF-β1 mRNAs, which return to normal after androgen replacement (10). TGFβ receptors are predominantly expressed in prostatic epithelial cells. The findings that the TGFβ receptor is under negative androgen control is also consistent with the proposed role of TGFβ as a growth-inhibitory factor in normal prostate. It had been documented that a 6- to 8-fold increase in TβRI and TβRII mRNA levels occurs shortly after castration in the rat ventral prostate (13). Sun *et al.* (22) had reported that epristeride could induce rat epithelial cell apoptosis *in vitro*. Our observation of a significant increase in TGFβ mRNA levels detected by *in situ* hybridization in those prostates treated with epristeride is at least a confirmation of this androgen regulation, and it points out a mediation of epristeride action by the TGFβ system. Immunohistochemistry results showed TGFβ receptor staining was strong and was mainly localized in epithelial cell treated with epristeride, whereas in untreated prostate, immunostaining was weak. It is possible that apoptosis induced by epristeride is due to up regulation of TGFβ. Our data demonstrate that epristeride might exert its action by altering the negative regulation by androgens on the TGFβ signaling system.

In summary, we have detected the effect of epristeride

on IGFs and TGFβ pathways in rat *in vivo*, and we have found that epristeride could decrease the expression IGF-1 and increase the expression TGFβ. Our data seems to indicate that the epristeride effect on BPH may be associated with suppression of the IGF-1 gene and with an increase in TGFβ gene expression. These findings are important in view of the widespread use of epristeride in the treatment of BPH.

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