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Developmental and Hormonal Regulation of Transforming Growth Factor-β1 (TGFβ1), -2, and -3 Gene Expression in Isolated Prostatic Epithelial and Stromal Cells: Epidermal Growth Factor and TGFβ Interactions*

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ABSTRACT

Growth factors are postulated to mediate stromal-epithelial interactions in the prostate to maintain normal tissue physiology. Transforming growth factor-β (TGFβ) has been shown to influence the prostate and probably mediate stromal-epithelial interactions. TGFβ1 messenger RNA (mRNA) expression is stimulated after castration and can be suppressed by *in vivo* treatment with androgens. The hypothesis tested is that TGFβ is regulated not only by androgen, but also by a network of locally produced growth factors that influence prostatic growth and differentiation. Epithelial and stromal cells from 20-day-old rat ventral prostate were isolated and used to test this hypothesis. The expression of mRNA for TGFβ1, -2, and -3 was analyzed by a quantitative RT-PCR procedure. Observations from this assay demonstrate that both epithelial and stromal cells express the mRNA for TGFβ1, -2, and -3. TGFβ1 mRNA expression was constant during development of the prostate. TGFβ2 mRNA expression was elevated at birth, then declined and elevated again at 100 days of age. TGFβ3 mRNA expression was high during puberty and young adult ages then declined at 100 days of age. TGFβ2 and TGFβ3 expression are inversely related during prostate development. After castration of 60-day-old rats, both TGFβ1 and TGFβ2 mRNA were enhanced. Interestingly, TGFβ3 mRNA was significantly suppressed after castration. Epidermal growth factor (EGF) stimulated TGFβ1 mRNA expression in stromal cells (6-fold increase), whereas keratinocyte growth factor stimulated TGFβ2 mRNA in epithelial cells. TGFβ inhibited both testosterone- and EGF-stimulated prostatic stromal and epithelial cell growth. EGF and TGFβ also inhibited prostatic ductal morphogenesis and growth in organ culture. Immunocytochemical localization of TGFβ in 20-day-old prostate demonstrated predominately stromal localization of the protein.

These results indicate that the isoforms of TGFβ2 and TGFβ3 are differentially regulated during prostate development, suggesting distinct regulatory mechanisms. Testosterone did not affect TGFβ expression in cultured prostatic cells. These observations suggest that the *in vivo* effects of castration on TGFβs are regulated indirectly through a complex network of growth factors, not simply by direct androgen depletion. The ability of EGF to inhibit prostatic ductal morphogenesis and growth in organ culture is postulated to be in part mediated by the increase in TGFβ1 expression. In summary, a network of growth factor-mediated stromal-epithelial interactions is needed to maintain prostate growth and development. TGFβ is postulated to have an important role in this process. (*Endocrinology* **139:** 1378–1388, 1998)

ANDROGENS are essential for the growth, development, and function of the prostate gland (1). The actions of androgen on the prostate appear to be in part mediated through stromal-epithelial interactions (2–4). Several growth factors that are produced by stromal cells in response to androgen can influence epithelial cells of the prostate (5). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), and insulin-like growth factors I and II can all stimulate growth of prostatic epithelial cells (6–8). In contrast, transforming growth factor-β (TGFβ) has been shown to have inhibitory effects on prostatic stromal cells and epithelial cells (6). Androgens have been shown to regulate growth factor gene expression in the prostate (9–12). In addition, growth factors influence growth factor expression. For example, TGFβ1 enhances bFGF expression in cultured human prostate stromal cells (13, 14). The hypothesis was developed that a network of locally produced growth factors is required for prostate growth and differentiation.

Three isoforms of TGFβ (TGFβ1, -2, and -3) have been identified in mammals, and their actions on cultured cells are similar in activity and potency (15). The biological actions of TGFβ are numerous and include control of cell proliferation, adhesion, and differentiation (15). TGFβs generally have growth inhibitory activity on epithelial, neuronal, and lymphoid cells. TGFβ activates the synthesis of extracellular matrix components (16). Recently, it was found that inhibition of cyclin-dependent kinases by TGFβ results in growth arrest of cells in the G1 phase of the cell cycle (17, 18). TGFβ has dramatic effects on the prostate (19, 20) through inhibiting the growth of both epithelial and stromal cells (13, 21, 22). These observations suggest that an alteration of TGFβ expression may cause an imbalance in growth regulation of stromal and epithelial cells in the prostate. For example, TGFβ may have a role in prostate cancer (23, 24). After ablation of androgen by castration, the expression of TGFβ1 in the prostate increases and can be suppressed by *in vivo* treatment with androgen (9, 25). This observation suggests

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that the expression of TGFβ may be regulated by androgen in normal prostate. TGFβ has been identified in stromal cells in rat ventral prostate and in an epithelial cell line derived from rat dorsal prostate (26, 27). Cultured human prostatic stromal and epithelial cells have been shown to express TGFβ1, -2, and -3 by RT-PCR (28). The developmental and hormonal regulation of TGFβ1, -2, and -3 has not been thoroughly investigated.

The current study investigates the development and hormonal regulation of TGFβ1, -2, and -3 expression. Isolated epithelial cells and stromal cells from rat ventral prostate were used in the *in vitro* experiments to investigate the actions of testosterone and growth factors.

Materials and Methods

Animals

Male Sprague-Dawley rats were purchased from Bantin-Kingman (Kent, WA). Animals of a variety of ages, including 1-, 20-, 60-, and 100-day-old rats, were used for the study of prostate development. Some of the 60-day-old rats were castrated under avertin anesthesia, which consists of 25 g tribromoethanol in 15.5 ml tertiary amyl alcohol. The prostate glands were removed 3 days after castration. Culture of both prostatic epithelial and stromal cells used 20-day-old rat prostates. Organ culture of ventral prostates used 0-day-old rat prostates. All animal procedures were approved by the university animal care committee.

Culture of ventral prostate cells

The isolated cell cultures used 20-day-old Sprague-Dawley rats; these animals were killed, and ventral prostates were removed. Tissue was incubated with Hanks' Balanced Salt Solution (Life Technologies, Gaithersburg, MD) containing 675 U collagenase activity/ml type II collagenase (Sigma Chemical Co., St. Louis, MO) and 0.04% deoxyribonuclease I (Sigma) at 37 C for 4 h. After incubation, tissue was separated by centrifugation. The mixture was spun at 30 × *g* for 4 min to pellet the epithelial cells. The remaining supernatant was centrifuged at 190 × *g* for 6 min to pellet the stromal cells. Both pellets were resuspended and spun at 30 × *g* for 4 min. The supernatant from stromal cells was removed and repelleted at 190 × *g* for 6 min. These were then subsequently placed in 10% bovine calf serum F-12 medium in six-well culture plates (Nunclon, Roskilde, Denmark) at 3×10^5 cells/well. After 24 h, the cells were washed in serum-free F-12 medium and incubated for 24 h. On day 3 of culture, the cells were maintained in serum-free conditions and incubated with or without the following treatments: from 10^{-6}-10^{-9} M testosterone (Sigma), 100 ng/ml EGF (Life Technologies), 25 ng/ml KGF (Life Technologies), 10 ng/ml human recombinant TGFβ1 (Life Technologies), 10% bovine calf serum (HyClone, Logan, UT), and a combination of TGFβ1 and testosterone (10^{-7} M), EGF, or 10% bovine calf serum. The treatments were administered for a period of 72 h. On the sixth day of culture, the medium was removed and stored for measurement of acid phosphatase at −20 C, and cells were harvested for RNA extraction.

Cell purity assay and immunocytochemistry

The purity of ventral prostate stromal and epithelial cells in culture was analyzed by immunohistochemical methods. Isolated stromal or epithelial cells were plated in four-well culture plates at 3×10^5 cells/well on Thermonox coverslips (Nunc, Inc., Naperville, IL). Two days postplating, cells were fixed in 100% methanol for 5 min, then washed in decreasing ethanol conditions and equilibrated in Tris-buffered saline (TBS; pH 7.4). Before antibody staining, the cells were treated with H_2O_2 to quench endogenous peroxidases and then incubated in TBS containing 1% BSA and 0.5% normal serum for 1 h at 20 C. After rinsing three times in TBS, cells were incubated for 2 h at 37 C with either rabbit polyclonal antikeratin (Dako Corp., Carpenteria, CA) or a monoclonal antivimentin (Sigma) antibody at dilutions of 1:250 and 1:300, respectively. Negative controls had no primary antiserum added, and positive controls used epithelial (MCF-10A, human breast) and stromal (SS-140, human fibroblast) cell lines. After primary antibody incubations, the cells were rinsed three times in TBS and incubated with either goat antirabbit or antimouse IgG horseradish peroxidase-conjugated secondary antibody (Sigma). Visualization was achieved using 50 mM Tris (pH 7.6) containing 0.6% diaminobenzine and 0.03% H_2O_2 for 10 min at 20 C. The cells were then counterstained in hematoxylin and mounted on slides using an aqueous mounting solution. Stained cells were counted in four separate areas of the slide using cells from five different experiments.

Ventral prostate sections were obtained from 20-day-old Sprague-Dawley rats. Tissue specimens were immediately fixed in Bouin's solution for 3–6 h. Tissue was cut into 5-μm thick sections, deparaffinized, rehydrated, and treated with an aqueous solution of 3.0% H_2O_2 to quench endogenous peroxidase. After several washes in TBS buffer, sections were incubated in TBS containing 1% BSA for 2 h at 37 C to reduce nonspecific staining. After rinsing three times in TBS, a sheep antirat pan TGFβ antibody (East Acres Biological, South Bridge, MA) was added at a 1:500 dilution and incubated overnight at 37 C followed by 2 h at 4 C. For controls, a nonimmune sheep IgG was used at a similar dilution. After three rinses in TBS, the primary antibody was detected with antisheep biotinylated secondary antibody, and the biotin was detected with an avidin-biotin-peroxidase kit (ABC-Elite, Vector Laboratories, Burlingame, CA). Diaminobenzidine tetrachloride was used as a chromagen, and serial sections were lightly counterstained with hematoxylin.

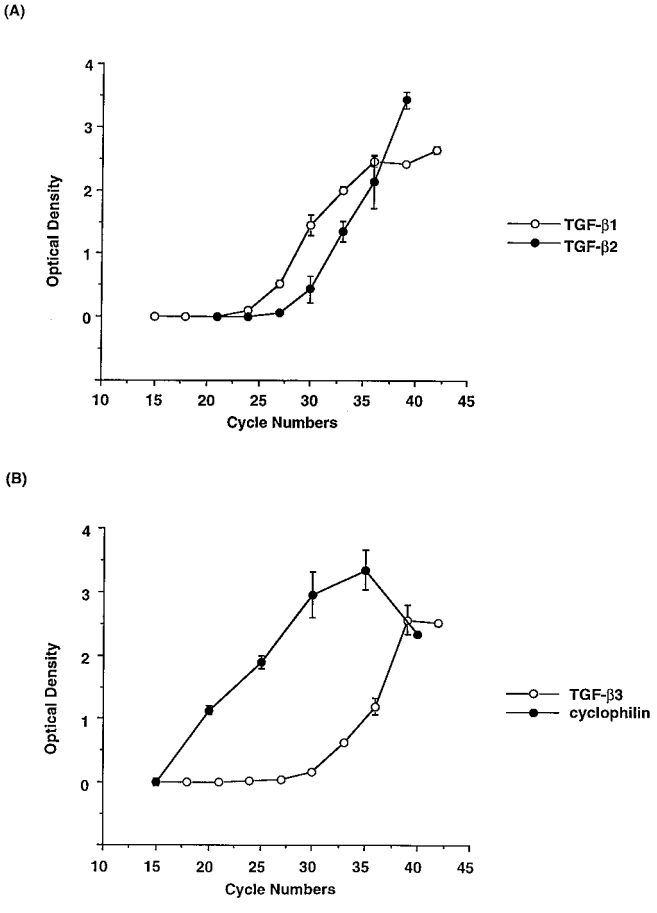

FIG. 1. Relationship between cycle numbers and optimal density as PCR products of TGFβ1, -β2, and -β3 and cyclophilin. Values are expressed as the mean ± SEM (n = 3). Linearity between cycle numbers and PCR products are shown in each growth factor as follows: TGFβ1; 25–35; TGFβ2; 27–40; TGFβ3; 30–40; and cyclophilin; 15–30.

RNA preparation

Total RNA was obtained using Tri Reagent (Sigma). Briefly, tissue or cells were lysed in Tri Reagent (1 ml/50–100 mg tissue or 1 ml/10 cm^2 of culture plate). After adding 0.2 ml chloroform/ml Tri Reagent, the mixture was centrifuged at 12,000 × *g* for 15 min at 4 C, the colorless upper aqueous phase was transferred to a fresh tube, and 0.5 ml isopropanol/ml Tri Reagent was added to pellet the RNA. The mixture was centrifuged at 12,000 × *g* for 10 min at 4 C. The RNA pellet was washed with 75% ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated H_2O. RNA was stored at −80 C until analysis.

RT

Complementary DNA (cDNA) was synthesized in a total 20-μl volume containing 1 μg total RNA, 0.05 μM of specific 3′-primers (TGFβ1, 5′-GGG GTG GCC ATG AGG AGC AGG-3′; TGFβ2, 5′-GCG CTG GGT GGG AGA TGT TAA-3′; TGFβ3, 5′-CCT TTG AAT TTG ATC TCC A-3′; cyclophilin, 5′-ATT TGC CAT GGA CAA GAT GCC-3′), 4 μl 5 × first strand PCR buffer (Life Technologies), 10 mM dithiothreitol (Life Technologies), 0.125 mM deoxy-NTPs, 100 U Moloney murine leukemia virus transcriptase (Life Technologies), 20 U ribonuclease inhibitor (Promega, Madison, WI), and DEPC-H_2O. Initially, RNA was denatured and annealed in the presence of each primer and DEPC-H_2O at 65 C for 15 min. The tube was placed on ice for 5 min. The other reagents were added to the tube and incubated at 42 C for 2 h. To decrease the high background signal, a higher RT reaction temperature (42 C) was used (29). The mixture was incubated at 95 C for 5 min to inactivate Moloney murine leukemia virus transcriptase. The mixture was diluted by UV-treated H_2O containing Bluescript plasmid DNA (Stratagene, La Jolla, CA) as carrier DNA. The final concentrations of cDNA and Bluescript plasmid were 1 ng/μl and 10 ng/μl, respectively. This concentration of Bluescript plasmid was included in all samples and standards.

Quantitative PCR assay

As a standard for the assay, PCR products of TGFβ1, -2, and -3 and cyclophilin amplified by each specific primer were subcloned into Blue-

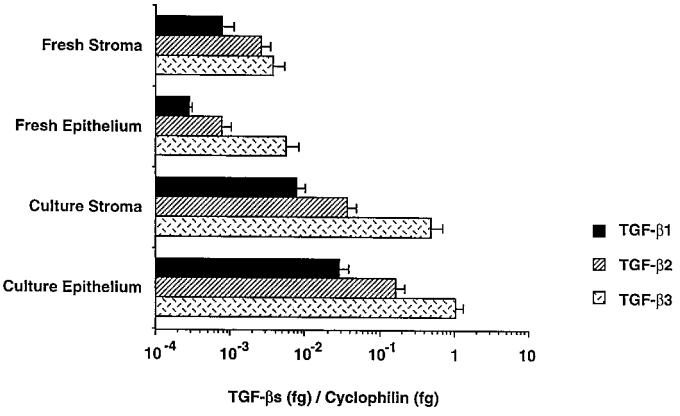

FIG. 3. Expression of mRNA of TGFβ1, -β2, and -β3 in both epithelial cells and stromal cells. Fresh cells that are separated into epithelium and stroma from 20-day-old rat ventral prostate and cells after 6-day culture are analyzed. Values are normalized with cyclophilin and are expressed as the mean ± SEM (n = 4).

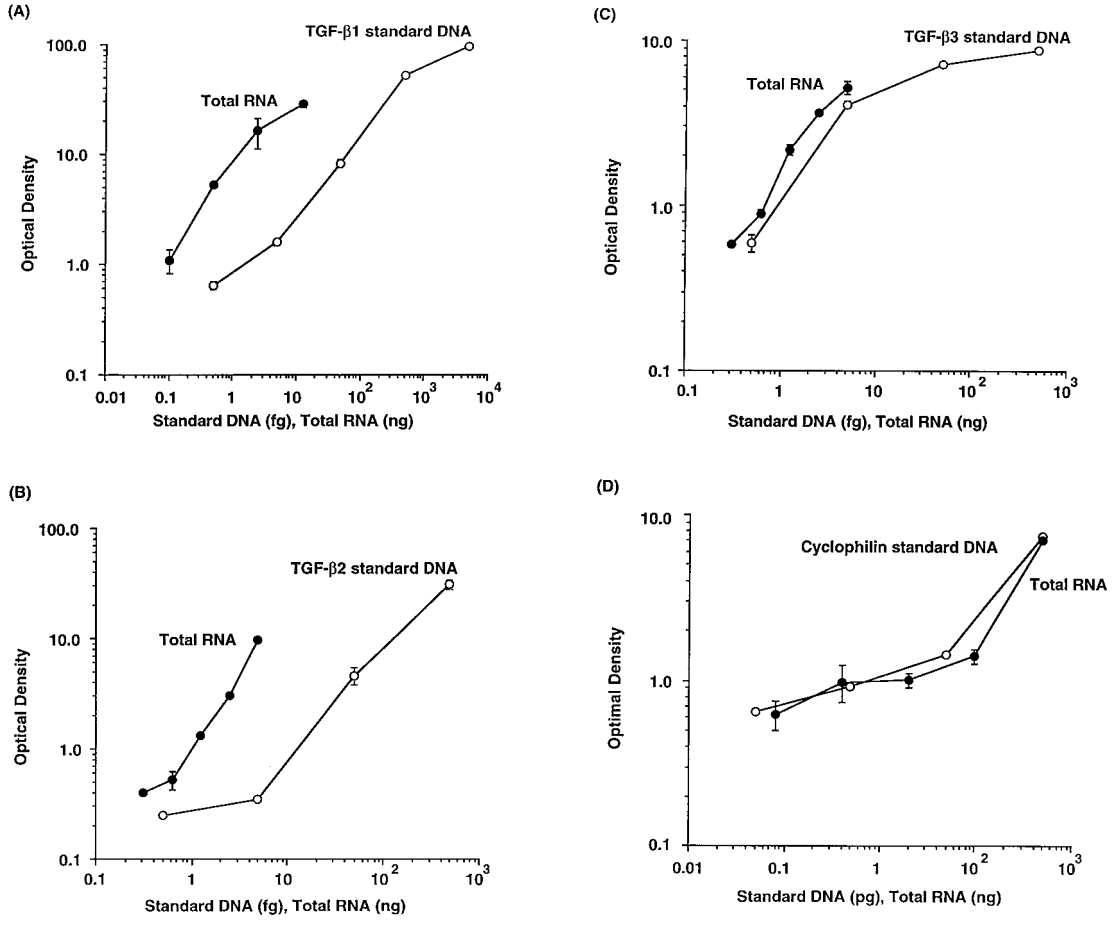

FIG. 2. Diluted curve of both plasmid DNA (standard) and total RNA in TGFβ1, -β2, and -β3 and cyclophilin. Values are expressed as the mean ± SEM (n = 2). Parallel relationships in PCR products between plasmid DNA and total RNA are shown.

script plasmid (Stratagene). Each subclone was sequenced in both directions and confirmed to be rat TGFβ1, -2, and -3 and cyclophilin. The size and base pair alignment of the PCR product generated were as follows: 200 bp size from 1003–1203 bp alignment on the coding sequence (accession no. 52498; TGFβ1), 194 bp size from 355–549 bp alignment on the coding sequence (TGFβ2), 288 bp size from 865-1153 bp alignment on the coding sequence (accession no. 403491; TGFβ3), and 105 bp size from 244–348 bp alignment on the coding sequence (accession no. M19533; cyclophilin). Plasmid DNA containing TGFβ subclones was used to generate standard curves ranging from 0.1 fg/μl to 1.0 pg/μl, and for cyclophilin from 10 fg/μl to 100 pg/μl. PCR was performed on a Perkin-Elmer GeneAmp PCR System 9600 (Perkin-Elmer, Branchburg, NJ) and was carried out in a total 25-μl reaction volume containing 5 μl plasmid DNA or RT reaction, 0.4 μM 3′-primer as shown above, 0.4 μM 5′-primer (TGFβ1, 5′-TCG ATT TTG ACG TCA CTG GAG TTG T-3′; TGFβ2, 5′-CCG CCC ACT TTC TAC AGA CCC-3′; TGFβ3, 5′-TGC CCA ACC CGA GCT CTA AGC G-3′; cyclophilin, 5′-ACA CGC CAT AAT GGC ACT GG-3′), 2.5 μl 10 × GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$; Perkin-Elmer), 25 μM deoxy-NTPs, 0.5 U *Taq* DNA polymerase (Perkin-Elmer), and 1 μCi [α-^{32}P]deoxy-CTP (Amersham Life Science, Arlington Heights, IL).

The TGFβ1 reaction cycle sequence comprised 5 min at 95 C, followed by 30 cycles of 1 min at 95 C, 1 min at 60 C, and 2 min at 72 C. The cyclophilin reaction cycle sequence comprised 5 min at 95%, followed by 25 cycles of 1 min at 95 C, 1 min at 60 C, and 2 min at 72 C. The TGFβ2 reaction cycle sequence comprised 5 min at 95 C, followed by 33 cycles of 1 min at 95 C, 2 min at 60 C, and 3 min at 72 C. The TGFβ3 reaction cycle sequence comprised 5 min at 95 C, followed by 35 cycles of 1 min at 95 C, 2 min at 55 C, and 3 min at 72 C. All reactions had a final extension for 10 min at 72 C.

After PCR, the products were electrophoretically separated on a 6% acrylamide gel. The gel was then dried and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) quantitatively. Each gene was assayed in separate PCR reactions from the same RT samples.

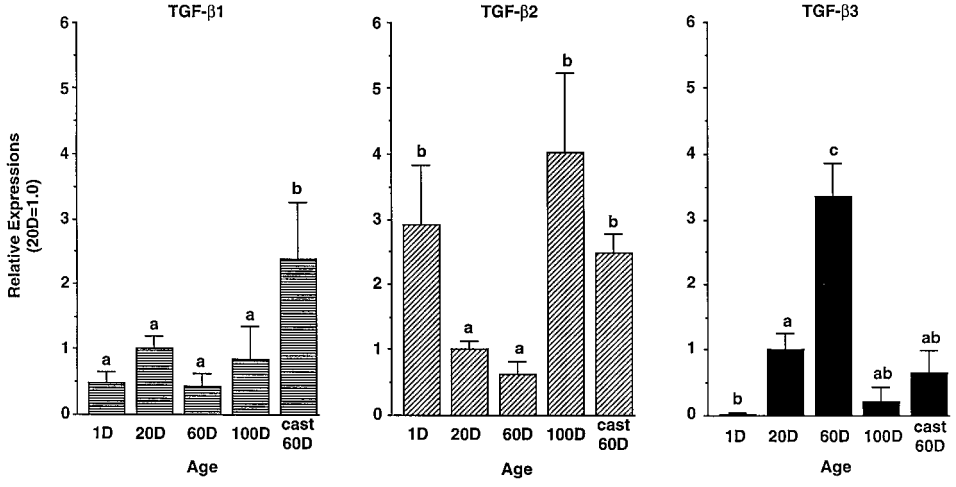

FIG. 4. Changes in the relative expression of TGFβ1, -β2, and -β3 during development of rat ventral prostate. Values are normalized with cyclophilin and compared with those of 20-day-old rats as relative expression and presented as the mean ± SEM (n = 3–5). Statistical difference is indicated by different letter superscripts ($P < 0.05$).

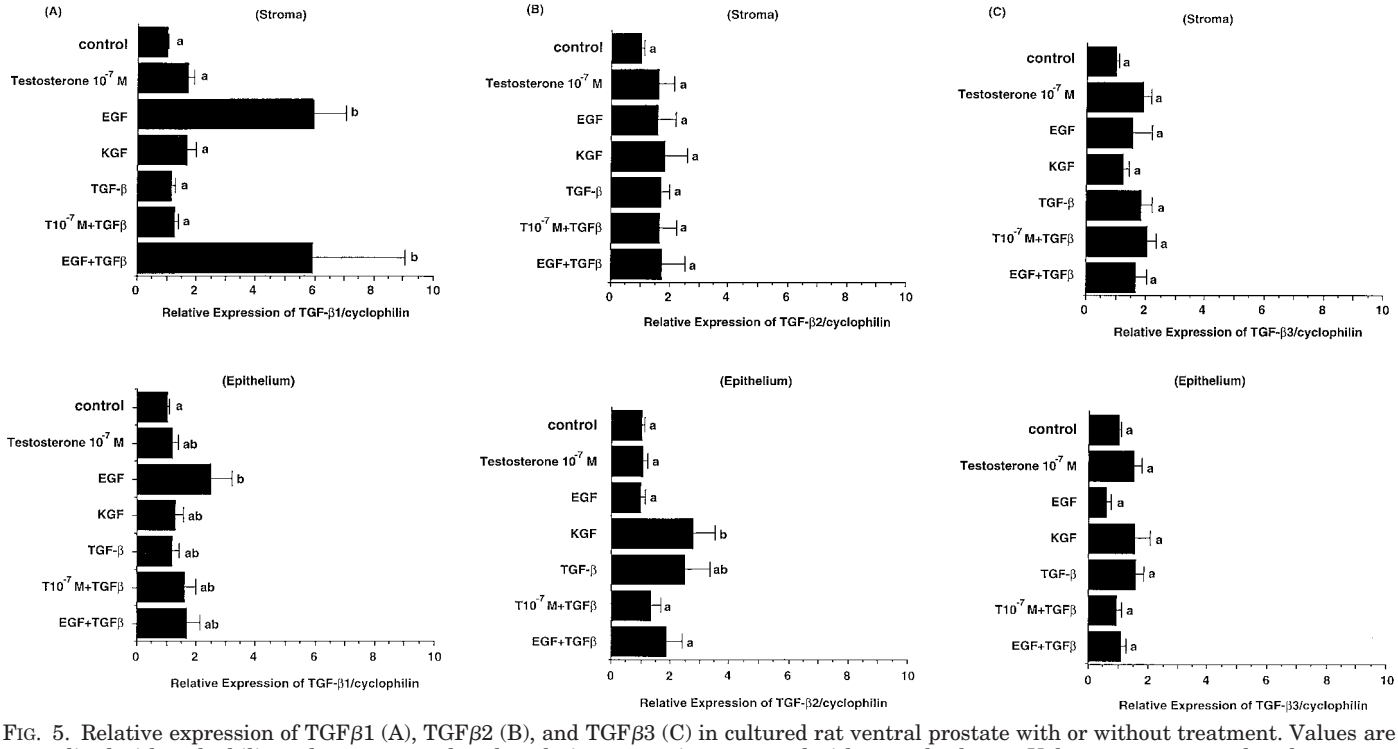

FIG. 5. Relative expression of TGFβ1 (A), TGFβ2 (B), and TGFβ3 (C) in cultured rat ventral prostate with or without treatment. Values are normalized with cyclophilin and are presented as the relative expression compared with control cultures. Values were expressed as the mean ± SEM (n = 4–12). Statistical difference is indicated by different letter superscripts ($P < 0.05$).

Equivalent steady state messenger RNA (mRNA) levels for each gene were determined by comparing each sample to the appropriate standard curve. TGFβ1, -2, and -3 were normalized for cyclophilin. For each assay, all samples were simultaneously measured in duplicate with intraassay variabilities of 7.5% (TGFβ1), 9.4% (TGFβ2), 9.9% (TGFβ3), and 9.2% (cyclophilin).

Cell growth assay

Cell growth was analyzed by examining [^{3}H]thymidine incorporation into newly synthesized DNA. Stromal and epithelial cells were placed at subconfluent densities (<1 million cells/cm^2) in 0.5 ml DMEM containing 0.1% calf serum. The low serum level does not stimulate growth and is required for progression factors (*i.e.* insulin-like growth factor) for the S phase of the cell cycle to detect the growth factor response. After 48–72 h of culture, the cells were treated with various agents for 24 h. After the 24-h treatment, 0.5 ml DMEM containing 2 μCi [^{3}H]thymidine was added to each well, and the cells were incubated for 4 h at 37 C before sonication. The quantity of [^{3}H]thymidine incorporated into DNA was determined as previously described (30). Data were normalized to total DNA per well using an ethidium bromide procedure, described previously (31). Under these subconfluent culture conditions, approximately 0.5–1.5 μg DNA were detected per well. [^{3}H]Thymidine incorporation was generally greater than 2×10^3 cpm/μg DNA.

Organ culture of rat ventral prostate

Ventral prostates were removed from 0-day-old rats and cultured in a drop of medium on a Millicell CM filter (Millipore, Bedford, MA) floating on the surface of 0.5–1.0 ml CMRL 1066 medium (Life Technologies, Gaithersburg, MD) supplemented with penicillin-streptomycin, insulin (10 μg/ml), and transferrin (10 μg/ml) with or without treatments. The cultures were performed in a four-well Nunclon surface dish (32). These plates were placed in the incubator at 37 C in an atmosphere of 5% CO_2 and 95% air. The tissues were cultured for 6 days. Treatments were testosterone (Sigma; 10^{-7} M), EGF (Life Technologies; 100 ng/ml), TGFβ1 (Life Technologies; 10 ng/ml), and a combination of testosterone and EGF or a combination of testosterone and TGFβ1. Images of the prostates were captured with an image analysis system (Pixera, Pixera Corp., Los Gatos, CA). At the end of the culture, the organs were fixed for histology. The analysis and quantitation of ductal branching morphogenesis were previously described (32).

Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using the Dunnett's test for comparison to controls and using the Tukey-Kramer honestly significant difference test for multiple comparisons. Statistical difference was confirmed at $P < 0.05$.

Results

Characterization of the quantitative RT-PCR (QRT-PCR) for TGFβ1, -2, and -3 and cyclophilin

Preliminary studies were performed to establish a reproducible and accurate QRT-PCR procedure. The linearity of generating a PCR product relative to cycle number was determined (33). The PCR product for TGFβ1 increased at 25 cycles and reached a plateau at 36 cycles. Thirty cycles was selected to be an appropriate cycle number for QRT-PCR (Fig. 1A). The TGFβ2 PCR product continued to be amplified at 42 cycles. For TGFβ2, 33 cycles was midpoint on the linear amplification of the PCR product and was selected for QRT-PCR (Fig. 1A). TGFβ3 product started to increase after 30 cycles and reached a plateau level at 40. Thirty-five cycles was determined appropriate to use for TGFβ3 (Fig. 1B). For cyclophilin, 25 cycles was determined to be the appropriate cycle number (Fig. 1B).

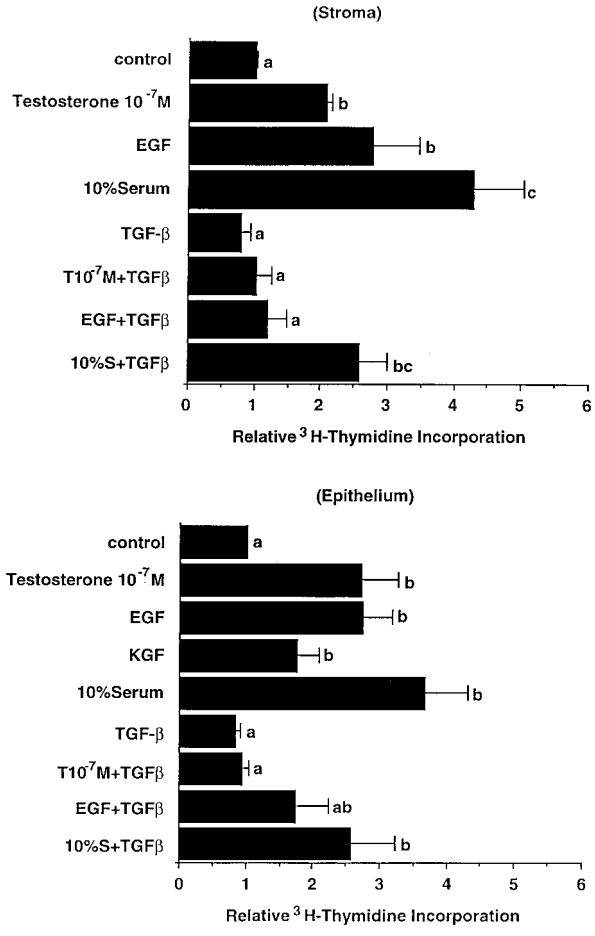

FIG. 6. The [^{3}H]thymidine incorporation growth assay on cultured rat ventral prostate cells. Values are expressed as the mean ± SEM (n = 3; control = 1). Statistical difference is indicated by different letter superscripts ($P < 0.05$).

Another aspect of the QRT-PCR procedure involves a comparison of standard DNA with unknown samples for parallel displacement. Plasmid DNA with subcloned PCR product and cDNA produced from unknown RNA samples were run in parallel. PCR products of diluted plasmid DNA and diluted unknown cDNA were compared. These two curves were parallel with each growth factor, indicating that the QRT-PCR assay could be used to quantitate mRNA levels (Fig. 2, A–D). The small error shown in each of the data points demonstrates minimal variability in the assay (Figs. 1 and 2).

Comparison of growth factor expression in fresh and cultured stromal and epithelial cells

Analysis of TGFβ1, -2, and -3 mRNA expression in both stromal and epithelial cells of rat ventral prostate was determined. The differences in the expression pattern of these products between freshly isolated ventral prostate cells and cultured cells was investigated. In freshly isolated cells from 20-day-old rat prostate, TGFβ expression normalized by cyclophilin ranged from $0.3–6 \times 10^{-3}$ fg TGFβ/fg cyclophilin (Fig. 3). Expression of TGFβ1 and -2 was higher in stromal

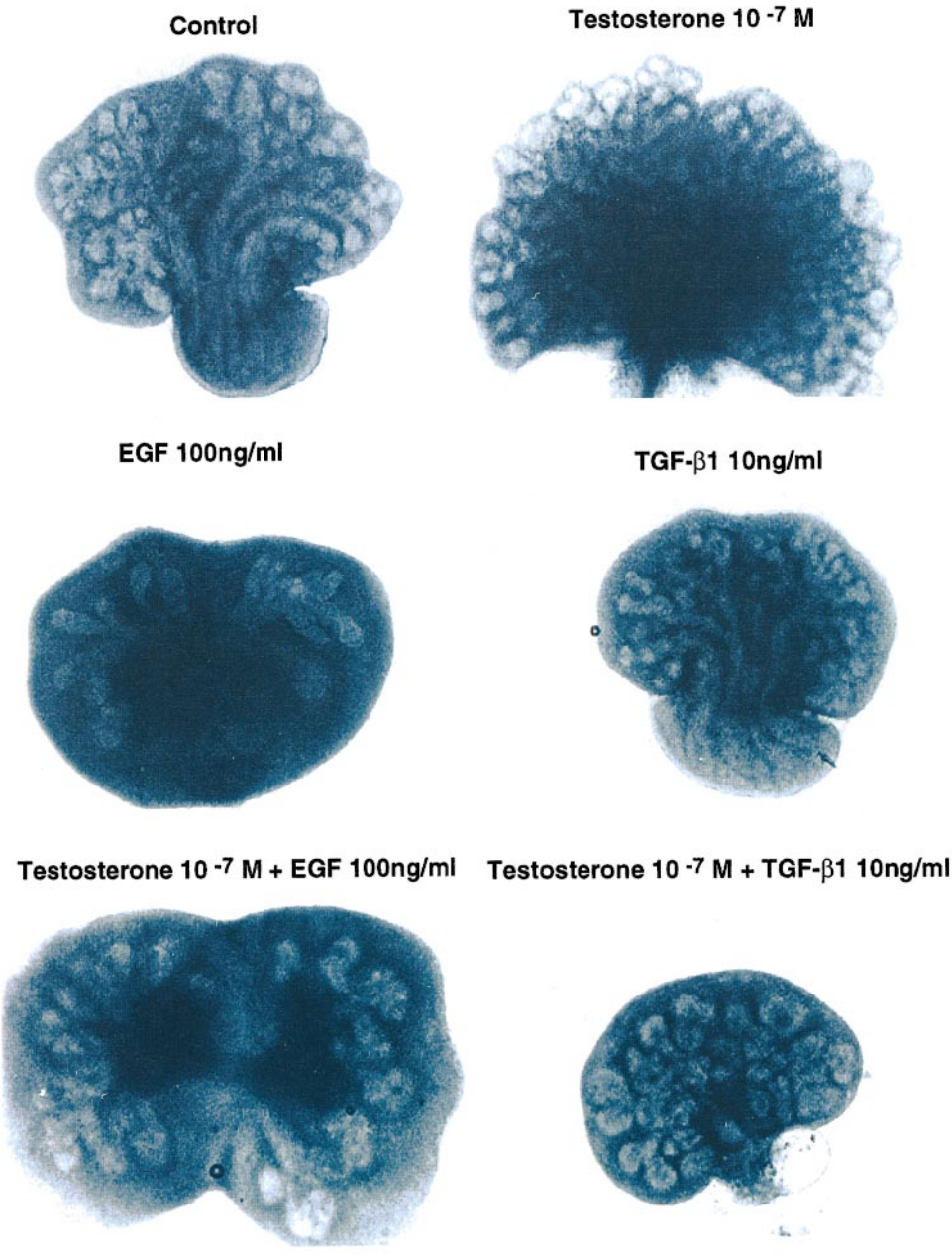

FIG. 7. Ventral prostate after 6 days of treatment with testosterone (10^{-7} M), EGF (100 ng/ml), TGFβ1 (10 ng/ml), or their combination in organ culture. These ventral prostates were removed from 0-day-old Sprague-Dawley rats. Data are representative of three different experiments performedin replicate.

cells than in epithelial cells. TGFβ3 expression in epithelial cells was the same as that in stromal cells. In cultured samples derived from 20-day-old rat ventral prostate, TGFβ1, -2, and -3 showed higher expression than fresh samples and ranged from 7–700 $\times$ 10^{-3} fg TGFβ/fg cyclophilin. Contrary to that in fresh samples, expression in epithelial cells in cultured samples was higher than that in stromal cells. In both epithelial and stromal cells, TGFβ1 had the lowest level of expression, and TGFβ3 had the highest level of expression (Fig. 3).

Developmental regulation of TGFβ expression in rat prostate

To investigate the changes in TGFβ1, -2, and -3 expression during rat development from the neonatal period (1-day-old) to adulthood (100-day-old), ventral prostates were removed from rats aged 1, 20, 60, and 100 days, and the mRNA expression of TGFβ was measured by QRT-PCR. The effects of androgen depletion on TGFβ1, -2, and -3 expression were analyzed by castration of 60-day-old rats. The expression of TGFβ isoforms was normalized by cyclophilin expression, and relative expression was compared with that in 20-day-old rats. TGFβ1 expression did not vary during development (Fig. 4). After castration of 60-day-old rats, TGFβ1 significantly increased to 5-fold that in 60-day-old rats. During the neonatal period, TGFβ2 expression was elevated, then decreased in pubertal and early adult periods (60 days of age) and was elevated in adult rats (100 days old). After castration, TGFβ2 was increased 4-fold, but remained lower than that in 100-day-old rats. In contrast to TGFβ2, TGFβ3 ex-

pression was very low in the 1- and 100-day-old prostates and was elevated during the pubertal and early adult periods (60 days of age). After castration, TGFβ3 expression was significantly suppressed from that in 60-day-old rats. Interestingly, TGFβ3 expression was inversely related to TGFβ2 expression (Fig. 4).

Hormonal regulation of TGFβ expression in cultured prostate cells

The effects of testosterone and growth factors on TGFβ1, -2, and -3 expression were investigated in stromal and epithelial cells derived from ventral prostate of 20-day-old rats. EGF and KGF were used as stimulatory growth factors, and human recombinant TGFβ1 was used as an inhibitory growth factor. TGFβ1 was combined with testosterone or EGF to study potential suppressive effects. All values were normalized with cyclophilin and expressed as relative expression compared with that in control cultures.

Analysis of cell purity indicated that isolated epithelial cells showed 93.2 ± 1.2% (mean ± SEM; n = 5) keratin-positive cells and less than 3% vimentin-positive cells. Isolated stromal cells showed 86.0 ± 3.5% (mean ± SEM; n = 5) vimentin-positive and less than 5% keratin-positive cells. These results demonstrate that cell purity in both isolated stromal and epithelial cells were appropriate for data interpretation.

EGF significantly stimulated (6-fold) TGFβ1 expression in stromal cells (Fig. 5A) and significantly stimulated the expression of TGFβ1 in epithelial cells at a reduced level (2.5-fold) compared to that in stromal cells. This effect was not significantly different compared with that of other treatments, but was different from the control. TGFβ1 had no effect on the expression of TGFβ1 in either stromal or epithelial cells. The effects of EGF on TGFβ1 expression were not influenced by exogenous administration of TGFβ1 in either cell type. Neither testosterone nor KGF had any effect on TGFβ1 expression in either cell type (Fig. 5A).

In stromal cells, the expression of TGFβ2 was elevated to approximately 1.5-fold by each treatment; however, these effects were not statistically significant (Fig. 5B). In epithelial cells, only KGF significantly stimulated (2.8-fold) TGFβ2 expression. TGFβ1 alone did stimulate TGFβ2 expression, but this stimulation was not statistically different from that in control cultures (Fig. 5B). Results indicate that prostate stromal cells were not influenced by any treatment, and epithelial cells were only influenced by KGF with regard to TGFβ2 expression.

No treatment affected TGFβ3 expression in either stromal or epithelial cells (Fig. 5C). Although small effects were observed with testosterone and TGFβ1, these were not statistically different from those in control cultures.

Combined observations indicate that EGF regulates TGFβ1 expression by stromal and epithelial cells, KGF regulates TGFβ2 expression by epithelial cells, and none of the treatments used in the current study influences TGFβ3 expression. Testosterone had no effect on TGFβ1, TGFβ2, or TGFβ3 in either cell type.

Regulation of prostate cell growth

Stromal and epithelial cells from rat ventral prostates were cultured to examine the effects of testosterone, EGF, KGF, and TGFβ1 on cell growth. A tritiated thymidine incorporation assay was performed using 10% bovine calf serum as a positive control. Calf serum significantly stimulated (~4-fold) DNA synthesis in both stromal and epithelial cells (Fig. 6). Testosterone and EGF stimulated (2.5- to 3-fold) DNA synthesis in stromal and epithelial cells. KGF had a stimulatory effect on epithelial cells. TGFβ had no direct effect on DNA synthesis alone (Fig. 6). However, TGFβ significantly inhibited the growth effects of testosterone and EGF on stromal cells as well as the growth effects of testosterone on epithelial cells. These results indicate that testosterone and EGF can stimulate the growth of both stromal cells and epithelial cells, whereas TGFβ can inhibit these actions and suppress growth. Although the growth response for testosterone was observed in less than 24 h, potential indirect effects of testosterone mediated by other peptide growth factors remain to be elucidated.

Regulation of ductal branching morphogenesis

To address the potential physiological function of TGFβ, an organ culture of 0-day-old rat prostate was used. Prostate organ cultures can be used to analyze the effects of growth factors on the whole organ, in which normal cell to cell interactions are intact (32). Testosterone (10^{-7} M) treatment of prostate organ cultures promoted significant branching morphogenesis compared with that in control cultures (Fig. 7). TGFβ1 alone caused a slight reduction in the number of branching ducts, but the number was similar to that in control cultures. However, TGFβ1 completely inhibited the stimulatory effect of testosterone on prostatic ductal branching morphogenesis (Figs. 7 and 8). EGF increased prostatic size; however, ductal branching was significantly reduced (Figs. 7 and 8), and ducts were swollen, with an increase in surrounding stromal layers (Fig. 7). The histology of these organ cultures is shown in Fig. 9 and demonstrates that EGF does increase the stromal/epithelial cell ratio as well as shows the

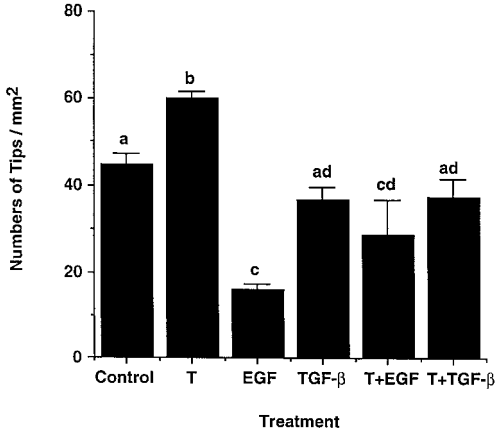

FIG. 8. Number of branching ducts in 0-day-old rat ventral prostate organ cultures, presented as the number of tips per mm^2. Data were obtained from images similar to those represented in Fig. 7. Data are expressed as the mean ± SEM (n = 3). Different superscipt letters indicate a statistical difference ($P < 0.05$).

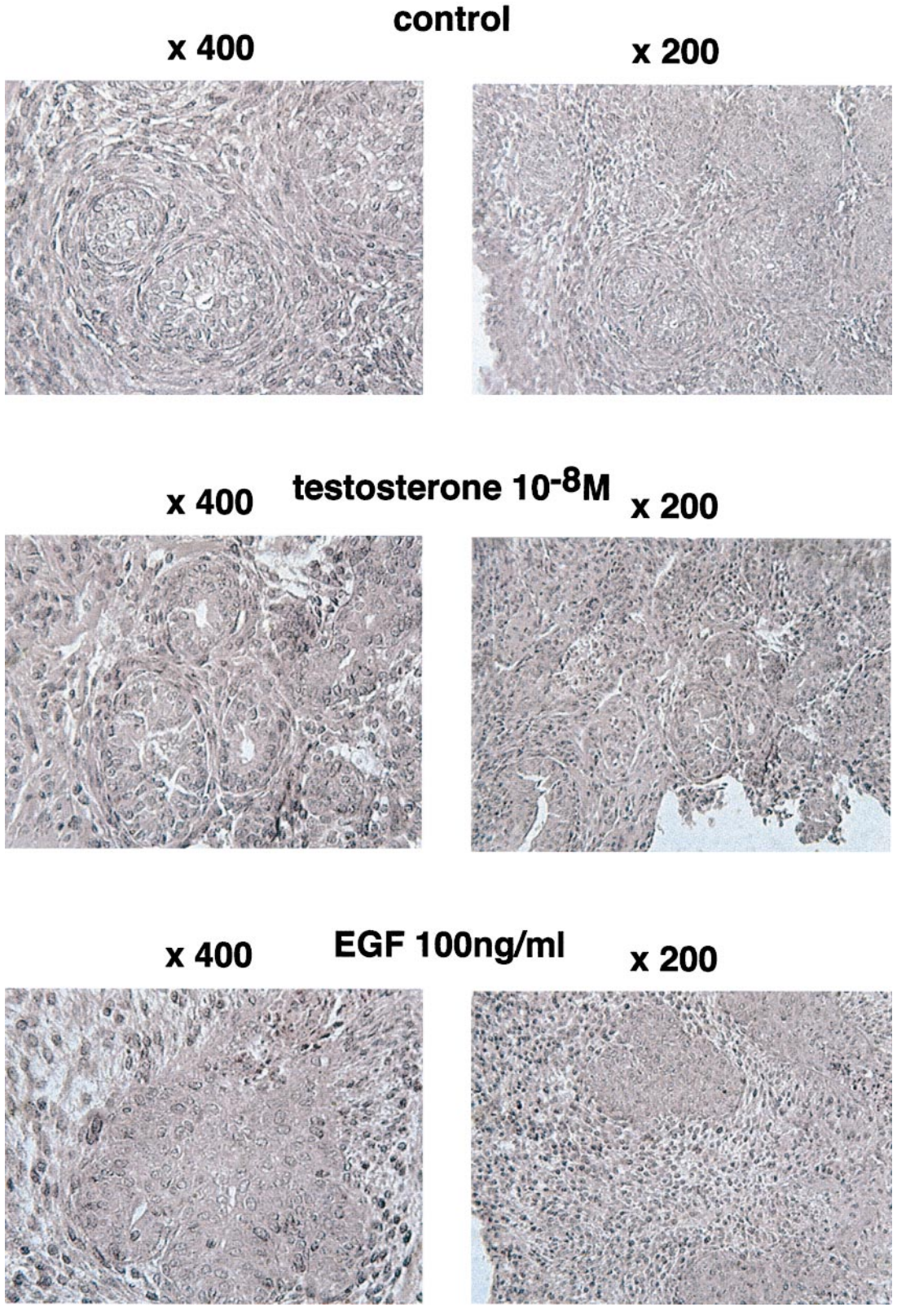

FIG. 9. Histology of 0-day-old rat ventral prostate organ cultures after 6 days of culture. Data are presented at two magnifications, as indicated with the treatments presented. Data are representative of three different experiments.

epithelial cells are less organized into ductal structures. Testosterone promotes a polarized epithelial cell morphology, whereas EGF promotes a more random proliferation of the epithelium (Fig. 9). A combination of testosterone and EGF caused enlargement of the prostate; however, this enlargement was not due to ductal formation and appears to be due to stromal enlargement (Figs. 7 and 8). The degree of ductal branching morphogenesis with testosterone and EGF was increased over that with EGF alone (Fig. 8). The ratio of stroma/epithelium was higher in the prostates treated with EGF than in control cultures. Combined results indicate that TGFβ can inhibit the stimulatory effects of testosterone on prostate morphogenesis. The ability of EGF to stimulate TGFβ may have a role in the inhibitory effects of EGF on prostate ductal branching morphogenesis.

TGFβ cellular localization

To address the *in vivo* localization of TGFβ, a pan-TGFβ antibody that recognizes all three forms of TGFβ was used in immunocytochemistry of 20-day-old rat prostate sections. TGFβ protein was predominantly found in the stromal cell population, with only sparse staining in the epithelium (Fig. 10). TGFβ staining was most intense in the stromal cells adjacent to the epithelial cells. Although the mRNA for TGFβ was found in both cell types, the protein is primarily localized in stromal cells. Whether this is due to localization in the extracellular environment of the stromal cells or is a reflection of production levels remains to be elucidated. Controls with nonimmune IgG had no staining detected (Fig. 10).

Discussion

The current study was designed to investigate the developmental and hormonal regulation of TGFβ expression in the prostate. Previously, the three isoforms of TGFβ were shown to be present in normal prostate, benign prostatic hyperplasia (BPH), and prostate cancer by Northern blot and ribonuclease protection assays (26, 27, 34). Recently, TGFβ1, -2, and -3 mRNA were detected in stromal and epithelial cells of normal human prostate and BPH by RT-PCR (28). For the current study, a sensitive QRT-PCR procedure was developed to accurately investigate the regulation of TGFβ expression. This QRT-PCR procedure was specific for the dif-

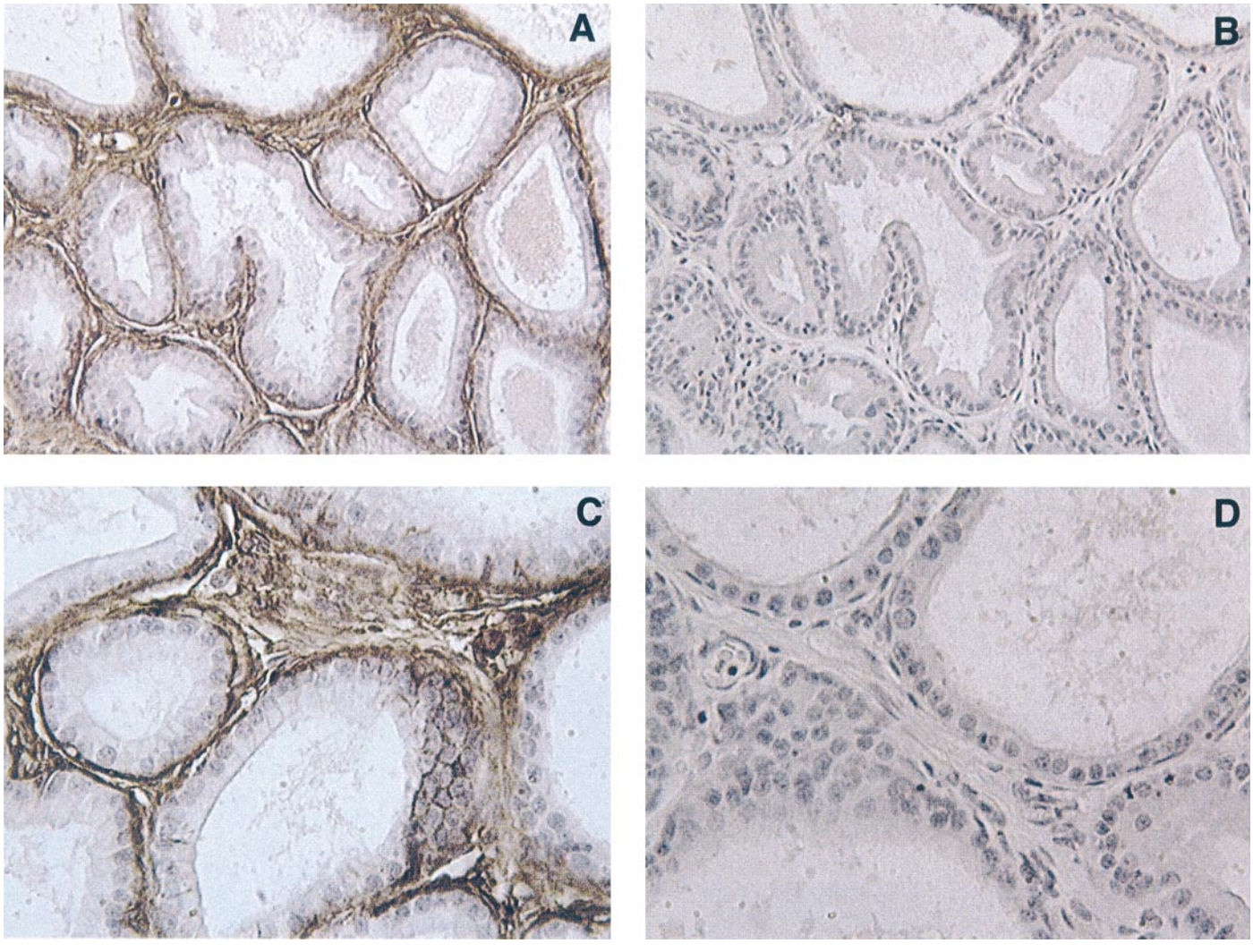

FIG. 10. Immunocytochemical localization of TGFβ in 20-day-old rat prostate sections. Sections were incubated in the presence of nonimmune antibody (B and D) or in the presence of a pan-TGFβ antibody (A and C). Data are representative of three different experiments.

ferent TGFβ isoforms and used small amounts of tissue or cells. All data obtained for TGFβ were normalized for a constitutively expressed gene, cyclophin. Cyclophilin expression did not change among the samples (data not shown) and was corrected for variations in RNA amount and integrity. The QRT-PCR procedure developed was useful to investigate TGFβ expression.

All three isoforms of TGFβ, TGFβ1, -2, and -3, were expressed in both stromal cells and epithelial cells from freshly isolated cells and cultured cells. Freshly isolated prostatic stromal cells have a higher level of TGFβ expression than epithelial cells. TGFβ3 has the highest level of expression, whereas TGFβ1 has the lowest. The high levels of TGFβ mRNA in freshly isolated stromal cells correlated to the immunocytochemical data, with TGFβ staining predominantly in the stromal cells. However, epithelial cells did express all isoforms of TGFβ. During cell culture, the levels of TGFβ1, -2, and -3 increased in both cell types. Interestingly, the epithelial cells developed a higher level of expression than stromal cells in culture. This increase in expression during culture may be due to the lack of regulatory agents that suppress expression in normal *in vivo* tissue. Previously, the type I and type II TGFβ receptors (15) have been shown to be expressed in the prostate (35). These observations suggest that both prostate stromal and epithelial cells have the capacity to express TGFβ1, -2, and -3, which may act as both paracrine and autocrine factors to influence prostate function.

The developmental regulation of TGFβ1, -2, and -3 expression was investigated with neonatal day 0 rats, pubertal day 20 rats, early adult day 60 rats, and adult day 100 rats. TGFβ1 expression (*i.e.* mRNA levels) remained constant during prostate development. TGFβ2 expression was high during the neonatal and adult (100 day) periods and low during the pubertal and early adult (60 day) periods. In contrast, TGFβ3 expression was low during the neonatal and adult (100 day) periods and high during the pubertal and early adult (60 day) periods. TGFβ2 and TGFβ3 expression appeared to be inversely related during prostate development. Recently, the ratio of stromal to epithelial cells was measured during prostate development and was constant throughout (36). Therefore, the changes in TGFβ1, -2, and -3 do not appear to be due to alterations in the cell populations. Although the three isoforms of TGFβ are highly homologous and have similar biological activities, the developmental expression during prostate development appears distinct. The inverse relation of TGFβ2 and TGFβ3 may reflect changes in the regulation of prostate function and may be required to coordinate local cell-cell interactions (37).

Androgens are postulated to be an essential factor to regulate TGFβ expression in the prostate (9, 25). Both TGFβ1 and TGFβ2 expression increased after castration. This supports previous observations with TGFβ1 (9, 10). Interestingly, TGFβ3 expression decreased after castration. Previously, TGFβ2 mRNA levels have been shown to be negatively correlated with androgen levels in both normal and malignant prostate (34). The changes in TGFβ expression during development cannot be directly correlated with serum androgen levels. The relationship of serum androgen levels and TGFβ expression remains to be elucidated. As discussed below, androgens have no effect on TGFβ expression using cultured prostate cells. Another factor to explain the changes after castration is the alteration in the stromal/epithelial cell populations (36). These observations suggest that the relationship of TGFβ expression and androgen levels remains to be elucidated and probably involves a combination of direct and indirect actions of androgens.

Several interesting observations came from the analysis of TGFβ expression in cultured prostatic stromal and epithelial cells. Both isolated cell populations were approximately 90–95% pure by an immunocytochemical analysis of the cell populations. Androgens did not influence TGFβ1, -2, or -3 in either stromal cell or epithelial cell cultures. The concentrations of androgen used ranged from 10^{-8}-10^{-6} M, with no effect at any concentration (data not shown). This observation suggests that androgen may not have a dramatic direct action on TGFβ expression. TGFβ3 expression was not influenced by any of the treatments used in the current study. TGFβ2 was also not affected in stromal cells by any treatment. KGF stimulated TGFβ2 in epithelial cells. KGF has been shown to mediate stromal-epithelial cell interactions in the prostate and to influence prostate morphogenesis (3, 32, 36). The ability of KGF to influence TGFβ2 expression in prostate epithelium will probably have a role in the actions of KGF on the prostate.

TGFβ had no effect on TGFβ1, -2, or -3 expression in either stromal cells or epithelial cells. Previously, TGFβ1 was found to enhance bFGF expression in cultured human prostatic stromal cells (13, 14). In rat kidney fibroblasts, TGFβ has been shown to stimulate TGFβ expression (38). In the current study, TGFβ did not have a major regulatory role in the control of TGFβ expression.

Perhaps one of the more interesting observations made was the effect of EGF on TGFβ1 expression. EGF stimulated the expression of TGFβ1 by both prostatic stromal and epithelial cells. EGF has been shown to be a potent growth stimulator of prostatic epithelium (39, 40). The observations made in the current study suggest that EGF may also have an important regulatory role. The actions of EGF on prostate function and development discussed below will probably be mediated in part by these effects on TGFβ1 expression.

The effects of TGFβ on cell growth were examined to investigate the functional significance of TGFβ in the prostate. Both EGF and testosterone dramatically stimulated prostate stromal and epithelial cell growth. The potential indirect actions of testosterone via peptide growth factors need to be considered. TGFβ inhibited the actions of testosterone and EGF on both cell types. This correlates with the previously identified function of TGFβ as a growth inhibitory substance. Previously, TGFβ has been shown to inhibit the growth of human prostate epithelial cells (21). An important function for TGFβ will probably be as a growth inhibitor in the prostate.

Another functional parameter of the prostate examined was ductal branching morphogenesis in prostate organ cultures. This organ culture system has been developed to investigate morphogenesis of the prostate and actions of agents such as androgens and KGF (36, 32). In the current study, androgen was found to promote ductal branching morphogenesis, and TGFβ was found to suppress the actions of

androgens. This suggests that TGFβ may have a role in regulating prostate morphogenesis. Interestingly, EGF also dramatically suppressed ductal branching morphogenesis. This was in part due to a proliferation of stromal cells and a disorganization of the epithelium. The ability of TGFβ to suppress ductal branching morphogenesis and inhibit EGF-stimulated growth suggests that TGFβ may have a role in this process. In support of this proposal is the ability of EGF to stimulate TGFβ1 expression. These observations suggest that TGFβ will have a role in prostate growth and morphogenesis. The actions of regulatory agents (*e.g.* EGF) on the prostate may be mediated indirectly through the production and action of TGFβ.

The combined observations suggest that a network of locally produced growth factors, such as KGF, EGF, and TGFβ, will coordinate prostate development and morphogenesis. This is supported by observations in the current study regarding the developmental and hormonal regulation of TGFβ1, -2, and -3 expression in prostate stromal and epithelial cells. Regulatory agents such as androgen probably involved both direct and indirect actions mediated through this network of growth factors to control prostate function. The abnormal phenotypes in prostate cancer and BPH will probably in part be due to the inability of this network of regulatory agents and growth factors to maintain normal prostate function. It is speculated that TGFβ will have a role in this process.

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