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Glucocorticoid Up-Regulates Transforming Growth Factor-β (TGF-β) Type II Receptor and Enhances TGF-β Signaling in Human Prostate Cancer PC-3 Cells

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Previous studies have shown that dexamethasone (Dex) induces the expression of TGF-β1 in androgen-independent prostate cancer both *in vitro* and *in vivo*. However, it is not clear whether Dex has a direct effect on the expression of TGF-β receptors. In this study, using the androgen-independent human prostate cancer cell line, PC-3 cells, we demonstrated that Dex increased the expression of TGF-β receptor type II (TβRII), but not TGF-β receptor type I (TβRI) in a time- and dose-dependent manner. The up-regulation of TβRII expression by Dex was mediated by glucocorticoid receptor and occurred at the transcriptional level. Dex also enhanced TGF-β1 signaling and increased the expression of cyclin-dependent kinase inhibitors $p15^{INK4B}$ (p15) and $p27^{KIP1}$ (p27), which are the target genes of TGF-β1 and have been identified as inducers of cell cycle arrest at the G1 checkpoint. The antiproliferative effect of Dex was partially blocked by anti-TβRII antibody, indicating that elevated TβRII and TGF-β1 signaling were involved in the antiproliferative effect of Dex. Because the TGF-β1 pathway could not fully explain the antiproliferative effect of Dex, we further examined the effects of Dex on the transcriptional activity of nuclear factor-κB (NF-κB) and the expression of IL-6 and found that Dex suppressed the transcriptional activity of NF-κB and IL-6 mRNA expression in PC-3 cells. These results demonstrated that glucocorticoid inhibited the proliferation of PC-3 cells not only through enhancing growth-inhibitory TGF-β1 signaling, but also through suppressing transcriptional activities of NF-κB. (*Endocrinology* 147: 5259–5267, 2006)

GLUCOCORTICOIDS ARE KNOWN to inhibit both *in vivo* and *in vitro* growth of certain normal and transformed cell lines. In addition to their well-documented cytotoxic effect on hematological cells (1), glucocorticoids also exert an antiproliferative effect on osteosarcoma cells (2–4), certain hepatoma cells (5, 6), and mammary (7), ovarian (8–10), and prostate cancer cells (11–13). However, the mechanism responsible for the antiproliferative effect of glucocorticoids on tumors remains unclear.

Previous studies showed that glucocorticoids such as dexamethasone (Dex) have antiproliferative effects on androgen-independent prostate cancer (AIPC) *in vitro* and *in vivo*, suggesting their therapeutic potential on AIPC. Indeed, glucocorticoids have been used in the treatment of advanced prostate cancer in several randomized studies (14). It is well known that glucocorticoid receptor (GR) mediates glucocorticoid action by regulating the expression of target genes (15–17). Although previous experiments have indicated that the majority of commonly used prostate cancer cell lines express functional GR, a few target genes have been known to mediate the antiproliferative effect of glucocorticoids on prostate cancer cells (13, 14).

TGF-β1 is an inhibitory growth factor in normal prostate epithelial cells and some prostate cancer cells (18, 19). It has two types of transmembrane receptors: TβRI and TβRII, and both have serine/threonine kinase activity (20). Binding of TGF-β1 to constitutively active TβRII leads to recruitment, interaction, and phosphorylation of TβRI, thus initiating a cascade of molecular events, such as phosphorylation of downstream Smad proteins, including Smad2 or Smad3. The phosphorylated Smad2 or Smad3 combines with Smad4, and then translocates to the nucleus to regulate the transcription of target genes (21–24). The growth inhibitory effect of TGF-β1 is mediated by regulating the expression of cell cycle regulators, such as some members of the cyclin-dependent kinase inhibitor (CKI) family, which in turn result in cell cycle lengthening and growth arrest of normal and neoplastic prostate cells.

Human prostate surface epithelial cells and stromal cells produce TGF-β1. The epithelial cells predominantly express TGF-β receptors, suggesting that proliferation and other activities of epithelial cells may be affected by autocrine and paracrine TGF-β1 signals (25–27). Moreover, several studies (10, 11) showed that glucocorticoids induced expression of TGF-β1 in androgen-insensitive prostate cancer cell lines, such as PA-III and PC-3 cell lines. Recently, we found that Dex not only inhibited the proliferation of PC-3 cells, which had been reported before by another laboratory (11), but also enhanced the antiproliferative effect of TGF-β1 on these cells. We therefore hypothesized that the antiproliferative effect of

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Abbreviations: AIPC, Androgen-independent prostate cancer; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; Dex, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; p15, $p15^{INK4B}$; p27, $p27^{KIP1}$; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II.

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Dex may be also through positively regulating TGF-β1 signaling and enhancing the sensitivity of PC-3 cells to TGF-β1.

In the present study, we examined effects of Dex on the expression of TGF-β1 receptors and target genes of the TGF-β1 signaling pathway in androgen-insensitive PC-3 cells to explore the mechanism for the antiproliferative effect of Dex on these cells.

Materials and Methods

Cell culture

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 and 37 C incubator. Dex (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol before addition to the culture medium. The final ethanol concentration was 0.1%. TGF-β1 (Peprotech, Rocky Hill, NJ) was diluted in the culture medium and stored at 4 C before addition to cultures.

Cell proliferation assay

The proliferation of PC-3 cells was determined by counting the cell number with a hemacytometer. Cells were seeded into 35-mm tissue culture dishes at a density of 4×10^4 cells/dish in 2 ml medium. Dex or TGF-β1 was added to the cultures 24 h later to give the final concentration of 100 nM (Dex) or 10 ng/ml (TGF-β1). Control cells were treated with the same volume of the vehicle (ethanol for Dex). At set time points, cells were trypsinized, resuspended in the medium, and counted.

Semiquantitative analysis of mRNA by RT-PCR

Cells were washed with ice-cold PBS once and total RNA was isolated with TRIzol (Invitrogen) according to the instructions of the manufacturer. About 2 μg RNA were treated with ribonuclease-free deoxyribonuclease, and cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and 2 μl of synthesized cDNA was subjected to 30 cycles of PCR that resulted in a single specific amplification product of the expected size. The PCR conditions were as follows: 30 sec denaturation at 94 C, 1 min annealing at 55 C (TβRI, TβRII, and p15) or 58 C (IL-6), and 45 sec extension at 72 C. PCR primers used in this study are as follows: TβRI, sense 5′-CGTGCTGACATCTATGCAAT-3′and antisense 5′-AGCTGCTCCATTGGCATAC-3′; TβRII, sense 5′-GCACGTTCAGAAGTCGGTT-3′ and antisense 5′-AGATATGGCAACTCCCAGTGGT-3′; p15, sense 5′-GGCGCGCGATCCAGGTCA-3′ and antisense 5′-GAACCTGGCGTCAGTCCC-3′; IL-6, sense 5′-TGACCCAACCACAAATGC-3′ and antisense 5′-CTGGCTCTGAAACAAAGGAT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of the PCR. The primers for GAPDH were sense 5′-CTGCACCACCAACTGCTTAG-3′ and antisense 5′-TTCAGCTCAGGGATGACCTT-3′.

To determine the specificity of RT-PCRs, we performed the amplifications three times and took the mean value of these three reactions. The RT-PCRs were in the linear range of amplification for the target mRNA as well as for the control. Each RT-PCR product was demonstrated on 1.4% agarose gel stained with ethidium bromide. The bands were documented, scanned, quantified using Quantity One software (PDI, New York, NY) and normalized with internal control GAPDH.

Western blot analysis

Cells were lysed in ice-cold lysis buffer [10 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM β-mercaptoethanol, containing 2 μg/ml of each of the protease inhibitors, leupeptin, aprotinin, and pepstatin] for 10 min. Protein concentrations in the cell lysates were measured using the Bio-Rad protein determination assay. Twenty micrograms of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Inc., Burlington, VT), and immunoblotted with various primary antibodies. Antibodies to TβRII and p27 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The blots were further incubated with alkaline phosphatase-conjugated goat antimouse or antirabbit IgG (Bio-Rad Laboratories, Hercules, CA) and colorized with phosphatase substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate)-Nitro Blue Tetrazolium.

TβRII antibody neutralizing assay

PC-3 cells were seeded into 96-well plates at a density of 1×10^3 cells/well in 0.2 ml medium with 20 μg/ml TβRII neutralizing antibody (R&D Systems, Minneapolis, MN) or with control IgG of the same subtype as the anti-TβRII antibody (R&D Systems). After a 3-h incubation, 100 nM Dex or 10 ng/ml TGF-β1 was added. Cells were allowed to grow for 6 d, and medium was renewed every other day. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method was used to determine cell proliferation.

Transfection and luciferase reporter assay

PC-3 cells were plated in triplicate into a 24-well plate at a density of 5×10^4 cells/well. After overnight culture, the cells were transiently transfected with 1 μg/well of TβRII promoter (−1883/+50) driven luciferase reporter plasmid (kindly provided by Dr. J. W. Freeman) using Lipofectamine plus transfection reagent (Invitrogen). To normalize the transfection efficiency, pRL-TK-Renilla-luc (30 ng/well; Promega, Madison, WI) was cotransfected into the cells. Cells were grown for 12 h and then treated with 1–100 nM Dex for another 24 h. For detecting TGF-β1 signaling activity, cells were cotransfected with 300 ng/well of p3TP-Luc (kindly provided by Dr. J. Massagué) and 30 ng/well of pRL-TK-Renilla-luc, and treated with 100 nM Dex or 10 ng/ml TGF-β1 for 24 h. The luciferase activities were determined using the dual luciferase assay system. Values were normalized by *Renilla* luciferase and reported as fold induction over control.

For assessing the transcriptional activity of nuclear factor-κB (NF-κB), PC-3 cells (2×10^5/well) were cotransfected in 24-well plates with 0.4 μg of NF-κB promoter driven firefly luciferase reporter construct and 1 ng of pRL-TK-Renilla-luc by a lipofectamine method (Invitrogen). After incubation for 12 h, cells were washed with fresh medium and treated with different concentrations of Dex or ethanol vehicle for 18 h or with 100 nM Dex for different periods of time.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Fisher's least significant difference test, and $P < 0.05$ was considered significant. Data are expressed as the means ± SD. * or #, $P < 0.05$.

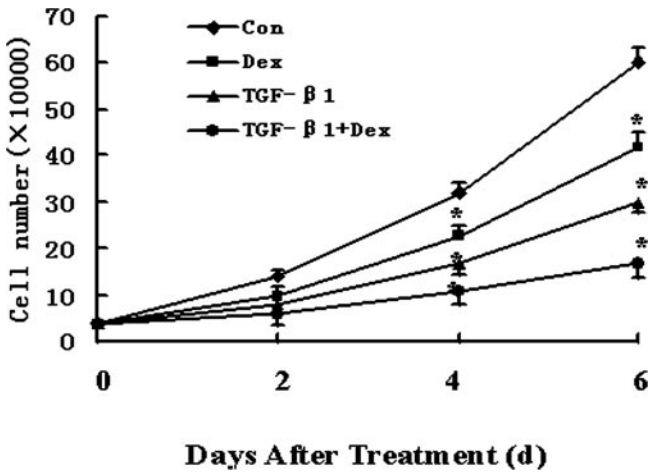

FIG. 1. Inhibitory effects of Dex and TGF-β1 on the growth of PC-3 prostate cancer cells. PC-3 cells cultured in 35-mm plates were treated with 100 nM Dex or/ and 10 ng/ml TGF-β1 for 2, 4 or 6 d. At set time points, cell number in cultures were counted as described in *Materials and Methods*. Cell viability (>98%) was determined by the trypan blue exclusion method. Each *point* represents the means ± SD of triplicate determinations, and $P < 0.05$. *, Statistically significant difference as compared with the control group.

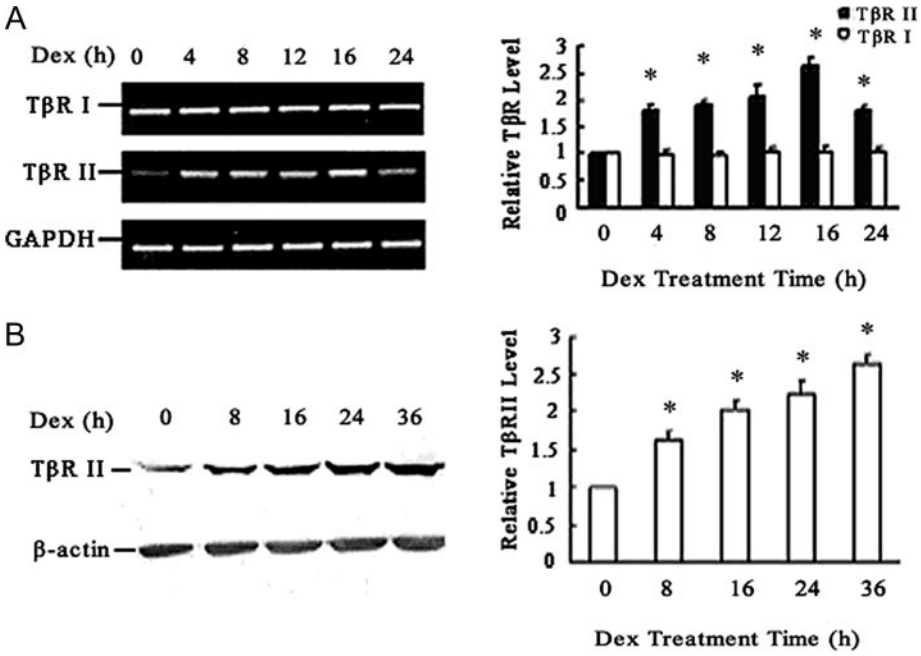

FIG. 2. Dex enhances expression of TβRII but not TβRI in PC-3 cells. A, TβR mRNA levels of PC-3 cells treated with 100 nM Dex or the vehicle for different periods of time were determined by RT-PCR analysis. B, TβRII protein levels of PC-3 cells treated with 100 nM Dex or the vehicle for different periods of time were determined by Western blot. Relative densitometric units of TβRs/GAPDH (A) and TβRII/β-actin (B) are shown in the *right panel*, with the density of the control bands set at 1.0. $P < 0.05$. *, Statistically significant difference between testing groups and the control group. These data are representative of the results from three independent experiments.

Results

Dex and TGF-β1 inhibit PC-3 cell proliferation

Effects of Dex or TGF-β1 on the growth of PC-3 prostate cancer cells were examined. As illustrated in Fig. 1, treatment of PC-3 cells with 100 nM Dex or 10 ng/ml TGF-β1 resulted in decreasing cell proliferation in a time-dependent manner. Cell numbers in Dex- and TGF-β1-treated groups at d 6 were 35% and 50% less than that in the control group, respectively. These observations indicated that both Dex and TGF-β1 exerted marked antiproliferative effects on prostate cancer cells, which was in agreement with a previous study (11). Cotreatment of PC-3 cells with Dex and TGF-β1 had an additive inhibitory effect on cell proliferation. The cell number decreased by up to 70% compared with that of the control group (Fig. 1).

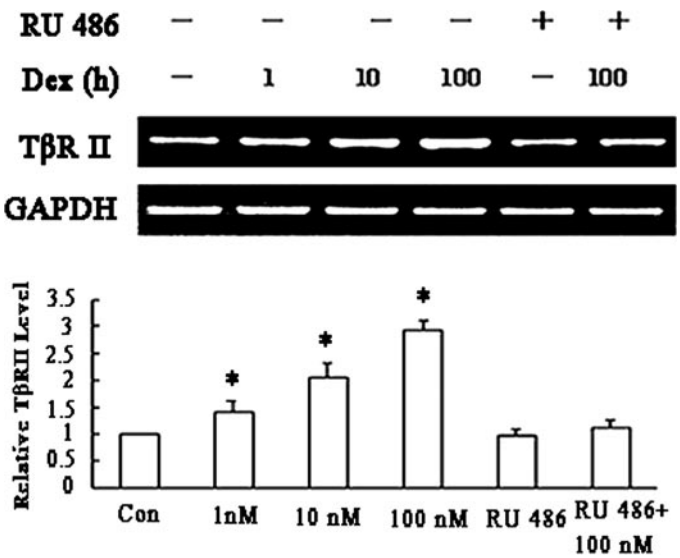

FIG. 3. Increased mRNA level of TβRII induced by Dex is blocked by GR antagonist RU486. PC-3 cells were treated with 1–100 nM Dex and/or 1 μM RU486 for 16 h. RT-PCR was performed as described *in Materials and Methods*. Relative densitometric units of TβRII/GAPDH are shown in the lower panel, with the density of the control bands set at 1.0. $P < 0.05$. *, Statistically significant difference between a testing group and the control group (Con, vehicle treated).

Dex increases the expression of TβRII in PC-3 cells

To determine whether Dex enhances the inhibitory effect of TGF-β1 by regulating the expression of TGF-β1 receptors, we firstly examined the effect of Dex on the mRNA level of TGF-β1 receptors in PC-3 cells by RT-PCR. Results showed that Dex did not alter the level of TβRI mRNA but increased the expression of TβRII in a time-

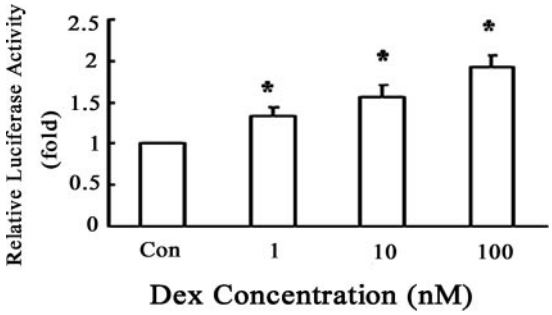

FIG. 4. Dex increases TβRII promoter activity in PC-3 cells. PC-3 cells were transiently cotransfected with 1 μg of TβRII promoter-Luc and 30 ng of TK-*Renilla* construct. Transfected cells were treated with ethanol vehicle or 1–100 nM Dex for 24 h, and the luciferase activities were measured. The *columns* indicate fold induction in TβRII promoter-Luc activity relative to the level in vehicle-treated control cells (Con). The activity in the control was set at 1.0. The results are means ± SD from three independent experiments. $P < 0.05$. *, Statistically significant difference between a Dex-treated group and the control.

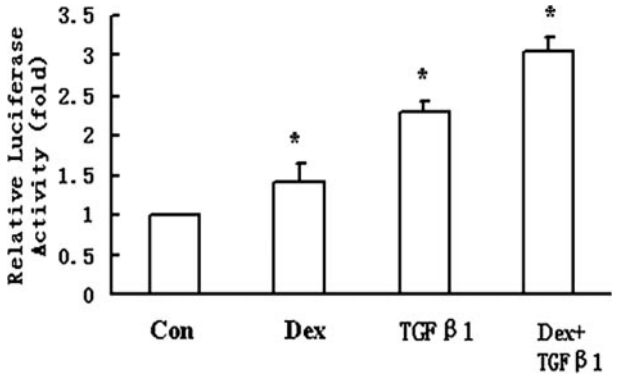

FIG. 5. Dex enhances TGF-β1-induced reporter gene expression. PC-3 cells were transiently transfected with a TGF-β1/Smad-responsive promoter construct (p3TP-Luc). Twelve hours later, cells were treated with 100 nM Dex or 10 ng/ml TGF-β1 for another 24 h and then harvested for detecting luciferase activity. The *columns* indicated means ± SD for 3TP-Luc activity relative to the level in vehicle-treated control cells (Con), which was set at 1.0. $P < 0.05$. *, Statistically significant difference between a treated group and the control.

dependent manner (Fig. 2A). TβRII mRNA increased about 2.5-fold after 16 h treatment with 100 nM Dex. A similar increase of TβRII expression at the protein level was observed after 36 h treatment with 100 nM Dex (Fig. 2B).

GR mediates the up-regulatory effect of Dex on TβRII expression

PC-3 cells were treated with ethanol vehicle, 1–100 nM Dex, 1 μM RU486 (a GR antagonist), or a combination of 100 nM Dex with 1 μM RU486 for 16 h. TβRII mRNA in the cells was measured by RT-PCR. As shown in Fig. 3, Dex increased the expression of TβRII mRNA in a dose-dependent manner, which could be blocked by RU486. The results indicated that the up-regulatory effect of Dex on the expression of TβRII was mediated by the GR.

Dex increases TβRII promoter activity in PC-3 cells

To determine whether increased expression of TβRII mRNA induced by Dex was due to GR-dependent transcriptional activation or stabilization of existing TβRII transcripts, a fusion construct containing the full-length TβRII promoter (−1883/+50) attached to a luciferase reporter was transiently transfected into PC-3 cells. Analysis of transcriptional regulation of TβRII in the cells was performed by the luciferase assay as described in *Materials and Methods*. Figure 4 shows the dose-dependent activation of luciferase resulted from the treatment with various concentrations of Dex. The luciferase activity of cells treated with 100 nM Dex was 2.0-fold compared with that of control cells. This induced expression of luciferase was similar to the increased level of

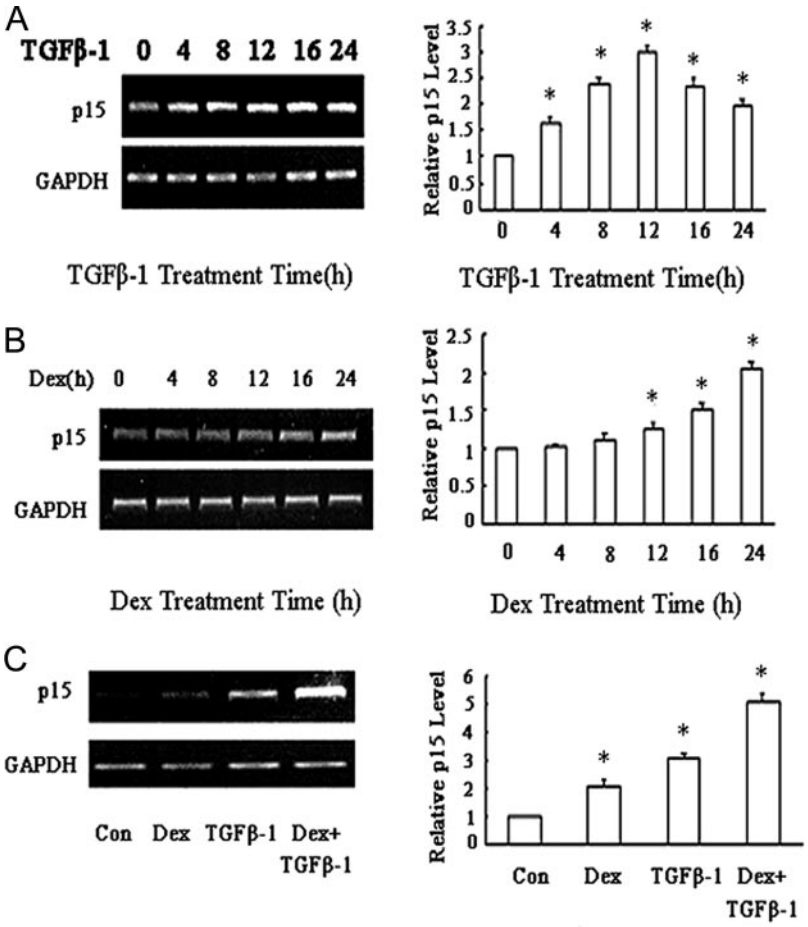

FIG. 6. Dex and TGF-β1 up-regulate expression of cell cycle-negative regulator p15. Cells were treated with either 10 ng/ml TGF-β1 (A) or 100 nM Dex (B) for different periods of time or with a combination of 10 ng/ml TGF-β1 and 100 nM Dex (C) for 12 h. RNA was isolated from the cells and the expression of p15 mRNA was analyzed by RT-PCR. The expression of p15 (relative densitometric units of GAPDH, which have been set at 1.0) is shown in *right panels*. $P < 0.05$. *, Statistically significant difference between a treated group and the control group.

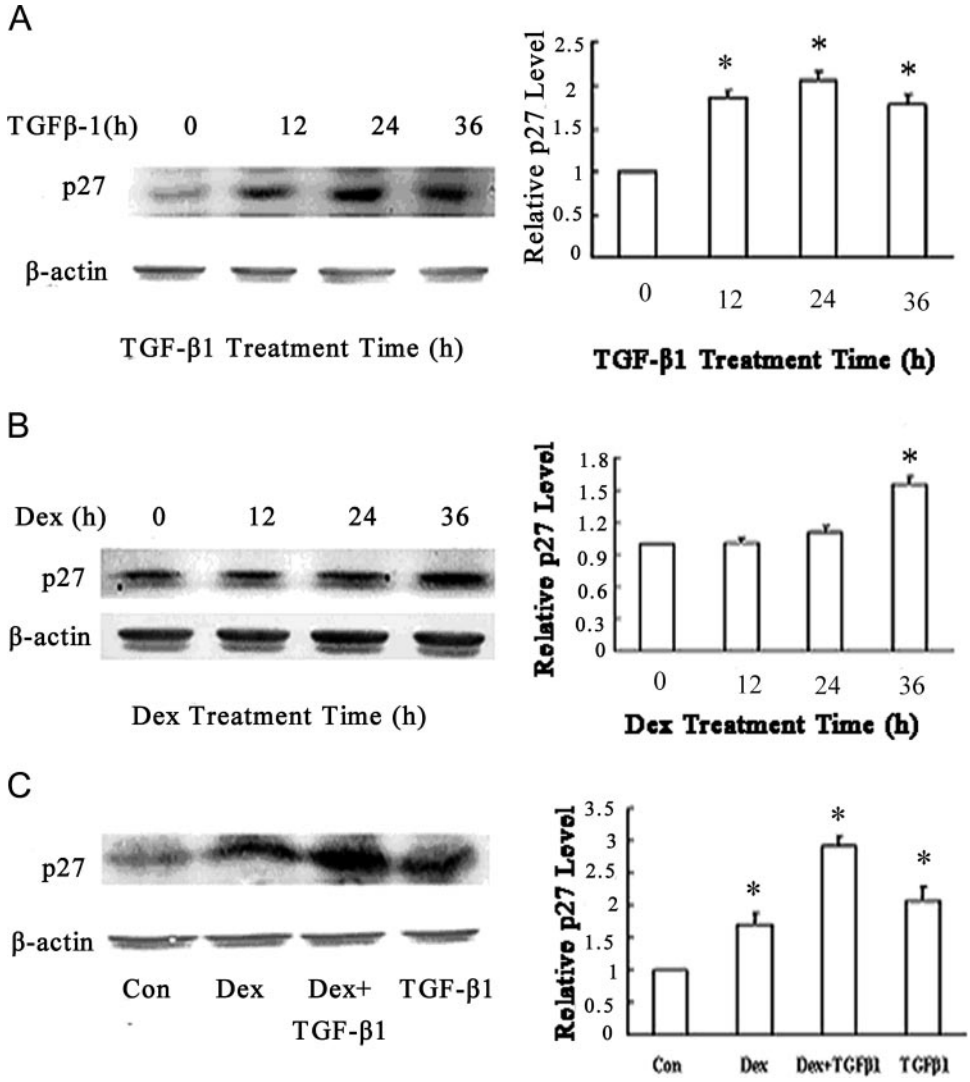

FIG. 7. Dex and TGF-β1 up-regulate expression of cell cycle-negative regulator p27. Cells were treated with either 10 ng/ml TGF-β1 (A) or 100 nM Dex (B) for different periods of time or with a combination of 10 ng/ml TGF-β1 and 100 nM Dex (C) for 24 h. Whole cell lysates were prepared at set time points. p27 Protein expression was determined by Western blot analysis. The expression of p27 (relative densitometric units of β-actin, which have been set at 1.0) is shown in the *right panels*. The results shown are means from three independent experiments. $P < 0.05$. *, Statistically significant difference between a treated group and the control.

TβRII mRNA after the Dex treatment, indicating that increased expression of TβRII mRNA induced by Dex occurred at the transcriptional level and through increasing TβRII promoter activity in PC-3 cells.

Dex enhances TGF-β signaling in PC-3 cells

Because the TβRII expression level was increased by Dex treatment, Dex might enhance TGF-β1 signaling and its downstream events, and increase the response of cells to TGF-β1. To test this hypothesis, we transiently transfected PC-3 cells with a TGF-β1/Smad reporter vector p3TP-Luc. This vector contains a luciferase gene whose promoter has a TGF-β1 response element. The effect of Dex on the luciferase expression mediated by TGF-β1 was determined by the luciferase assay. As shown in Fig. 5, 100 nM Dex alone only induced a 1.5-fold increase of luciferase activity, but it further increased TGF-β1-induced luciferase activity up to 3-fold when combined with 10 ng/ml TGF-β1.

Dex and TGF-β1 up-regulate the expression of cell cycle-negative regulators p15 and p27 in PC-3 cells

TGF-β1 exerts its antiproliferative effect by inducing the expression of CKIs, such as p15 and p27, which have been identified as inductors of cell cycle arrest at the G1 checkpoint. To further elucidate the mechanism of enhanced TGF-β1 inducing growth inhibitory effect of Dex, effects of Dex or TGF-β1 alone, or both on the expression of p15 and p27 in PC-3 cells were examined.

RT-PCR analysis showed that 10 ng/ml TGF-β1 induced the expression of p15 mRNA in a time-dependent fashion (Fig. 6). The maximum induction was approximately 3.0-fold higher than that in the control at 12 h. Dex alone (100 nM) also had a weak and time-delayed induction effect on the expression of p15 mRNA, compared with that of TGF-β1 (Fig. 6B). Treatment of PC-3 cells with 100 nM Dex markedly increased TGF-β1-induced expression of p15 mRNA (up to 5-fold) (Fig. 6C, lane 4).

Previous studies have indicated that the regulatory mechanism of p27 expression was complex and several levels of

the regulation were involved. For example, some agents, such as 1, 25-$(OH)_2$ vitamin D_3, exert antiproliferative effects on a human prostate cancer cell line LN-CaP through stabilizing p27 protein instead of increasing mRNA level (28). Therefore, the expression of p27 was examined by Western blot analysis. A similar induction of p27 protein by TGF-β1 and Dex was observed in this study. As shown in Fig. 7A, exposure of PC-3 cells to TGF-β1 resulted in a time-dependent increase (~2.0-fold) of p27 protein expression, compared with that in control cells at 24 h. Dex at 100 nM alone also slightly increased the expression of p27 protein in a time-delayed manner (Fig. 7B). Combined treatment with Dex and TGF-β1 resulted in a 3.0-fold increase of p27 expression (Fig. 7C, lane 3), significantly higher than that with TGF-β1 alone.

TβRII-neutralizing antibody partially blocks the growth-inhibitory action of Dex

We further tested the ability of a neutralizing antibody specifically against the extracellular domain of TβRII to block the growth-inhibitory action of Dex on PC-3 cells using the MTT reduction cell proliferation assay. Results showed that either 100 nM Dex or 10 ng/ml TGF-β1 markedly inhibited the proliferation of PC-3 cells. Treatment of PC-3 cells with the anti-TβRII polyclonal antibody at a concentration of 20 μg/ml completely blocked the inhibitory effect of 10 ng/ml TGF-β1, but partially blocked the growth inhibitory action of 100 nM Dex in these cells (Fig. 8), indicating that TGF-β1 signaling was involved in the proliferative inhibition of PC-3 cells by Dex, but there might be other mechanisms responsible for the antiproliferative effect of glucocorticoids.

Dex inhibits the transcriptional activity of NF-κB and IL-6 expression in PC-3 cells

Recently, NF-κB is hypothesized to contribute to the development and/or progression of androgen-independent prostate cancer cells by regulating the expression of genes involved in cell growth, antiapoptosis and metastasis (29). Therefore, we examined whether Dex exerted its antitumor effect by suppressing NF-κB activation and inhibiting the expression of its target genes, IL-6 in human PC-3 cells. PC-3 cells were transfected with NF-κB-specific reporter plasmid and were incubated for 12 h. Then, the cells were treated with different concentrations of Dex for 18 h or treated with 100 nM Dex for different time periods for NF-κB activity assay. The results showed that Dex decreased the transcriptional activity of NF-κB in a dose- and time-dependent manner. Inhibition of 38 ± 1.8% was observed in PC-3 cells treated with 100 nM Dex for 18 h (Fig. 9). Dex also inhibited the expression of the NF-κB-dependent cytokine IL-6 in PC-3 cells. Treatment of PC-3 cells with 100 nM Dex for 12 h resulted in a 34.6% decrease of IL-6 mRNA level tested by RT-PCR (Fig. 10).

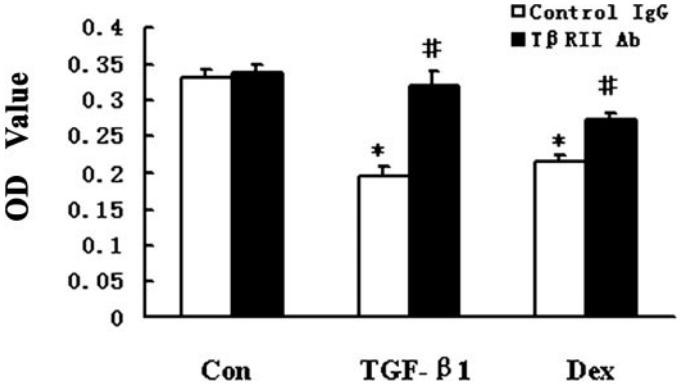

FIG. 8. TβRII-neutralizing antibody blocks inhibitory effects of Dex on the growth of PC-3 cells. PC-3 cells were pretreated with 20 μg/ml TβRII neutralizing antibody or 20 μg/ml control IgG for 3 h, and then treated with 10 ng/ml TGF-β1 or 100 nM Dex. Cells were allowed to grow for 6 d with renewing the medium every other day. MTT reduction method was used to determined cell proliferation. Results are expressed as means ± SD of quadruple determinations. *, Significantly inhibitory effect ($P < 0.05$) of TGF-β or Dex on the growth of PC-3 cells. #, Significant blocking of the inhibitory effect ($P < 0.05$) of TGF-β or Dex on the growth of PC-3 cells by TβRII neutralizing antibody.

Discussion

Previous studies (14) have demonstrated that glucocorticoids have inhibitory effects on AIPC *in vitro* and *in vivo*. The

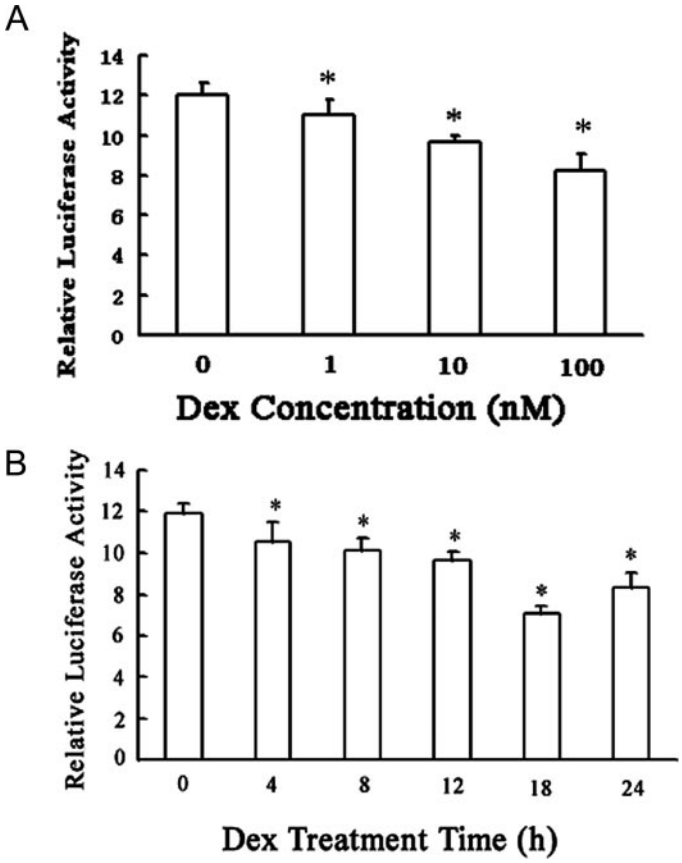

FIG. 9. Dex decreases the transcriptional activity of NF-κB in a dose- and time-dependent manner in PC-3 cells. PC-3 cells were cotransiently transfected with 0.4 μg of pGL3-NF-κB-luc and 1ng of pRL-TK-Renilla-luc. Transfected cells were treated with ethanol vehicle or 1–100 nM Dex for 18 h (A), or 100 nM Dex for 0–24 h (B), then the luciferase activities were measured. Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of *Renilla* luciferase. The results shown are means from three independent experiments. $P < 0.05$. *, Statistically significant difference between Dex-treated group and the control.

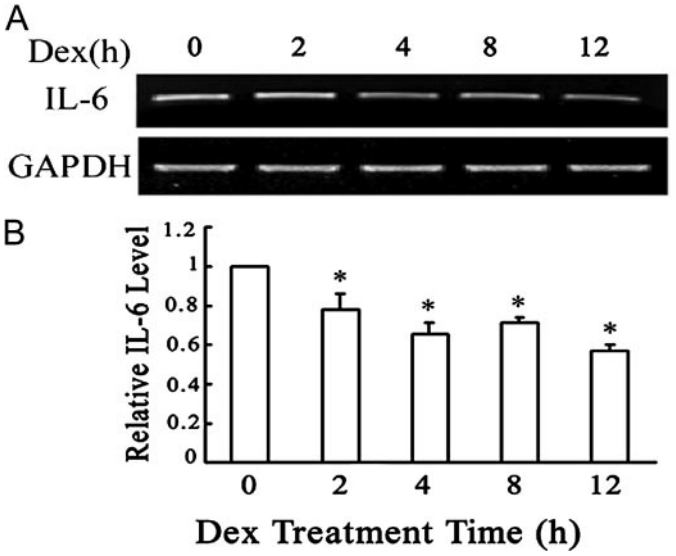

FIG. 10. Dex inhibits the expression of IL-6 mRNA in PC-3 cells. A, RT-PCR analysis of IL-6 mRNA in PC-3 cells treated with the vehicle or 100 nM Dex for different times. GAPDH was included as an internal control. B, Densitometric analysis of IL-6 mRNA expression shown in A. Relative densitometric units of IL-6/GAPDH are shown in the *lower panel*, with the density of the control bands set at 1.0. $P < 0.05$. *, Statistically significant difference between Dex treatment and the vehicle treatment.

effects are associated with several potential mechanisms: down-regulation of adrenal androgens, modulation of cellular growth factors, such as up-regulation of TGF-β1 and down-regulation of androgen receptor-dependent transcription. However, our understanding about the mechanisms by which glucocorticoids affect AIPC is far from complete.

Previous work of other researchers (11) has demonstrated that PC-3 cells contain functional GR and Dex inhibits the proliferation of these cells. Moreover, Dex up-regulates TGF-β1 that partially mediates the growth inhibitory effect of Dex on PC-3 cells. We found in the present study that cotreatment of PC-3 cells with Dex and TGF-β1 resulted in a further decrease in cell proliferation compared with treatment with exogenous TGF-β1 alone, suggesting that Dex significantly potentiates the antiproliferative effect of TGF-β1 on PC-3 cells. This result also presents a possibility that Dex may increase the sensitivity of PC-3 cells to the growth inhibitory effect of TGF-β1 or enhance the response of PC-3 cells to TGF-β1.

Treatment of PC-3 cells with Dex increased the expression of TβRII mRNA and protein, but not TβRI. The similar effects were also observed in an ovarian cancer cell line HO-8910 (30), indicating that this regulation was not cell type specific. TβRII, but not TβRI, is also up-regulated by androgen, retinoid acid, and $1,25(OH)_2D_3$ (31–35). Moreover, the expression and function of TβRII are frequently altered in many human cancers including prostate cancer (36–39), suggesting that TβRII was a critical signaling effector of TGF-β1 and regulatory mechanisms of TβRII and TβRI were different.

To determine whether the up-regulation of TβRII mRNA by Dex was achieved through increasing new mRNA synthesis, or through stabilizing the existing TβRII transcripts, the analysis of the transcriptional regulation of TβRII in PC-3 cells was performed by the TβRII promoter luciferase assay. The results showed that Dex treatment of PC-3 cells transiently transfected with TβRII promoter driven luciferase reporter gene caused a dose-dependent increase in luciferase activity, indicating that Dex increased the expression of TβRII at a transcriptional level. GR-mediated stimulation of the gene expression usually takes place when the ligand-activated GR directly interacts with a glucocorticoid response element (GRE), present in the target gene. There are several potential GREs in the TβRII enhancer and promoter region scanned with the BioInformatics & Molecular Analysis Section system from the web site http://www-bimas.cit.nih.gov/molbio/signal/. Glucocorticoids may up-regulate TβRII gene expression by inducing the binding of the GR to GRE in this region. Further work is needed to clarify this postulation.

Because TβRII is a critical signaling effector in the TGF-β signal transduction pathway, increase of TβRII expression by Dex should enhance TGF-β1 signaling and therefore change the molecular events of the downstream of TGF-β1 signaling, such as increasing target gene expression. In this study, we showed that TGF-β1 signaling in PC-3 cells was increased after treatment with Dex, as determined by a TGF-β/Smad-responsive reporter gene (3TP-Luc) assay that has been extensively used for estimating TGF-β responsiveness.

We also found that cotreatment with Dex and TGF-β1 resulted in a further increase of the expression of p15 mRNA and p27 protein in PC-3 cells compared with the treatment with TGF-β1 alone. Both p15 and p27 play critical roles in lengthening G1 phase in the cell cycle and TGF-β1 mediated cell growth arrest (40, 41). p15 Is a member of the Ink4 family that exclusively inhibits cyclin D-CDK (cyclin-dependent kinase) 4/CDK6 complexes, which prevents the phosphorylation and inactivation of the RB protein, thereby resulting in arrest of the cell cycle at the G1 phase. In contrast, p27 belongs to the Kip/Cip family that are considered as universal inhibitors because they can down-regulate the activities of cyclin D-CDK4/CDK6, cyclin E-CDK2, and cyclin A-CDK2 complexes. These results are consistent with our observation that Dex enhances the growth inhibitory effect of TGF-β1. The mechanism whereby Dex increases these two CKIs levels is unclear. It is interesting to know whether the up-regulation of p15 and p27 by Dex is the direct effect of glucocorticoid-GR complex or via inducing the endocrine TGF-β1 signaling pathway or some other pathway.

We further examined effects of anti-TβRII antibody on Dex-induced inhibition on the proliferation of PC-3 cells and found that the inhibitory effect of Dex on PC-3 cells was partially reversed by a neutralizing antibody specific to the extracellular domain of TβRII. A similar result using antibody to TGF-β1 was also reported by Carlos Reyes-Moreno (11), indicating that Dex suppressed proliferation of PC-3 cells not only by the up-regulation of TGF-β1, but also by inducing its receptor, TβRII, thereby enhancing the cell sensitivity to TGF-β1. In this study, we observed that the antiproliferative effect of glucocorticoid could only partially blocked by antibody TβRII; thus, there might exist other mechanisms or pathways involved in the growth inhibitory effect of glucocorticoid.

Previous studies (42–45) showed that transcription factor

NF-κB activity was constitutively activated in androgen receptor-negative prostate cancer cells, such as PC-3 cells. IL-6, an NF-κB inducible gene, was an autocrine growth factor for prostate cancer cells, and androgen-independent prostate cancer cells were dependent on the cytokine IL-6 for their growth. We found that Dex inhibited the transcriptional activity of NF-κB and decreased IL-6 mRNA levels in PC-3 cells. These observations suggested that the inhibition of the NF-κB-IL-6 pathway might be another mechanism besides the TGF-β1 signaling pathway responsible for the antitumor activity of Dex against AIPC.

Taken together, our results suggested that glucocorticoid exerts its role of antiproliferation through a complex process. There were at least two mechanisms, by which glucocorticoid inhibits the proliferation in PC-3 cells. One mechanism is by inducing growth-inhibitory factors and/or their receptors, such as up-regulation of TβRII and enhancing TGF-β1 signaling. Another mechanism is by decreasing the expression of growth-stimulatory factors such as IL-6, through suppressing the transcriptional activities of other transcription factors, including NF-κB.

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