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Insulin-Like Growth Factor Binding Protein-3 and -5 Are Regulated by Transforming Growth Factor-β and Retinoic Acid in the Human Prostate Adenocarcinoma Cell Line PC-3

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The family of insulin-like growth factor binding proteins (IGFBPs) can affect cell proliferation by modulating the availability and bioactivity of insulin-like growth factors (IGFs), or by mechanisms independent of IGFs. To understand better the role(s) of IGFBPs in prostate growth and malignancy, we examined the regulation of IGFBPs in PC-3 cells, a human prostatic adenocarcinoma epithelial cell line that is androgen-insensitive. Both transforming growth factor-β (TGF-β) and retinoic acid (RA), known inhibitors of cellular proliferation, significantly changed the IGFBP profile in PC-3 cells. In cells that were treated with transforming growth factor β-2 (TGF-β2) (0.5–10 ng/mL), IGFBP-3, and IGFBP-5 protein and mRNA increased in a time- and dose-dependent manner. At 10 ng/mL TGF-β, IGFBP-3, and IGFBP-5 protein concentrations were 14- and 9-fold, respectively, over that of controls. Cells treated with RA (0–1 μ*M*) also showed a time- and dose-dependent increase in IGFBP-3 protein and mRNA levels. However, in contrast to TGF-β2, high concentrations of RA (1 μ*M*) negatively regulated IGFBP-5 expression, with IGFBP-5 mRNA levels downregulated to 20% of that of the control, and protein levels were decreased by 50%. Since both TGF-β and RA increased IGFBP-3 expression and both are known to inhibit prostate cell growth, we speculate that the inhibition of growth is mediated, at least in part, by IGFBP-3.

Key Words: IGFBP-3; IGFBP-5; prostate; TGF-β, retinoic acid.

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Introduction

Prostate cancer is the most commonly diagnosed cancer in males over the age of 50 *(1)*. Cancer growth involves loss of the normal growth regulatory machinery, which encompasses the complex regulation of androgenic hormones and multiple growth factors. Circulating androgenic hormones, which are normal endocrine effectors of prostate cell growth, are believed to be pathogenic in prostatic cancer *(2)*. Therefore, treatment for advanced stages of prostate cancer involves androgen ablation, either surgically or chemically, through the use of estrogens or antiandrogen therapy *(3)*. However, regression in tumor growth is often only temporary, as the proliferation of hormone-insensitive or hormone-unresponsive phenotypes can develop within a few months of treatment. Involvement of paracrine or autocrine factors may, therefore, be critical for tumor growth at this stage.

Some factors known to affect prostatic cellular proliferation include transforming growth factor-β (TGF-β; 4), epidermal growth factor (EGF; *5*), fibroblast growth factors (FGFs; *6*), nerve growth factor (NGF; *7*), and insulin-like growth factors (IGFs; *8*). IGFs (IGF-I and II) are peptide hormones of approx 7 kDa mol wt that are mitogenic for many cell types, including normal and malignant prostate cells *(8–11)*. The mitogenic effects of IGFs are mediated mainly through interaction with the type I IGF receptor signaling pathway. In biological fluids, IGFs are sequestered by a family of IGF binding proteins (IGFBPs), of which there are six known (IGFBP-1–6; *12*). A seventh IGFBP (IGFBP-7) has recently been identified *(13,14)*. IGFBPs function not only as carriers for IGFs, but as modulators of IGF availability and bioactivity, either by inhibiting *(15,16)* or enhancing *(17)* IGF action. In addition to these IGF-dependent actions, IGFBPs also have biological functions independent of their ability to bind IGFs. The functions of IGFBPs can be antiproliferative *(18–20)* or growth-stimulatory *(21)*.

In many cell types, IGFBP production is regulated by a variety of mitogenic and inhibitory factors, and IGFBPs are thought to mediate the activities of these factors. In breast cancer cells, for example, it was shown that IGFBP-3 expression is upregulated by known growth inhibitors TGF-β *(18,22)*, RA *(18)*, and antiestrogens *(20)*. Furthermore, blockage of IGFBP-3 expression using IGFBP-3 antisense oligodeoxynucleotides abrogated the growth inhibitory effects of these factors, thus demonstrating that IGFBP-3 can mediate the inhibitory actions of these factors. In the prostate, little is known about the regulation of IGFBPs, although expression of IGFBPs is altered in malignancy. IGFBP-2 expression is increased in prostate cancer *(23,24)*, as are IGFBP-4 and -5 *(25)*, while IGFBP-3 protein levels are decreased owing to proteolysis *(23,24,26,27)*. The stimuli leading to altered IGFBP expression, the mechanism(s) by which this occurs and the means by which altered IGFBP expression affect prostate carcinoma are not known.

To understand better the role(s) of IGFBPs in prostate growth and malignancy, we have chosen to examine the regulation of IGFBPs in prostate cancer cells. In this article, we demonstrate, for the first time, that expression of IGFBP-3 and IGFBP-5 in prostate cancer cells is altered by known epithelial cell growth inhibitors, TGF-β and RA. TGF-β is important in prostate growth and differentiation, and accumulation of TGF-β has been associated with human prostate adenocarcinoma *(28,29)*. Interestingly, endogenous production of retinoids (of which RA is the biologically active metabolite) in the prostate was reduced in prostate carcinoma tissues, suggesting retinoids also play a role in the pathophysiology of prostate cancer *(30)*. For these studies, we used the well-established androgen-independent prostate adenocarcinoma epithelial cell line, PC-3 *(31)* as the model system, and demonstrate that TGF-β regulates not only the expression of IGFBP-3, but that TGF-β also positively regulates IGFBP-5. In addition, we show that RA also upregulates the expression of IGFBP-3 with concomitant downregulation of IGFBP-5. We therefore speculate that IGFBPs have roles, to be further defined, in the regulation of prostatic cellular growth.

Results

IGFBPs Detectable in Serum-Free Media Conditioned by PC-3 Cells

Western ligand blot analysis was used to determine which IGF-binding proteins were present in serum-free media conditioned for 48 h by PC-3 cells. As shown in Fig. 1, the molecular weights of the bands detectable by Western ligand blot corresponded to those known for IGFBP-3 (doublet band at 40–45 kDa), IGFBP-5 (doublet band at approx 30 kDa), and IGFBP-4 (single band at approx 24 kDa). Immunoblots confirmed that the doublet

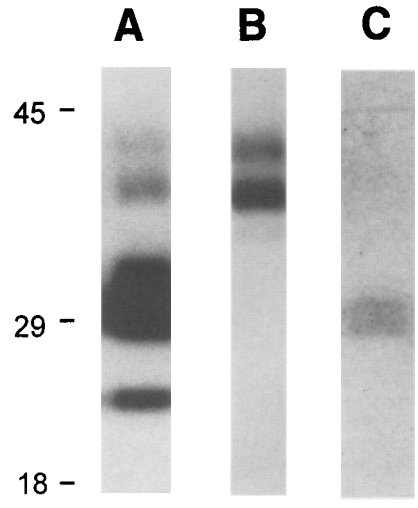

Fig. 1. IGFBPs detected in serum-free media conditioned by PC-3 cells for 48 h. The molecular weights, in kilodaltons, are indicated on the left-hand side. **(A)** IGFBPs detected by Western ligand blot; this blot, which is representative of more than 10 similar experiments, was deliberately overexposed to X-ray film to permit visualization of all bands; **(B)** immunoblot using antihuman IGFBP-3; **(C)** immunoblot using antihuman IGFBP-5.

at 40–45 kDa was IGFBP-3 (Fig.1, lane B) and that the doublet at 30 kDa was IGFBP-5 (Fig. 1, lane C).

PC-3 Cells Treated with TGF-β2 or RA

Growth factors added exogenously to cultured cells can change the profile and/or the concentrations of IGFBPs in conditioned medium (CM). To determine if TGF-β2 or RA could affect the expression of the IGFBPs in PC-3 cells, cells were initially treated with 5 ng/mL TGF-β2 or 1 μM RA. After 24 or 48 h, the CM were collected and subjected to Western ligand blot analysis (Fig. 2). Results indicate that compared to control cells, both TGF-β2 and RA stimulated a reproducible increase in the level of detectable proteins in the 40–45 kDa range (corresponding to IGFBP-3). TGF-β2 also stimulated a marked increase in the doublet at 30 kDa (corresponding to IGFBP-5) in PC-3 cells. The same results were obtained with CM collected from cells treated with TGFβ1 (data not shown), indicating that these two isoforms of TGF-β regulate IGFBPs in PC-3 cells in a similar manner. All subsequent work was done using TGF-β2 only. Interestingly, RA not only failed to stimulate IGFBP-5, but resulted in decreased concentrations of IGFBP-5 in the CM (Fig. 2).

The changes in IGFBP-3 and -5 concentrations in CM induced by either TGF-β2 or RA were confirmed by immunoblotting (Fig. 2, panels B and C). Antibody specific for IGFBP-3 clearly indicated that TGF-β2 and RA increased the concentrations of detectable IGFBP-3 in the CM collected at both 24 and 48 h. Immunoblots using antibody against IGFBP-5 showed that only TGF-β2 increased the level of IGFBP-5 in CM compared to con-

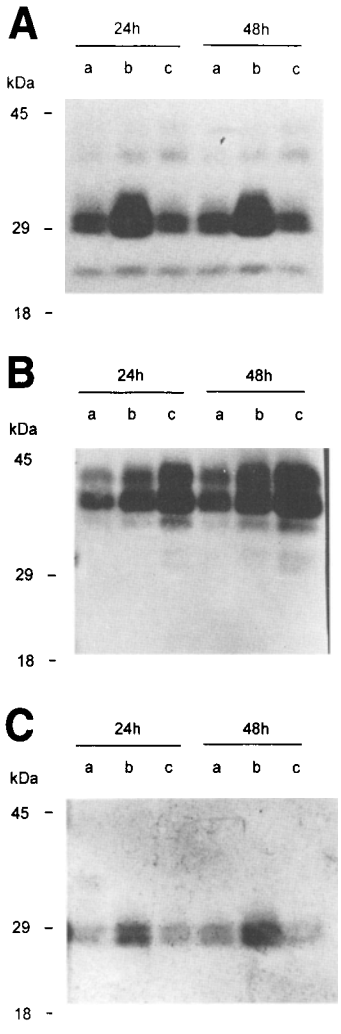

Fig. 2. The effect of TGF-β2 or RA on the IGFBP profile in PC-3 cell-conditioned media. PC-3 cells were treated with TGF-β2 (5 ng/mL) or RA (1 μ*M*) for 24 or 48 h, and the CM collected and subjected to analysis by Western ligand blot and immunoblot. The molecular weights, in kilodaltons, are indicated on the left-hand side of each panel. For each panel: a, control (untreated cells); b, TGF-β2-treated cells; c, RA-treated cells. (**A**) Western ligand blot; this blot, which is representative of at least three similar experiments, was deliberately overexposed to X-ray film to permit visualization of all bands. Shorter exposures were used when quantitation by densitometry was required. (**B**) Immunoblot using antihuman IGFBP-3. (**C**) Immunoblot using antihuman IGFBP-5.

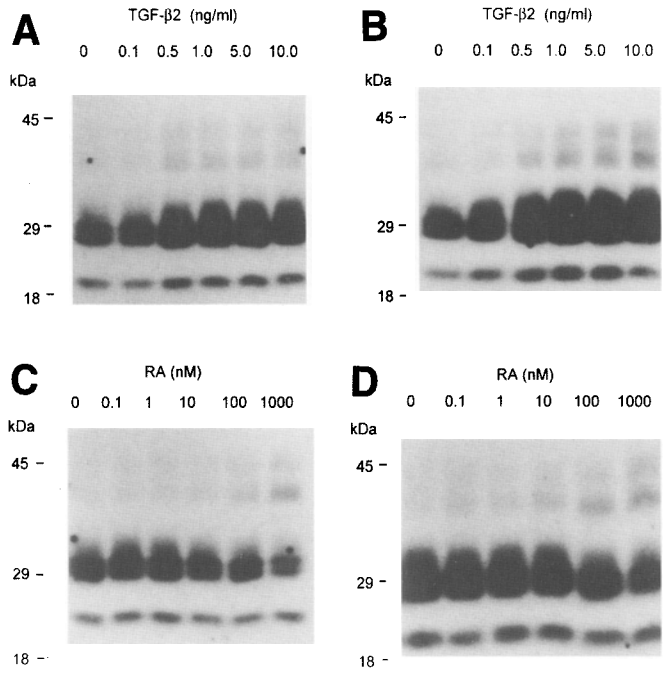

Fig. 3. Dose-dependent effect of TGF-β2 or RA on IGFBP-3 and 5 concentrations in PC-3 cells. Western ligand blots of CM collected from PC-3 cells treated with increasing concentrations of TGF-β2 or RA for 24 or 48 h. Blots have been deliberately overexposed in order to visualize the fainter bands. For densitometric quantitation, the Western ligand blots were exposed to X-ray film for a shorter period of time. Molecular-weight standards (kilodaltons) are indicated on the left-hand side of each panel. (**A**) Cells exposed to 0–10 ng/mL TGFβ2 for 24 h; (**B**) as in A, except cells were treated for 48 h; (**C**) cells exposed to 0–1000 n*M* RA for 24 h; (**D**) as for C, except cells were treated for 48 h.

trol concentrations (untreated cells). As observed in ligand blots, RA reduced the concentrations of IGFBP-5 below control levels in CM collected at 48 h. It is of note that no proteolytic fragments of IGFBP-3 or -5 were detected, under either basal or stimulated conditions.

PC-3 cells were treated with varying concentrations of TGF-β2 (0–10 ng/mL), or RA (0–1000 n*M*) for 24h or 48h, to see if the effects on the IGFBP profiles were dose-dependent. The results of the Western ligand blot analyses are shown in Fig. 3. TGF-β2 increased the concentrations of both IGFBP-3 and -5 in the CM in a dose-dependent manner (Fig. 3, panels A and B). This increase was detectable after both 24 h and 48 h of exposure to TGF-β2. The relative increases in concentration of IGFBP-3 in CM collected at 48 h ranged from a 6-fold increase in cells treated with 0.5 ng/mL TGF-β2, to a 14-fold increase in cells exposed to 10 ng/mL TGF-β2 (Fig. 4A). Concomitantly, IGFBP-5 concentrations were increased from five-fold (0.5 ng/mL TGF-β2) to nine-fold (5 and 10 ng/mL TGF-β2). In cells treated with RA, the increase in concentration of IGFBP-3 in CM was observed at RA concentrations as low as 0.1 n*M* (Fig. 3, panels C and D). At an RA concentration of 1000 n*M*, a 12-fold increase in IGFBP-3 was found (Fig. 4B). Unlike TGF-β2, which also stimulated IGFBP-5, and consistent with the results shown earlier, RA at concentrations of 100 and 1000 n*M* resulted in up to a 50% decrease in IGFBP-5 in CM (Fig. 4B).

The effects on IGFBP-3 and -5 concentrations in CM of PC-3 cells resulting from treatment with either TGF-β2 (5 ng/mL) or RA (1 m*M*) were detectable at 24 h *(see* above*)*. Earlier time points taken showed that the alterations in IGFBP-3 and -5 concentrations were detectable in CM as early as 8 h after treatment (data not shown).

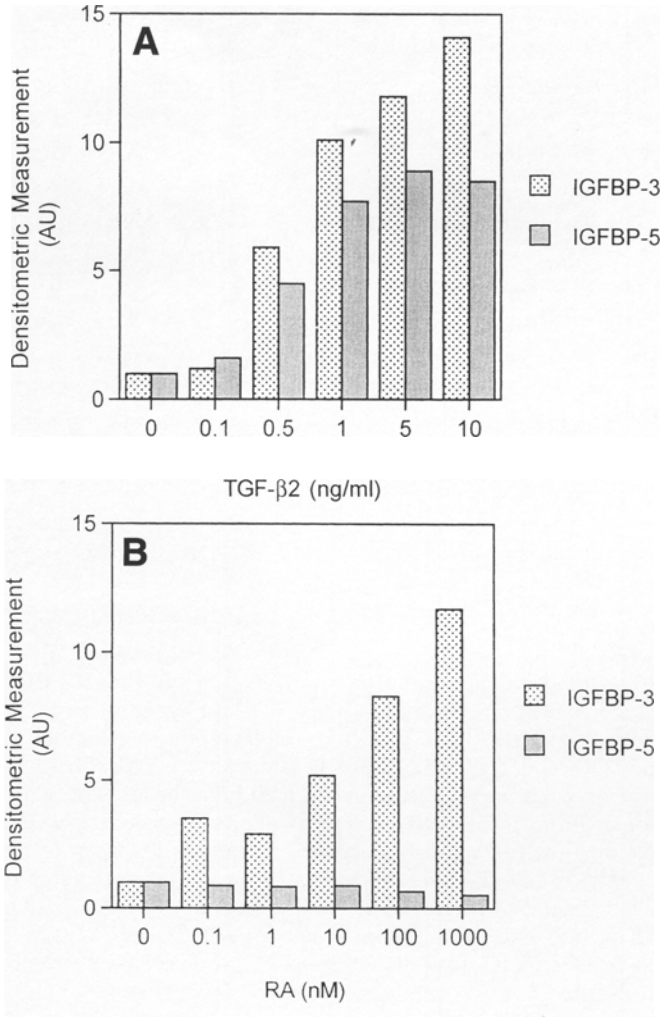

Fig. 4. Densitometric determination of the concentrations of IGFBP-3 and -5 in PC-3 cells treated for 48 h with TGF-β2 **(A)** or RA **(B)** compared to untreated cells (*see* Fig. 3). The bar graph is representative of two similar experiments. The concentration of IGFBP-3 or -5 in control (untreated) cells was given an arbitrary unit (AU) of 1, and the effects of TGF-β2 or RA are shown relative to control cells. (A) Dose effect of TGF-β2 on IGFBP-3 and 5. (B) Dose effect of RA on IGFBP-3 and -5.

As a comparision to the effects seen with TGF-β2 and RA, cells were also treated with varying concentrations of IGF-I (0–100 ng/mL), IGF-II (0–100 ng/mL), or EGF (0–100 ng/mL), all of which are known to induce epithelial cell proliferation. Interestingly, the IGFBP profiles in the CM of PC-3 cells did not significantly change under these conditions (data not shown). However, at high concentrations of IGF-I (100 ng/mL) or IGF-II (100 ng/mL), a slight increase in IGFBP-5 was detected (data not shown).

TGF-β2 and RA Affects IGFBP-3 and -5 mRNA Levels

To determine whether the changes observed at the protein level were reflective of increased mRNA, Northern blot analysis of PC-3 cells treated with TGF-β2 (5 ng/mL) or RA (1 μ*M*) for 8–25 h was performed; the results are shown in Figs. 5 and 6. Consistent with the increase in

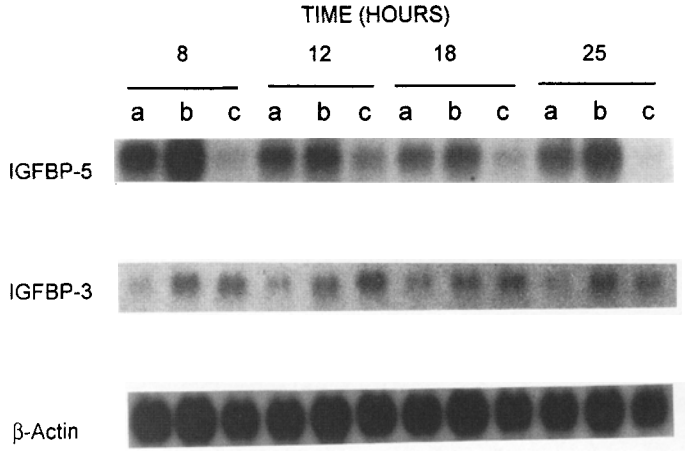

Fig. 5. Northern blot analysis of the effect of TGF-β2 or RA on IGFBP-3 and 5 mRNA levels over time. Total RNA was extracted and processed as indicated in the Materials and Methods section. The IGFBP-5 mRNA (6 kb), IGFBP-3 mRNA (2.6 kb), and β-actin mRNA (1.4 kb) are as indicated. For each time-point: a, control (untreated) cells; b, TGF-b2 (5 ng/mL) treated cells; c, RA (1 μM) treated cells. Time-course experiments were done at least two times under each condition.

IGFBP-3 detected in the CM, increases in IGFBP-3 mRNA were also observed in cells treated with TGF-β2 or RA (Fig. 5A). The increase in IGFBP-3 mRNA was detectable within 8 h of treatment with either TGF-β2 or RA. In TGF-β2 treated cells, IGFBP-3 mRNA levels were ninefold higher than control mRNA concentrations at the 25 h time point (Fig. 6A). IGFBP-3 mRNA from RA-treated cells was six-fold higher at the same time-point (25 h).

IGFBP-5 mRNA was also affected by exposure of PC-3 cells to TGF-β2 or RA (Fig. 5B). In cells treated with TGF-β2, an increase in IGFBP-5 mRNA of up to twofold was detected (Fig. 6B). In contrast, RA-treated cells showed a decrease in IGFBP-5 mRNA, by as much as 80% compared to control cells. The effects of both TGF-β2 and RA on IGFBP-5 mRNA were detected within 8 h of exposure.

Discussion

In many cell types, the concentrations of secreted IGFBPs are regulated by treatment of cells with mitogenic factors, such as IGFs and EGF. For example, IGFBP-5 expression is increased in a dose-dependent manner on stimulation by IGF-I or IGF-II in rat articular chondrocytes *(32)* and in porcine aortic smooth muscle cells *(33)*; in breast cancer cell lines, IGF-I increases the levels of IGFBP-2, -3, and -5 in conditioned media *(34)*; and in cervical epithelial cells, EGF was shown to reduce IGFBP-3 production *(35)*. Very little data exist describing such changes in PC-3 cells, although the effects of various growth factors (e.g., IGF-I, IGF-II, EGF, TGF-β) on the proliferation of PC-3 cells have been extensively described *(10,36–38)*. In this study, we show that TGF-β2 and RA, both known

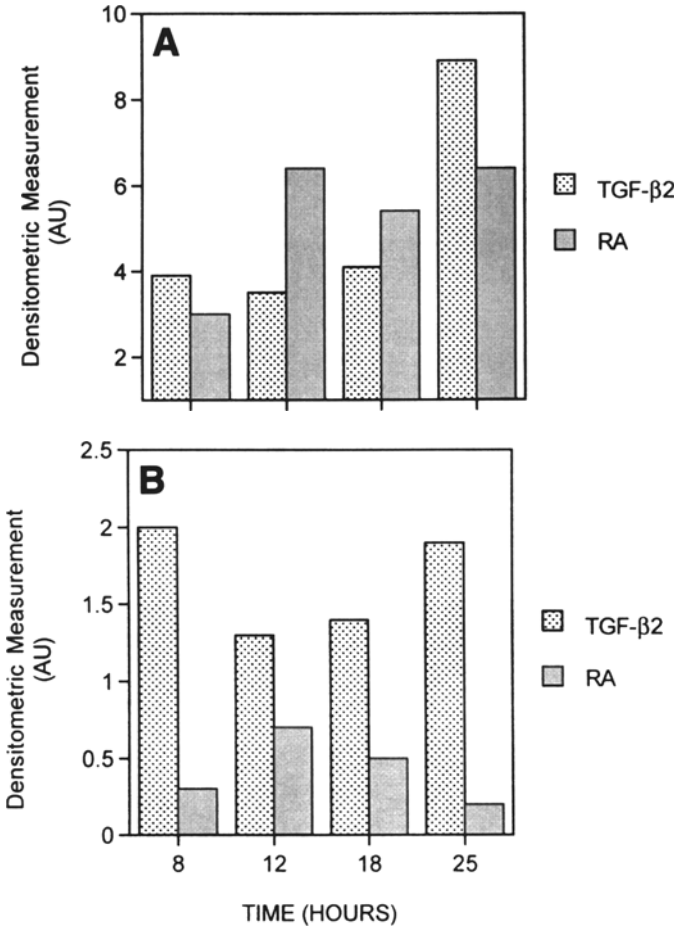

Fig. 6. Summary of the effects of TGF-β2 (5 ng/mL) and RA (1 μ*M*) on IGFBP-3 and 5 mRNA levels shown in Fig. 5, as determined by densitometry. Graphs are representative of three similar experiments. IGFBP-3 and -5 mRNA concentrations from control cells were given an arbitrary unit (AU) of 1 for each time-point and are not shown. Concentrations were also normalized to the β-actin control. (**A**) Effects of TGF-β2 and RA on IGFBP-3 mRNA levels. (**B**) Effects of TGF-β2 and RA on IGFBP-5 mRNA levels.

to inhibit growth of prostate cells, significantly affect the concentrations of detectable IGFBPs, specifically IGFBP-3 and IGFBP-5.

TGF-β2 and RA both positively regulated IGFBP-3 in a dose-dependent manner. The increases in IGFBP-3 concentrations detected in CM of TGF-β2 or RA treated cells were reflective of enhanced steady-state levels of IGFBP-3 mRNA. The effects of TGF-β2 and RA on IGFBP-3 mRNA and protein were detected as early as 8 h after treatment. All together, these results suggest that IGFBP-3 is transcriptionally regulated by both TGF-β2 and RA. However, it is still not entirely clear whether the increase in mRNA, with subsequent increase in protein concentrations, is owing to increased mRNA synthesis, or to increased stability of mRNA. Experiments investigating the half-life of IGFBP-3 mRNA, as well as the requirement for *de novo* synthesis of proteins, in treated and untreated PC-3 cells are under way to clarify this point.

Interestingly, in PC-3 cells, IGFBP-3 can also be regulated posttranslationally, owing to the presence of the urokinase-type plasminogen activator, which can proteolyse IGFBP-3 *(39)*, and it is conceivable that TGF-β2 or RA could inhibit proteolytic degradation of IGFBP-3. However, no obvious proteolysis of IGFBP-3 was detected in this study. Immunoblots using a polyclonal antibody against IGFBP-3 showed that IGFBP-3 detected in the CM of control cells or from cells treated with TGF-β2 or RA was primarily intact. Furthermore, when the CM was subjected to analysis by IGFBP-3 protease assays *(40)*, no IGFBP-3 protease activity was detected in CM, under either basal or stimulated conditions (Hwa, unpublished data). Therefore, no evidence of altered post-translational processing of IGFBP-3, resulting from TGFβ2 or RA exposure, was identified, and the increased protein concentrations appear to be entirely attributable to changes in mRNA levels.

In addition to positively regulating IGFBP-3, TGF-β also positively regulated expression of IGFBP-5 in a dose-dependent manner. To date, only IGF-I and II have been shown to upregulate IGFBP-5 expression *(32,33)*. In bone cells *(41)*, rat skeletal cells, and mouse myocytes *(42)*, TGF-β has been shown to inhibit IGFBP-5 expression. Therefore this is the first demonstration that IGFBP-5 can also be positively regulated by TGF-β. As with IGFBP-3, regulation of IGFBP-5 appeared to be at the mRNA level, since IGFBP-5 mRNA was also increased by TGF-β2 treatment, although the maximal increase was only twofold.

In contrast to TGF-β2, low concentrations of RA had minimal effects on IGFBP-5 expression, but expression was negatively affected by high concentrations of RA (100 n*M* and 1 m*M*). IGFBP-5 concentrations in CM of cells treated with 1 μ*M* RA were reduced by 50% compared to control. As with IGFBP-3, this alteration in IGFBP-5 was not likely owing to proteolysis, since only intact IGFBP-5 was detected in immunoblots. Correlated to the decrease in IGFBP-5 concentrations in CM of RA-treated cells was the observation that IGFBP-5 mRNA levels were reduced to approx 20% of control. Similar observations have been made recently in human osteoblast cells, where it was shown that RA downregulated IGFBP-5 expression at both the mRNA and protein levels, but simultaneously upregulated expression of IGFBP-3, -4, and -6 *(43)*. RA has also been shown to decrease IGFBP-5 mRNA levels in T47D human breast cancer cells *(44)*. Therefore, RA differentially regulates IGFBPs in a number of systems, including prostate cancer cells.

The biological significance of the regulation of IGFBP-3 and -5 by either TGF-β2 or RA in PC-3 cells has yet to be determined. TGF-βs are known negative regulators of epithelial cell growth *(4)*, and RA also has been shown to inhibit proliferation of many cell lines *(45,46)*.

Only in breast cancer cell lines, such as Hs578T and MDA231, has it been clearly demonstrated that TGF-β2-dependent cell growth inhibition is mediated by increased expression of IGFBP-3 *(18,22)*. When TGF-β-induced IGFBP-3 transcription was specifically blocked by IGFBP-3 antisense oligonucleotides, cell growth was no longer inhibited by TGFβ. Furthermore, the inhibition in HS578T cells is owing to specific binding of IGFBP-3 to the cell surface and is IGF-independent *(47)*. Gucev et al. *(18)* similarly showed that the growth inhibitory effect of RA on breast cancer cells is also mediated by regulation of IGFBP-3 gene transcription. In the prostate, the mechanism of TGF-β and RA effects on prostate cell growth are not known. However, the observation that these two factors regulate IGFBP expression suggests that these regulated proteins may play a central role(s) in the control of cell proliferation.

Of the IGFBPs detectable in PC-3 cells, only IGFBP-3 was increased by both TGF-β2 and RA. It is, therefore, possible that the growth-inhibitory effect of both TGF-β2 and RA is via a common mechanism that involves IGFBP-3. As has been shown for breast cancer cells, IGFBP-3 could inhibit cell growth through an IGF-independent pathway *(47)*, or, since PC-3 cells produce low concentrations of IGF-II, which acts as an autocrine growth factor under serum-free conditions *(39)*, the increased IGFBP-3 could bind the endogenously produced IGF-II, and thus modulate its availability for cell growth. However, it is also possible that TGF-β2 inhibits PC-3 growth by a different pathway, involving IGFBP-5, since TGF-β2 also increased IGFBP-5 expression in PC-3 cells. It is of interest to note that in a rat model for prostate cancer, castration was shown to induce de novo synthesis of IGFBP-5 *(48)*. Furthermore, recent *in situ* hybridization and immunohistochemistry studies of human prostate tissues indicate that IGFBP-5 expression was elevated in prostate adenocarcinoma *(25)*. The biological significance of increased IGFBP-5 expression in prostate malignancy remains to be elucidated.

In summary, we have shown that in PC-3 cells TGF-β and RA both regulate expression of IGFBP-3 and -5 mRNA and protein. Further clarification of the roles IGFBP-3 and IGFBP-5 play in TGF-β and RA regulation of PC-3 growth should increase our understanding of the way in which these factors modulate prostate growth.

Materials And Methods

Growth Factors

Recombinant human IGF-II was provided by Eli Lilly Research Laboratories (Indianapolis, IN). Recombinant human IGF-I was obtained from Bachem (Torrance, CA) and recombinant TGF-β2 was a generous gift of Celtrix, Inc. (Santa Clara, CA). TGF-β1 was purchased from Austral Biologicals (San Ramon, CA). EGF and all-trans-RA were purchased from Sigma Chemical Corporation (St. Louis, MO). Iodination of IGF-I and IGF-II to a specific activity of 350–500 μCi/μg was performed by a modification of the chloramine-T technique *(49)*.

Cell Culture

The human prostate adenocarcinoma cell line, PC-3, was obtained from ATCC (Rockville, MD). PC-3 cells *(31)* are prostate epithelial cells that are androgen-independent. The cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 IU/mL and Streptomycin 100 μg/mL). All tissue culture media were from Gibco-BRL (Grand Island, NY). Cells were grown at 37°C in an atmosphere of 5% carbon dioxide.

Preparation of CM

Exponentially growing cells were trypsinized and seeded in 12-well plates and grown until 95% confluent in serum-containing medium. Cells were then washed with phosphate-buffered saline (PBS, Gibco-BRL), and switched to serum-free RPMI-1640 for 16 h of incubation. Fresh serum-free RPMI-1640 (0.5 mL) was then added, with or without growth factor supplements, and further incubated for the indicated times. The growth factors, when required, were added to the following final concentrations: IGF-I, 0–100 ng/mL; IGF-II, 0–100 ng/mL; EGF, 0–100 ng/mL; TGF-β2, 0–10 ng/mL; TGF-β1, 0–10 ng/mL; and RA, 0–1000 n*M*. The resultant CM was collected, centrifuged at 1000*g* for 5 min to remove cell debris, and kept frozen at –20°C. The conditioned media were analyzed for IGFBPs by both Western ligand blotting or immunoblotting.

Western Ligand Blots

Proteins from CM samples were size fractionated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, and electophoretically transferred (Semi-Transphor, Hoeffer, San Francisco, CA) to nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell, Keene, NH). The membranes were incubated overnight with 1.0×10^6 cpm of ^{125}I-labeled IGF-II and 1.0×10^6 cpm of ^{125}I-labeled IGF-I, washed, dried, and exposed to film, as previously described *(50)*. Densitometric analysis was performed using ImageMaster Software for 1-D evaluation from Pharmacia LKB Biotechnology (Piscataway, NJ).

Immunoblots

CM samples were run on SDS polyacrylamide gels and electroblotted to nitrocellulose membranes in the same way as for Western ligand blots. The membranes were blocked with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) made up in Tris-buffered saline (TBS), pH 7.6; 20 μ*M* Tris base, 137 μ*M* sodium chloride, adjusted to pH 7.6 with 1 *M* hydrochloric acid). Primary antibodies, rabbit antirecombinant IGFBP-3 *(26,51)* diluted

1:1000 in 1% BSA, rabbit antihuman IGFBP-5 (Austral Biologicals, San Ramon, CA), diluted 1:750, or rabbit antihuman IGFBP-2 *(52)* diluted 1:500, were then added to the membranes for 2 h (room temperature with gentle shaking). The membranes were washed four times with TBS before the addition of the secondary antibody (goat antirabbit IgG alkaline phosphatase conjugate; Promega, Madison, WI). After 1 h of incubation at room temperature, the membranes were developed with a mixture of 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitroh-blue tetrazolium (NBT), according to the manufacturer's instructions (Promega). The reaction was stopped with PBS containing 20 m*M* EDTA.

Northern Blots

PC-3 cells were grown to 95% confluency in 35 mm wells (6-well trays; Costar Corporation, Cambridge, MA) and treated with TGF-β2 (5 ng/mL) or RA (1 μ*M*) for the indicated times before the RNA extraction process. Total cellular RNA was isolated using RNA STAT-60 (TEL-TEST "B," Inc., Friendswood, TX) according to the manufacturer's instructions. Samples of isolated RNA (20 μg) were electrophoresed in 1.2% agarose-formaldehyde gels and transferred to nylon membranes (GeneScreen Plus, NEN Research Products, Boston, MA) by capilliary wicking. Prehybridizations and hybridizations were done using solutions purchased from 5 Prime-3 Prime, Inc. (Boulder, CO), according to the manufacturer's protocol. The probes used were a 2.5 kb *Eco*RI human IGFBP-3 cDNA *(53)*, and a 317-bp *Sac*II-*Sac*I fragment of the human IGFBP-5 cDNA *(54)*. The cDNA fragments were gel-purified using the QiaexII agarose gel extraction kit (Qiagen Inc., Chatsworth, CA), and the DNA was labeled with the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA) using ^{32}P α-dCTP (NEN). Band densities were analyzed using the area under the curve as calculated using the ImageMaster Software for 1-D evaluation from Pharmacia LKB Biotechnology (Piscataway, NJ).

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