

Combination Therapy of Insulin-Like Growth Factor Binding Protein-3 and Retinoid X Receptor Ligands Synergize on Prostate Cancer Cell Apoptosis *In vitro* and *In vivo*

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Abstract We have previously identified the retinoid X receptor- α (RXR α) as an insulin-like growth factor binding protein-3 (IGFBP-3) nuclear binding partner, which is required for IGFBP-3-induced apoptosis. In the current study, we investigated the biological interactions of the RXR ligand, VTP194204 and rhIGFBP-3, *in vitro* and *in vivo*. *In vitro*, IGFBP-3 and VTP194204 individually induced apoptosis, and suppressed cell growth in prostate cancer cell lines in an additive manner. *In vivo*, LAPC-4 xenograft-bearing severe combined immunodeficiency mice treated daily with saline, IGFBP-3, and/or VTP194204 for 3 weeks showed no effect of individual treatments with IGFBP-3 or VTP194204 on tumor growth. However, the combination of IGFBP-3 and VTP194204 treatments inhibited tumor growth by 50% and induced a significant reduction in serum prostate-specific antigen levels. In terminal nucleotidyl transferase-mediated nick end labeling immunohistochemistry of LAPC-4 xenografts, there was modest induction of apoptosis with either IGFBP-3 or VTP194204 individual treatment, but combination therapy resulted in massive cell death, indicating that IGFBP-3 and VTP194204 have a synergistic effect in preventing tumor growth by apoptosis induction. In summary, this is an initial description of the successful therapeutic use of IGFBP-3 as a cancer therapy *in vivo*, and shows that combination treatment of IGFBP-3 and RXR ligand has a synergistic effect on apoptosis induction leading to substantial inhibition of prostate cancer xenograft growth. Taken together, these observations suggest that combination therapy with IGFBP-3 and RXR ligands may have therapeutic potential for prostate cancer treatment.

The insulin-like growth factor (IGF) system is well recognized to play a critical role in cancer development. In particular, IGF binding proteins (IGFBP) have been proposed to act as growth inhibitory molecules through their IGF inhibitory effects. However, a direct IGF-independent role of IGFBPs, particularly IGFBP-3 in the regulation of apoptosis, has emerged over the last decade (1). A key intracellular role for IGFBP-3 was shown in 2000 with the discovery of the nuclear retinoid X receptor α (RXR α) as a binding partner for IGFBP-3. In that study, IGFBP-3 cotransfections potently and dose-dependently inhibited retinoic acid signaling via retinoic acid response element, but enhanced RXR-specific ligand signaling via the retinoid X

response element, supporting a co-activator/repressor role for IGFBP-3 in transcription. In addition, IGFBP-3 had no discernible effects in an RXR knock-out line, indicating that RXR is required for IGFBP-3-induced apoptosis. In both androgen-dependent and -independent prostate cancer cell lines, additive effects of IGFBP-3 and RXR ligand on apoptosis were shown (2).

Retinoids (including RXR ligands) are prime candidates for cancer chemoprevention because cancer is characterized by abnormal growth with lack of differentiation, which could be modified by retinoids. The nuclear retinoic acid receptors and RXRs mediate the biological effects of the retinoids via transcriptional regulation (3) and various ligands have been used in prostate cancer models to induce apoptosis (4–6).

Because both IGFBP-3 and RXR ligands have shown efficacy *in vitro* on cancer cell apoptosis, we undertook a human xenograft mouse model to assess these effects *in vivo*. We show significant reduction in tumor weight by combination treatment associated with a significant increase in tumor apoptosis, and show the initial therapeutic use of IGFBP-3 and RXR ligand as cancer therapy.

Materials and Methods

Materials. Recombinant human IGFBP-3 was generously provided by D. Mascarenhas of Celtrix, Inc. (Mountain View, CA). Vitae Pharmaceuticals (Irvine, CA) provided the RXR ligand VTP194204

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Received 10/21/04; revised 4/4/05; accepted 4/13/05.

Grant support: Supported in part by NIH grants R01AG20954, P50CA92131, and R01CA100938, a CaP CURE/Prostate Cancer Foundation award (P. Cohen) as well as a fellowship award from the Giannini Foundation (K-W. Lee).

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Note: Presented in part in the Endocrine Society Meeting in New Orleans 2003.

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(7). Commercial antibodies included anti-human IGFBP-3 from DSL (Webster, TX). SDS-PAGE reagents, Tween, and fat-free milk were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were from Amersham (Sunnyvale, CA). All other chemicals were from Sigma-Aldrich, St. Louis, MO.

Tissue culture. 22RV1 cells were from American Type Culture Collection (Manassas, VA), and maintained in DMEM containing 10% FCS (Life Technologies, Carlsbad, CA), 100 units/mL of penicillin, and 100 units/mL of streptomycin in a humidified environment with 5% CO₂. LAPC-4 cells were a kind gift of Dr. C. Sawyers (University of California at Los Angeles) and cultured in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 1% L-glutamine, 1 nmol/L R1181 (NEN Life Science Products, Boston, MA).

Proliferation assays. Cells (1,000 cells/cm²) were seeded on 96-well plates. Following overnight attachment of the cells, each well was washed with 100 μ L PBS, serum-starved for 2 hours, then incubated with 100 μ L of experimental conditions. CellTiter 96 AQ nonradioactive cell proliferation assay (Promega Corp., Madison, WI) was done according to the manufacturer's instructions. The assay uses a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Owen's reagent, a tetrazolium compound) and phenazine methosulfate, an electron-coupling reagent. Twenty microliters of this solution were added to each well. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bioreduced to soluble formazan by metabolically active cells, which we quantified by ELISA. All experiments were done in triplicate. Reaction products in each 96-well plate were quantified using the Bio-Rad Laboratories, model 3550-UV microplate reader. Mean \pm SD values of the raw absorbance detected at 490 nm were plotted.

Caspase assays. For caspase assays, cells were plated at equal density (104 cells/mL) on 96-well plates (Costar, Corning, NY) in medium containing 10% fetal bovine serum and antibiotics. Cells were cultured overnight and changed to serum-free medium for the experiments. rhIGFBP-3 was used at the indicated concentrations. A caspase 3/7-specific fluorometric assay (Apo-ONE, Promega) was used according to the manufacturer's instructions to measure the degree of apoptosis induction by IGFBP-3, RXR ligand, and combination thereof. The Caspase-Glo 8 assay system (Promega) was used to measure caspase 8 activation.

Tumor xenografts. The LAPC-4 human prostate cancer cell line expresses a wild-type androgen receptor and secretes prostate-specific antigen (PSA). LAPC-4 xenograft tumors were generated by injection of 1×10^6 cells in 200 μ L mixed at a 1:1 dilution with Matrigel in the right flank of male severe combined immunodeficiency mice. Tumors were established for 2 weeks before the start of treatment. Ten severe combined immunodeficiency mice with LAPC-4 tumors were treated daily with saline, IGFBP-3 (4 mg/kg/d), RXR ligand (VTP 194204, 4 mg/kg/d), or combination given by daily i.p. injections. The length and width of the mass located at the site of injection of the LAPC-4 cells were measured with calipers and recorded once a week. The mice were euthanized at 21 days. Tumors were harvested, weighed, fixed in formaldehyde, and embedded in paraffin. At the termination of tumor xenograft experiment, blood was collected by an intracardiac route from mice in heparinized tubes, and plasma was separated for analyses. Animal care was in accordance with current regulations and standards of the NIH, as well as our institutional guidelines for animal care.

Terminal nucleotidyl transferase-mediated nick end labeling and proliferating cell nuclear antigen staining. Paraffin-embedded sections were prepared from LAPC-4 tumors harvested on day 21. After deparaffinization of tissue section, apoptotic DNA fragments were labeled by terminal deoxynucleotidyl transferase, and detected by antidigoxigenin antibody conjugated to fluorescein (ApopTag fluorescein *in situ* apoptosis detection kit, Chemicon, Temecula, CA). Cells were examined at $\times 20$ using an inverted fluorescence microscope (Axiovert 135M, Carl Zeiss, New York, NY). Apoptotic staining was quantified by pixel histogram (Adobe Systems, Mountain View, CA) and confirmed by manual counting ($r = 0.98$) by counting

the positive cells (brown-stained), as well as the total number of cells in 10 arbitrarily selected fields by an independent observer. Proliferating cell nuclear antigen antibody was from Zymed Laboratories (South San Francisco, CA). Indirect immunohistochemistry was done with Vectastain ABC kit (Vector Labs, Burlingame, CA) using 3,3'-diaminobenzidine as a chromogen and quantitated as per terminal nucleotidyl transferase-mediated nick end labeling assay above.

Quantitation of serum human PSA and mouse IGF-I. PSA ELISA was obtained from American Qualix (San Clemente, CA) and done according to the manufacturer's instructions. Briefly, 25 μ L of sample serum was incubated in plastic wells coated with anti-PSA antibodies. Horseradish peroxidase conjugate was added, unbound conjugate was washed from the wells and chromogen solution added. Absorbance is read at 450 nm. Mouse IGF-I was quantitated with mouse IGF-I RIA (Diagnostic Systems Laboratories, Webster, TX).

Statistical analysis. All *in vitro* experiments were repeated at least thrice. Means \pm SD are shown. Statistical analyses were done using ANOVA tests using InStat (GraphPad, San Diego, CA). Differences were considered statistically significant when $*P < 0.05$ and when $**P < 0.005$.

Results

Additive inhibitory effects of RXR ligand and IGFBP-3 on CaP proliferation are associated with an induction of apoptosis. The effects of IGFBP-3 and RXR ligand treatment alone and in combination on 22RV1 CaP proliferation at 24 hours was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the results are shown in Fig. 1A. Proliferation of 22RV1 cells was not significantly affected by IGFBP-3, RXR ligand, or combination thereof at 24 hours. However, growth of 22RV1 CaP cells was significantly reduced (to about 80% of control) after a 72-hour incubation with 1 mg/mL of IGFBP-3 (Fig. 1B). RXR ligand (500 nmol/L; VTP194204) treatment alone also resulted in inhibition of cell growth (to about 70% of control). Additionally, combination treatment with both compounds resulted in a >50% inhibition of cell growth over control.

Next, we addressed the question of whether the growth-suppressive effects of this particular RXR ligand and IGFBP-3 correlated with apoptosis induction in this cell line, which we had previously reported in the CaP cell lines, LAPC-4 and LNCaP, using the RXR ligand LG1069 (2). Apoptosis was assessed using an apoptosis ELISA assay as previously described (8, 9). Incubation of 22RV1 CaP with either agent alone for 24 hours increased the level of apoptosis in 24 hours. Notably, there is a synergistic effect (2- to 3-fold induction) of IGFBP-3 and RXR ligand in combination with CaP apoptosis after 24 hours (Fig. 1C). This effect is reflected, albeit to a smaller degree, at 48 hours (Fig. 1D).

Nonactivation of the extrinsic apoptotic pathway by IGFBP-3. Caspase activation by IGFBP-3 has now been described in several papers (8, 9). Specifically, IGFBP-3-induced apoptosis in MCF-7 breast cancer cells was suggested to involve a caspase 8-dependent (extrinsic), mitochondria-independent pathway (9). We assessed the death receptor-dependent extrinsic pathway in 22RV1 CaP cells by assessing caspase 3/7 as well as caspase 8 activation. As shown in Fig. 2A, a 24-hour treatment of either IGFBP-3 or RXR ligand as single agents significantly induced caspase 3/7 activity. Cotreatment induced a 3-fold increase of caspase activation in the same time period. The extrinsic pathway (death receptor activation of caspase 8)

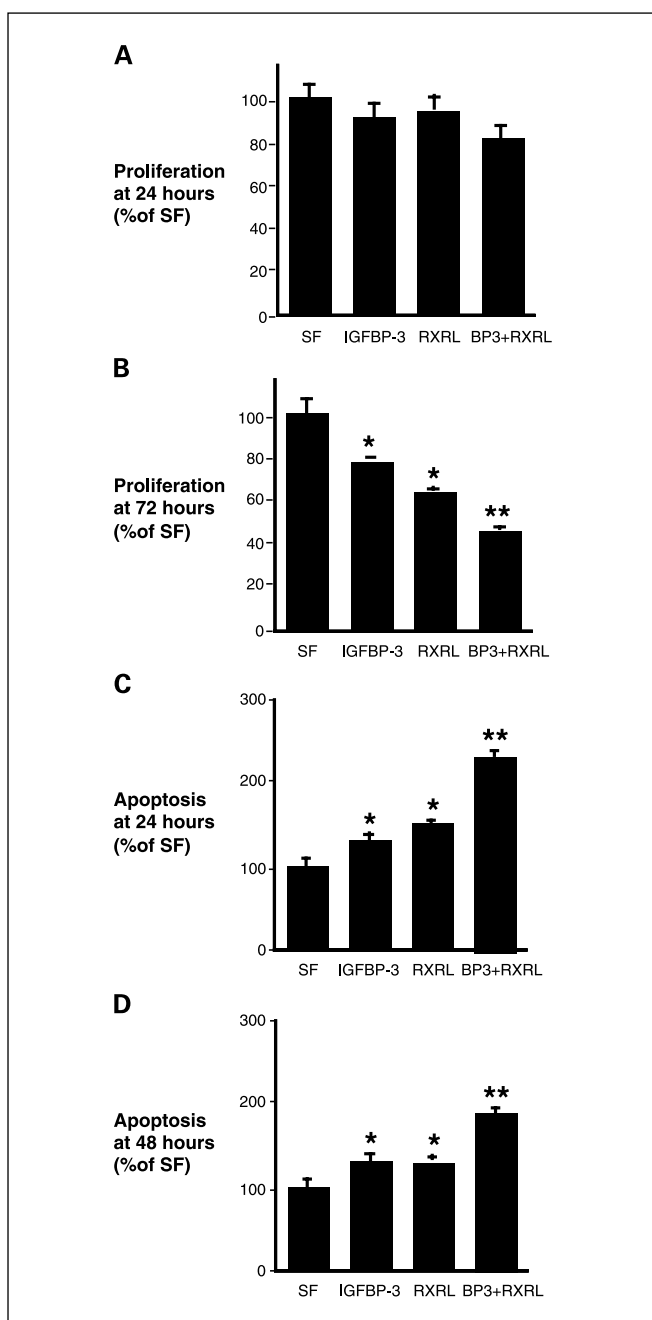


Fig. 1. Additive inhibitory effects of RXR ligand and IGFBP-3 on CaP proliferation are associated with an induction of apoptosis. *A*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay of 22RV1 CaP cells. Cells were treated with or without IGFBP-3 (1 μ g/mL), RXR ligand (500 nm), or the combination for 24 hours. Values are expressed as the percentage of serum-free (SF) medium. *B*, corresponding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay at a 72-hour time point. *, $P < 0.05$ compared with SF; **, $P < 0.005$ compared with SF. *C*, apoptosis ELISA assay of 22RV1 CaP cells at 24 hours. Cells were treated with or without IGFBP-3 (1 μ g/mL), RXR ligand (500 nm), or the combination for 24 hours. Notably, there is a synergistic effect of IGFBP-3 and RXR ligand on apoptosis. *, $P < 0.05$ compared with SF; **, $P < 0.005$ compared with SF. *D*, corresponding apoptosis ELISA assay of 22RV1 CaP cells at 48 hours.

was evaluated using luminometric caspase activation assay. As shown in Fig. 2B, there were no differences in caspase 8 activation by IGFBP-3, RXR ligand, or combination thereof. These results indicate that in 22RV1 prostate cancer cells,

caspase activation by IGFBP-3 is not via a death receptor-related pathway but involves the intrinsic (mitochondrial) apoptosis pathway.

Combination therapy of IGFBP-3 and RXR ligand inhibits the growth of LAPC-4 prostate cancer xenografts and reduces serum PSA levels. To examine the effect of IGFBP-3, RXR ligand, and combination treatment on tumor cell growth, animals with established androgen-dependent LAPC-4 prostate cancer xenografts were given saline, IGFBP-3 (4 mg/kg/d i.p.), RXR ligand (4 mg/kg/d i.p.), or combination thereof for 3 weeks. At this dose, no lethal toxicity or weight loss (>10% of body weight) was observed among treated animals (data not shown). Single therapy with either IGFBP-3 or RXR ligand did not show any significant reduction in calculated tumor volume over the treatment period (Fig. 3A). However, combination treatment with both IGFBP-3 and RXR ligand resulted in significant tumor size inhibition (33% growth inhibition, $P < 0.005$; $n = 10$) relative to control animals (Fig. 3A). A greater effect (55% inhibition) was seen for combination therapy in tumor weight (Fig. 3B). These studies show that combination therapy of IGFBP-3 and RXR ligand inhibits the growth of androgen-dependent LAPC-4 prostate cancer xenografts.

PSA secretion from tumors is an important marker of prostate cancer disease activity in humans and is a feature of

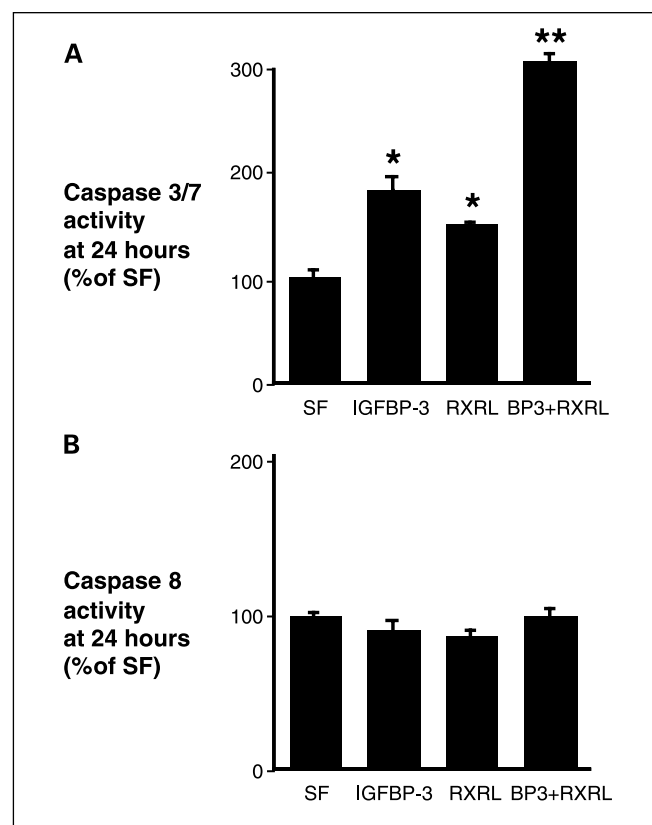


Fig. 2. Nonactivation of the extrinsic apoptotic pathway by IGFBP-3. *A*, fluorometric assessment of caspase 3/7 activation by IGFBP-3. Cells were treated with or without IGFBP-3 (1 μ g/mL), RXR ligand (500 nm), and combination for 24 hours. Values are expressed as percentage of serum-free. *, $P < 0.05$ compared with SF; **, $P < 0.005$ compared with SF. *B*, luminometric assessment of caspase 8 activation by IGFBP-3. Cells were treated with or without IGFBP-3 (1 μ g/mL), RXR ligand (500 nm), and combination for 24 hours. Values are expressed as percentage of serum-free.

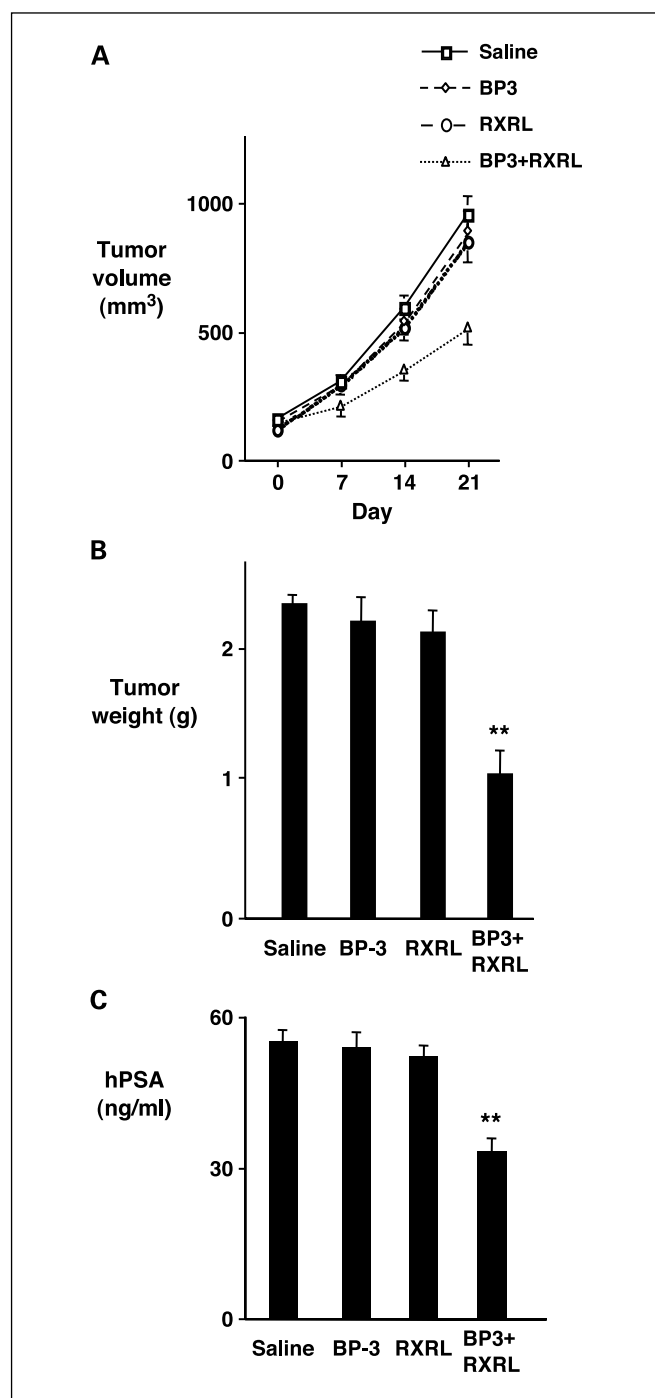


Fig. 3. Combination therapy of IGFBP-3 and RXR ligand inhibits the growth of LAPC-4 prostate cancer xenografts and reduces serum PSA levels. *A*, calculated tumor volume of LAPC-4 CaP xenografts treated with or without IGFBP-3 (4 mg/kg/d), RXR ligand (4 mg/kg/d), and combination for 3 weeks. **, $P < 0.005$ as compared with saline treatment. *B*, tumor weights of LAPC-4 CaP xenografts in this same experiment. *C*, serum PSA levels as measured by ELISA in treatment groups.

the LAPC-4 system. As shown in Fig. 3C, animals that were treated with IGFBP-3/RXR ligand had reduced PSA serum levels (33 ± 3.3 ng/mL, $n = 10$) as compared with vehicle-treated mice (55.7 ± 3.3 ng/mL, $n = 10$). Single therapy with either agent alone was not significantly different from that of control animals in the time interval tested. The observed significant

decrease in serum PSA levels is most likely a consequence of the decrease in tumor size in combination therapy-treated animals, because LAPC-4 cells are the only source of PSA in this model. Serum mouse IGF-I was unchanged among treatment groups compared with saline injection (data not shown).

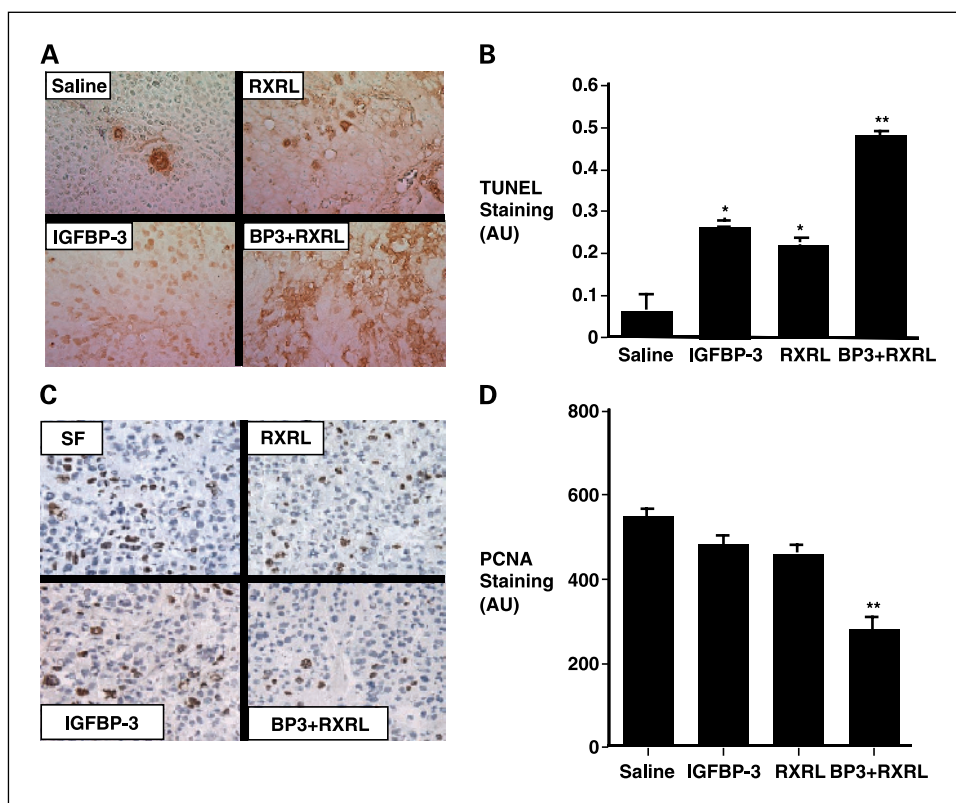
Inhibition of xenograft growth by combination therapy is associated with an increase in apoptosis and decrease in proliferation. The effect of IGFBP-3, RXR ligand, and combination therapy on apoptosis in this xenograft model was examined by light microscopic terminal nucleotidyl transferase-mediated nick end labeling assay. Representative photographs are shown in Fig. 4A. As shown in Fig. 4B, quantitation of terminal nucleotidyl transferase-mediated nick end labeling-positive cells was statistically increased >2-fold in IGFBP-3 and RXR ligand single treatment groups. Furthermore, combination therapy with IGFBP-3 and RXR ligand induced massive apoptosis, resulting in an almost 5-fold increase in apoptosis over saline-treated tumors. We next assessed whether this effect of IGFBP-3 is associated with an *in vivo* alteration in cell proliferation. Microscopic examination of tumor sections stained for proliferating cell nuclear antigen clearly showed a decreased number of proliferating cell nuclear antigen-positive cells in the combination therapy group as compared with the control group (Fig. 4C). Quantification of this data showed that combination therapy decreases proliferation over saline injection by >40%. Whereas single therapy was not significantly different in this time course, the trend was to lower proliferation (Fig. 4D). Taken together, these results suggest the possible involvement of both anti-proliferative as well as apoptotic effects of combination therapy of IGFBP-3 and RXR ligand in the *in vivo* inhibition of prostate tumor growth.

Discussion

The present study shows that combination therapy of IGFBP-3 and RXR ligand inhibits the growth of prostate cancer cells *in vitro* and *in vivo*. The growth-suppressive effects of combination therapy correlated with its ability to induce apoptosis. Although IGFBP-3 has been studied extensively as a proapoptotic agent *in vitro*, this is the first report to use recombinant IGFBP-3 *in vivo* over a substantial period of time, and to show a significant biological effect on tumor growth.

Two recent reviews have profiled population studies examining the association between IGFBP-3 and cancer risk (10, 11) and conclude that data for association is interesting but not consistent, highlighting the fact that this may reflect differences among different cancer types and variation in assays and measurement error in assessing the various IGFBP-3 proteolytic fragments present in serum (12). Nevertheless, the largest nested case-control study to date shows a compelling inverse relationship between serum IGFBP-3 and prostate cancer risk, one that is independent of serum IGF-I levels and becomes even more prominent in late-stage disease (13, 14). Tissue specimens from patients who underwent neoadjuvant hormonal therapy showed a statistically significant increase in IGFBP-3 expression compared with baseline. This increase in expression correlated significantly with the induction of apoptosis, and patients with high IGFBP-3 expression in the post-hormonal therapy specimens had a good prognosis, suggesting that IGFBP-3 is a

Fig. 4. Inhibition of xenograft growth by combination therapy is associated with an increase in apoptosis and decrease in cell proliferation. **A**, terminal nucleotidyl transferase – mediated nick end labeling immunohistochemistry of xenografts, representative slides are shown. **B**, terminal nucleotidyl transferase – mediated nick end labeling pixel histogram quantitation. *, $P < 0.05$; **, $P < 0.005$ as compared with SF. **C**, proliferating cell nuclear antigen immunohistochemistry of xenografts, representative slides are shown. **D**, proliferating cell nuclear antigen pixel histogram quantitation. *, $P < 0.05$; **, $P < 0.005$ as compared with SF.



potentially useful predictor of biochemical recurrence in patients with prostate cancer (15). In addition, a specific IGFBP-3 polymorphism associated with low IGFBP-3 levels in serum is correlated with advanced disease status in prostate cancer (16). In some tumors, IGFBP-3 promoter activity is silenced (17). In animal models, elevated levels of IGFBP-3 are associated with prostate apoptosis following castration (18, 19), as well as prostate tumor-suppressive effects of inositol hexaphosphate (20), grape seed extract (21), silibinin (22), and green tea phenols (23).

The *in vivo* data presented here complements a compendium of *in vitro* data in prostate cancer models, showing that IGFBP-3 induces apoptosis, in a manner that could be unrelated to its IGF binding (24, 25); and that it mediates many tumor-suppressive effects of cytokines and hormones (26). IGFBP-3 is secreted and reuptaken rapidly to the nucleus via distinct endocytic pathways (7) and nuclear localization is mediated by importin- β pathway via a bipartite basic consensus sequence near its COOH terminus (27, 28). Phosphorylation seems to affect its ability to bind IGF-I, but reports conflict on the nature of this effect (29, 30).

Retinoids classically transactivate retinoic acid receptors and retinoid X receptors, although recent reports have described synthetic related molecules that induce cancer apoptosis via transcriptional and nontranscriptional mechanisms involving mitochondrial and nonmitochondrial pathways with caspase activation (31, 32). In our initial observation of IGFBP-3 directly interacting with RXR α , we also showed that LG1069, a RXR ligand, had additive effects with IGFBP-3 on prostate cancer cell apoptosis *in vitro* (2). This same compound was used *in vivo* in two different

mammary tumorigenesis models and shown to be an effective chemopreventive agent without toxicity (33, 34). The specific compound described herein: the novel, high-affinity, highly selective, RXR-retinoid, VTP 194204, has recently been described to suppress human pancreatic cancer cell proliferation (35). In addition, RXR-selective retinoids have been proposed to be useful candidate combination therapy agents, as they may display less toxicity than retinoic acid receptor-selective retinoids (e.g., mucocutaneous irritation, hypertriglyceridemia, and teratogenicity; ref. 36.)

We have previously described enhancement of nuclear localization with RXR ligand. Presumably, this would lead to enhanced signaling via RXR responsive genes. Our initial studies showed endogenous IGFBP-3 colocalized with RXR in both the cytoplasm and nucleus (2). A recent report describes IGFBP-3 association with retinoic acid receptor *in vitro* (37). IGFBP-3 is a potent inducer of apoptosis via several pathways (9). The mechanism of action of IGFBP-3 may involve additional IGF-independent components involving binding to additional partners involved in cancer progression (38), but clearly, RXR ligand synergism is an important feature of IGFBP-3 action.

In this study, we showed that IGFBP-3 and RXR ligands have an *in vivo* synergistic effect on suppressing the growth of prostate cancer xenografts. Both of these agents are in various stages of clinical development and may hold promise as anticancer molecules. Further investigation into these nuclear and extranuclear events, coupled with a better understanding of the IGFBP-3/RXR interaction modulated by specific ligands, may reveal new targets to be exploited in the development of improved combination therapy for prostate cancer.

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