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Ligand for Peroxisome Proliferator-activated Receptor γ (Troglitazone) Has Potent Antitumor Effect against Human Prostate Cancer Both *in Vitro* and *in Vivo*[1]

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ABSTRACT

Troglitazone, a thiazolidinedione derivative, is a widely used antidiabetic drug that binds and activates peroxisome proliferator-activated receptor γ (PPARγ) and enhances insulin sensitivity. It induces differentiation of adipocytes, which highly express PPARγ. We report that human prostate cancer cells expressed PPARγ at prominent levels and normal prostate tissues had very low expression. Dose-response clonogenic assays of the PC-3 prostate cancer cell line with troglitazone showed an antiproliferative effect (ED_{50}, 3×10^{-7} M) and other PPARγ ligands (BRL49653: ED_{50}, 8×10^{-8} M; 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2: ED_{50}, 2×10^{-6} M; ciglitizone: ED_{50}, not reached; indomethacin: ED_{50}, not reached) showed similar effects. Combinations of troglitazone and a ligand specific for either retinoid X receptor or retinoic acid receptor did not show a synergistic effect. Pulse-exposure to troglitazone (10^{-5} M) for different durations showed that 4 days of pulse-exposure to the agent irreversibly inhibited 50% clonal growth of PC-3 cells. Interestingly, PC-3 cells cultured with troglitazone (10^{-5} M) showed dramatic morphological changes both by light and electron microscopy, suggesting that the cells became less malignant. Nevertheless, troglitazone did not affect either the cell cycle or several markers of differentiation. LNCaP cells constitutively produced prostate-specific antigen, and levels were markedly enhanced by all-*trans*-retinoic acid. Troglitazone (10^{-5} M, 4 days) decreased by 50% the levels of prostate-specific antigen produced by these cells. *In vivo* treatment of PC-3 tumors growing in male BNX triple immunodeficient mice with oral troglitazone (500 mg/kg/day) produced significant inhibition of tumor growth ($P = 0.01$). The only objective side effect of troglitazone in mice was the elevation of serum transaminases. Short-term culture of four surgically obtained human prostate cancer tumors with troglitazone (10^{-5} M, 4 days) produced marked and selective necrosis of the cancer cells (about 60%) but not the adjacent normal prostate cells. Taken together, these results suggest that troglitazone may be a useful therapeutic agent for the treatment of prostate cancer, especially in the setting of low disease burden.

INTRODUCTION

Prostate cancer has become the most common malignant disease among men in the United States (1, 2). More than 40,000 men in America die from this malignancy every year, constituting the second leading cause of cancer mortality among males. Although surgical resection or radiotherapy are potentially curative for localized disease, advanced prostate cancer is associated with a poor prognosis. Conventional chemotherapy and radiotherapy are still of limited effectiveness. Blockade of androgen stimulation often leads to either a partial or full remission; however, subsequent relapse often occurs and the disease re-emerges within a few years in a poorly differentiated, androgen-independent form. The shortage of curative therapies for advanced disease has resulted in a large impetus to develop novel therapies.

Induction of differentiation represents an alternative to conventional therapies. Differentiation therapy with ATRA,[3] ligand for RARα, for example, has become one of the standard treatments for acute promyelocytic leukemia (3, 4). Like RARα, other nuclear receptors that regulate differentiation may become the targets for novel therapies of some malignancies.

PPARγ, a member of the nuclear receptor superfamily, functions as a master regulator of adipogenesis (5). PPARγ is highly expressed in adipocytes and induces differentiation of several preadipocyte cell lines (6). Forced expression of PPARγ in fibroblast and myoblast cell lines results in adipocyte differentiation (5, 7). PPARγ and RXRα form a heterodimer, which in the presence of ligand, bind to DNA response elements that help regulate expression of target genes (8–10). Previous studies have shown that PPARγ is activated by selected prostaglandins, prostaglandin-like molecules, and arachidonic acid metabolites (9, 11–15). Studies have also shown that adipocyte differentiation is promoted by CCAAT/enhancer-binding protein transcriptional factors (16).

A new class of antidiabetic drugs termed thiazolidinediones has been developed over the last decade (17) and are now beginning to be widely used for insulin-resistant diabetes mellitus. They improve insulin resistance and reduce elevated plasma glucose, perhaps by stimulating the activity of the glucose transporter in cells (18, 19). Thiazolidinedione has also been identified as a specific ligand for PPARγ (14, 20). When the PPARγ heterodimerizes to RXR and each is bound by their ligands, the complex can prominently transactivate target genes and *in vivo* they can potently lower blood sugar in diabetic mice (21). Recently, studies have shown that thiazolidinediones and RXR agonists can induce terminal differentiation of liposarcoma cells and breast cancer cells *in vitro* (22, 23). These findings suggest that PPARγ and RXR ligands might induce differentiation of other cell types. We have evaluated prostate cancer cells as a potential target for therapy with troglitazone, one of the thiazolidinedione derivatives, because we found that these cells expressed PPARγ. In addition, RAR- and RXR-selective retinoids inhibit proliferation of prostate cancer cells both *in vitro* and *in vivo* (24–26). Therefore, the combined effect of troglitazone and retinoids was also studied. Troglitazone itself demonstrated a powerful antiproliferative effect on very aggressive, androgen-independent PC-3 prostate cancer cells, both *in vitro* and *in vivo* using a murine model; no synergistic activity occurred with a combination of troglitazone and a retinoid. In addition, other PPARγ ligands showed similar effects. Importantly, troglitazone also had a strong necrotic effect on fresh human prostate cancers without any demonstrable activity on normal human prostate tissues *in vitro*. Taken together, troglitazone may be a promising adjuvant in the treatment of prostate cancer.

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[3] The abbreviations used are: ATRA, all-*trans*-retinoic acid; RA, retinoic acid; RAR, RA receptor; PPARγ, peroxisome proliferator-activated receptor γ; RXR, retinoid X receptor; 15dPGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; LDL, low-density lipoprotein.

MATERIALS AND METHODS

Cells and Samples. Prostate cancer cell lines (PC-3, DU145, and LNCaP) and myeloid leukemia cell lines (KG-1 and U937) were obtained from American Type Culture Collection (Rockville, MD) and maintained according to their recommendations. PC-3 and DU145 were maintained in DMEM with 10% FCS. LNCaP, KG-1, and U937 were grown in RPMI 1640 with 10% FCS. Troglitazone, BRL49653 (Parke-Davis/Warner-Lambert), ciglitizone (BIOMOL Research Laboratories, Inc.), indomethacin (Sigma Chemical Co.), and 15dPGJ$_2$ (Calbiochem, La Jolla, CA) were dissolved in a solution containing 50% DMSO and 50% ethanol; 9-*cis*-retinoic acid (Sigma), LG100268 (Ligand Pharmaceuticals, Inc., San Diego, CA), SR11345 (SRI International, Menlo Park, CA), ATRA (Sigma), and ALRT1550 (Ligand Pharmaceuticals, Inc., San Diego) were dissolved in 100% ethanol and applied to cells at a concentration of <0.1% of the media volume. Fresh prostate cancer tissues and adjacent normal prostate tissues (n = 12) were surgically obtained after informed consent from patients. These samples were rinsed and dissected immediately into several pieces (approximately 3 mm × 3 mm × 3 mm) and cultured in DMEM with either 10^{-5} M of troglitazone or culture media with diluant in the presence of 10% FCS for 4 days. These samples were fixed by standard method for subsequent morphological analysis.

Soft Agar Colony Assay. Trypsinized and washed single-cell suspensions of cell lines were enumerated and plated into 24-well flat-bottomed plates using a two-layer soft agar system with a total of 1×10^3 cells/well in a volume of 400 μl/well, as described previously (27). The feeder layer was prepared with agar that had been equilibrated at 42°C. Prior to this step, compounds were pipetted into wells. After 14 days of incubation, the colonies were counted. All experiments were done at least three times using triplicate plates per experimental point.

Pulse-Exposure, Time Course Studies. PC-3 cells were incubated in liquid culture with either 10^{-5} M troglitazone, 10^{-7} M LG100268, or both ligands for various durations. After incubation, these cells were extensively washed several times; viable cells were counted and plated in agar into 24-well plates for colony assay as described previously (28).

Cell Cycle Analysis. Cells (5×10^5) were exposed to either 10^{-5} M troglitazone, 10^{-7} M LG100268, or both ligands for 4 days in liquid culture. Total cells, both in suspension and adherent, were collected, washed, suspended in cold PBS, and stained with trypan blue; both blue and non-blue (viable) cells were counted. The cells were adjusted to 1×10^6 viable cells/ml and fixed in a 2:1 ratio (v/v) in chilled methanol overnight before staining with propidium iodide in the presence of RNase (Promega) as described previously (29). Cell cycle status was analyzed on a Becton Dickinson Flow Cytometer and CellFIT Cell-Cycle Analysis software.

Differentiation Marker Analyses. PSA is secreted by the mature prostate gland, and increased production has been construed as a marker of differentiation (30). PC-3 and LNCaP cells (1×10^5) were plated in six-well dishes in 3 ml of media with either troglitazone (10^{-5} M) or diluant alone (control). Media were harvested, and total cells, both in suspension and adherent, were enumerated at days 4 and 8, and fresh media and troglitazone were added at day 4. PSA levels were measured on days 4 and 8 by a TANDEM-E ELISA method (28) and RT-PCR (31), as described previously. Another marker of differentiation is the cell surface binding protein known as E-cadherin. Increased expression of E-cadherin has been identified as a differentiation marker (32). PC-3 and LNCaP cells were cultured as above, and E-cadherin was examined by immunohistochemistry and Western blot with an antibody to E-cadherin (Transduction Laboratories). To examine for lipid expression, PC-3 and LNCaP cells were cultured as above and stained with oil red O using a standard method (33). In addition, high levels of CD36 expression are found in triglyceride storing and secreting cells such as differentiated adipocytes; therefore, CD36 has been used as a marker of adipose differentiation (34). It can function as a receptor of oxidized low density lipoprotein. PC-3 and LNCaP cells were examined for CD36 expression by flow cytometry using anti-CD36 antibody (Immunotech, Inc.), as described previously (29).

Ultrastructural Analysis. PC-3 cells were incubated either with or without troglitazone as above. Cell pellets were fixed in glutaraldehyde and embedded in Epon blocks for electron microscopy, as described previously (35). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL transmission electron microscope.

RT-PCR. Total RNAs were isolated from prostate cancer cell lines with Trizol (Life Technologies, Inc.). The nucleotide bases used were 5′-TCTGGCCCACCAACTTTGGG-3′ (nucleotides 113–132) as an upstream primer and 5′-CTTCACAAGCATGAACTCCA-3′ (nucleotides 453–472) as a downstream primer for human PPARγ (36). One μg of RNA was reverse transcribed with Molony murine leukemia virus reverse transcriptase (Life Technologies, Inc.) at 42°C for 60 min in a 20-μl mixture in the presence of random hexamers (Life Technologies, Inc.). Two μl of a reverse-transcribed mixture was subject to PCR in a 20-μl mixture [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 20 mM deoxynucleotide triphosphate, 0.5 unit of Taq polymerase (Life Technologies, Inc.), and 0.25 pmol of primers]. Thirty-five cycles of reaction at 94°C for 50 s, 60°C for 50 s, and 72°C for 90 s, respectively, were carried out by a DNA thermal cycler (Perkin-Elmer Cetus). Efficiency of RT was controlled in each samples by PCR amplification of β_2-microglobulin using amplimers yielding a 135-bp product (sense, 5′-GGAAAAGATGAGTATGCCTG-3′; antisense, 5′-TTCACTCAATCCAAATGCGG-3′).

Western Blot Analyses. Lysates were made by standard method. Samples were equalized to 20 μg/lane and run on a 12% polyacrylamide gel. Proteins were Western blotted onto Immobilon polyvinylidene difluoride membrane (Millipore). The filter was blocked in PBS, 0.05% Tween 20, and 5% nonfat milk at room temperature for 90 min. All antibodies were diluted in the same buffer. Antibodies were used at a dilution of 1:2000 (anti-PPARγ; Calbiochem), 1:2500 (anti-E-cadherin; Transduction laboratories), 1:1000 (anti-α-actin; Oncogene Research Products), and incubated at room temperature for 90 min. The secondary antibodies were used at a dilution of 1:1500 and incubated at room temperature for 90 min. The signals were detected by enhanced chemiluminescence (Amersham). The stripping procedures were performed using standard methods.

***In Vivo* Murine Studies.** Forty male BNX *nu/nu* nude mice at 8 weeks of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in pathogen-free conditions with irradiated chow. Mice received 300 rads of whole-body irradiation prior to inoculation. A total of 5×10^6 PC-3 cells in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) were injected s.c. into bilateral sides of each mouse, forming two tumors/mouse. Mice were randomly divided into four groups of 10 mice each: group A, control; group B, troglitazone; group C, ATRA; and group D, combination of troglitazone and ATRA. Troglitazone (500 mg/kg/day) was administered p.o. as a suspension in 1.5% carboxymethylcellulose with 0.2% Tween 20 (Sigma) for 5 days a week. ATRA (10 mg/kg/day) was also administered p.o. as a suspension in sesame oil (Sigma) every other day. In the control group, both vehicles were administered p.o. The doses used in this study were chosen after a series of initial experiments determining the highest dose that could be given without side effects. Treatments were started on the day after the injection of the PC-3 cells. Tumor sizes were measured every week with vernier calipers and calculated by the formula: A (length) × B (width) × C (height) × 0.5236. During the experiment, one mouse in the combination group died of bleeding caused by the gavage procedure. At the end of the experiment (6 weeks), bloods were collected for chemistries and hematopoietic analyses. All mice were euthanized, and autopsies were performed; tumors were removed, weighed, and fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. The statistical significance of the differences was analyzed by the nonparametric Mann-Whitney U test.

RESULTS

Expression of PPARγ. We initially examined the expression of PPARγ in prostate cancer cell lines using RT-PCR (Fig. 1*A*). All three prostate cancer cell lines, LNCaP, PC-3, and DU145, expressed PPARγ at significant levels as compared with the negative control. The U937 was used as a previously reported positive control (37). To quantify the expression levels of PPARγ, we performed Western blot analysis using a specific antibody to PPARγ (Fig. 1*B*). Expression levels were corrected for the differences in protein loading according to probing with antibody to α-actin, and PC-3 cells were used as the reference point. The expression level of LNCaP was ~70% of PC-3 cells, and DU145 expressed comparable levels of PPARγ as PC-3. A

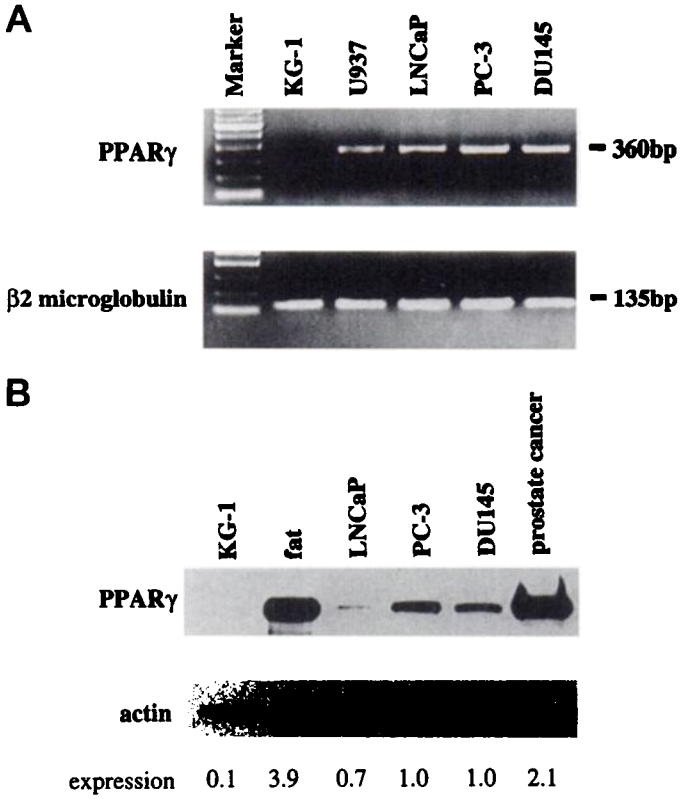

Fig. 1. Expression of the PPARγ gene in prostate cancers. *A*, RT-PCR. RNAs were isolated from prostate (LNCaP, PC-3, and DU145) and myeloid leukemia (KG-1 and U937) cell lines, reverse transcribed using random hexamers, and amplified by PCR using specific primers for PPARγ and β_2-microglobulin. The RT-PCR products were electrophoresed on ethidium bromide-containing agarose gels. The 360-bp band corresponds to nucleotides 113–472 of the PPARγ cDNA (*upper panel*). RT-PCR products of β_2-microglobulin were used as a control for RNA quantity and integrity (*lower panel*). *B*, Western blot. Lysates were extracted from KG-1 myeloblasts (negative), normal human fat cells (positive), prostate cancer cell lines, and a fresh prostate cancer. The lysates were electrophoresed, blotted onto membrane, and reacted with a specific antibody to PPARγ. The antibody was removed from the membrane and incubated with an antibody to α-actin. The expression levels were corrected for the difference in protein loading according to the actin probing, and PC-3 was used as the reference point.

fresh prostate cancer expressed levels twice as high as PC-3. In addition, we evaluated the expression levels of PPARγ in normal and cancer prostate tissue by immunohistochemistry. Cancer cells were expressed at a significant level; in contrast, normal prostate cells were expressed at a very low or negligible level (data not shown).

Effect of Ligands on Clonal Proliferation of Prostate Cancer Cell Lines. The PC-3, DU145, and LNCaP cells were cloned in soft agar in the presence of either troglitazone (10^{-8}–10^{-5} M), RXR-selective ligands: SR11345 and LG100268 (10^{-8}–10^{-5} M); RAR-selective ligands: ATRA and ALRT1550 (10^{-8}–10^{-5} M); RAR- and RXR-pan-agonist: 9-*cis*-RA (10^{-8}–10^{-5} M); or the combination of troglitazone and each of these compounds (10^{-8}–10^{-5} M). The means of the dose-response experiments were plotted on semilogarithm graph paper, and the effective dose that inhibited 50% colony formation (ED_{50}) was determined (Fig. 2 and Table 1). Troglitazone inhibited 50% of the clonal proliferation of PC-3 cells at 3×10^{-7} M. An ED_{50} for DU145 and LNCaP cells was not reached with troglitazone ($\leq 10^{-5}$ M).

The RXR- and RAR-selective ligands were effective in inhibition of clonal proliferation of PC-3 and LNCaP cells. For example, LG100268 (RXR) and ATRA (RAR) achieved ED_{50}s of 3×10^{-6} M and 1×10^{-6} M, respectively, for PC-3 $<1 \times 10^{-8}$ M and 2×10^{-7} M, respectively, for LNCaP cells. DU145 cells were fairly resistant to retinoids; only ATRA and 9-*cis*-RA achieved ED_{50}s for these cells at 7×10^{-7} M and 3×10^{-7} M, respectively.

When PPARγ heterodimerizes with RXR, both receptors can bind to their respective ligands, and prior experiments suggested that ligand binding of both receptors can enhance transactivation of target genes (21). Therefore, we examined the effects of the simultaneous exposure of target cells to troglitazone plus a RXR-selective ligand, LG100268. The combination of troglitazone and LG100268 showed a slightly additive inhibition of clonal growth of PC-3 cells (Fig. 2). The ED_{50} for PC-3 with this combination was 5×10^{-8} M, which was more potent than either troglitazone (3×10^{-7} M) or LG100268 alone (3×10^{-6} M). No additive effects of troglitazone and a retinoid were detected with either LNCaP or DU145 cells. In addition to LG100268, other retinoids (SR11345, ATRA, ALRT1550, and 9-*cis*-RA) were also tested in a dose-response fashion (10^{-8}–10^{-5} M) with an equivalent molar concentration of troglitazone, and each of these combinations lacked either an additive or synergistic activity on the three prostate cancer cell lines (Table 1).

Other PPARγ ligands were also examined in soft agar colony assay using the three prostate cancer cell lines, and similar results were found. The PPARγ-specific ligand, BRL49653, inhibited the clonal growth of PC-3 cells with an ED_{50} of 8×10^{-8} M, which was more potent than troglitazone. A natural PPARγ ligand, $15dPGJ_2$, also inhibited PC-3 growth with an ED_{50} of 2×10^{-6} M (Fig. 3*A* and Table 1). Two other ligands, ciglitizone and indomethacin, showed only a slight antiproliferative effect on PC-3 cells. Experiments with LNCaP found that BRL49653 mediated a slight inhibition of clonal growth of LNCaP cells (up to 20% inhibition). $15dPGJ_2$ (6×10^{-6} M) achieved a 50% inhibition of LNCaP cells. Other ligands (ciglitizone and indomethacin) showed only a slight inhibition of LNCaP cells similar to what was observed with troglitazone. DU145 cells were fairly resistant to all of these ligands (data not shown).

To examine whether the clonogenic growth arrest of PC-3 cells by troglitazone was reversible, the cells were cultured in liquid media with either 10^{-5} M troglitazone, 10^{-7} M LG100268, or both for different durations, thoroughly washed, and plated in soft agar in the absence of ligands. Colony numbers were enumerated on day 14. The mean time-response curves indicated that >50% of the clonogenic cells were inhibited by 4 days of exposure to troglitazone, suggesting that the ligand was capable of mediating an irreversible inhibition of growth of PC-3 cells (Fig. 3*B*). LG100268 showed an irreversible clonal inhibition, and the two agents together were slightly additive (Fig. 3*B*).

Cell Cycle Analysis. Analyses of the cell cycle of PC-3, DU145, and LNCaP cells after their exposure to either troglitazone (10^{-5} M), LG100268 (10^{-7} M), or a combination of both ligands were performed. These cell lines showed no significant increase in the number of cells in the G_0-G_1 phase of cell cycle (PC-3: control 59%, troglitazone 53%, LG100268 57%, and combination of both ligands 55%; DU145: control 47%, troglitazone 47%, LG100268 44%, and combination of both agents 47%; LNCaP: control 55%, troglitazone 54%, LG100268 57%, and combination of both 56%, respectively). Furthermore, no significant decrease occurred in the S phase of the cell cycle (PC-3: control 23%, troglitazone 27%, LG100268 24%, and combination of both agents 26%; DU145: control 28%, troglitazone 30%, LG100268 33%, and combination of both 30%; LNCaP: control 22%, troglitazone 24%, LG100268 21%, and combination of both 23%).

Differentiation Markers. PSA is a secreted protease of the normal prostate that has also been used as a prostate-specific tumor marker, and it may be a differentiation marker of normal development of the prostate (30). The culture media of LNCaP cells showed ~50% reduction of PSA levels when the cells were treated with troglitazone (10^{-5} M) compared with LNCaP cultures with no troglitazone (day 4 of culture: control, 2.7 ± 0.2 ng/10^5 cells; troglitazone, 1.4 ± 0.3

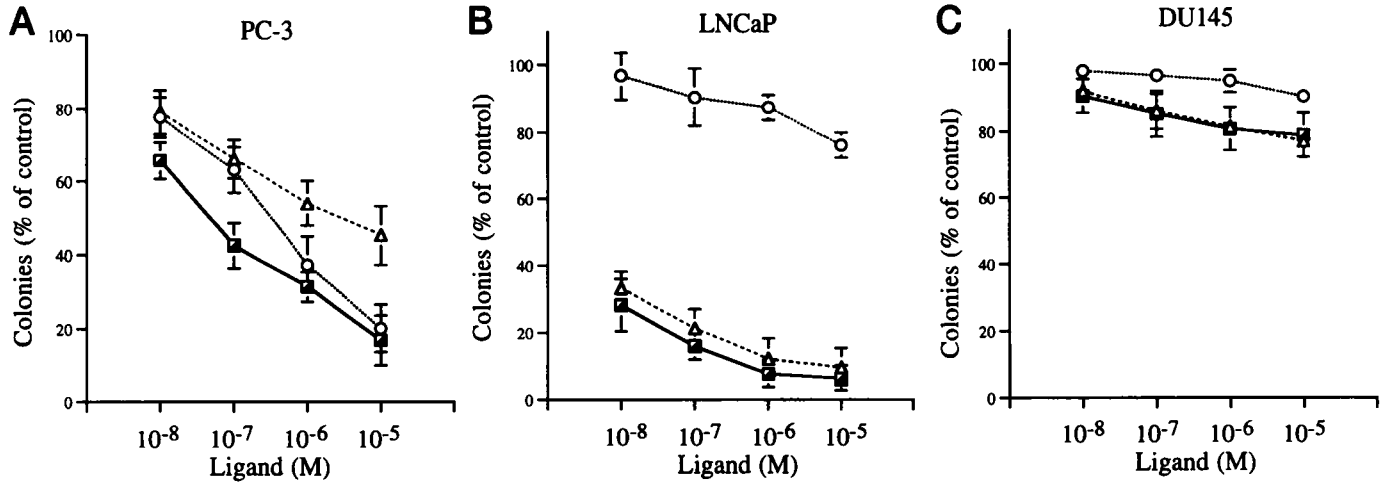

Fig. 2. Dose-response effects of troglitazone and RXR-specific ligand (LG100268) on clonal proliferation of PC-3, LNCaP, and DU145 prostate cancer cells. Results are expressed as a mean percentage of control plates containing no ligand. Each point represents a mean of three independent experiments with triplicate dishes; *bars*, SD. ○, troglitazone; △, LG100268; ◪, troglitazone + LG100268.

ng/10^5 cells; day 8 of culture: control, 7.4 ± 1.1 ng/10^5 cells; troglitazone, 3.5 ± 0.5 ng/10^5 cells, respectively). RT-PCR analyses could not detect PSA transcripts from PC-3 and DU145 cells cultured with and without troglitazone (10^{-5} M; 4 days; 45 cycles; data not shown).

E-cadherin is a cell surface adhesion molecule. Its expression often decreases associated with metastatic prostate cancer (32), and some investigators have used it as a differentiation marker. Western blot (Fig. 4) and immunohistochemistry (data not shown) showed no significant difference of E-cadherin expression between PC-3 cells cultured with and without troglitazone (10^{-5} M; 4 days). In addition, E-cadherin expression did not change in PC-3 cells cultured with retinoids (10^{-7} M of either ATRA or LG100268 for 4 days) or the combinations of troglitazone and either of the retinoids (Fig. 4). Also, troglitazone did not affect the expression of E-cadherin in LNCaP cells (Western blot analysis; data not shown). Because troglitazone can enhance adipocyte differentiation, we examined the expression of lipids in PC-3 and LNCaP cells by staining them using oil red O. Troglitazone (10^{-5} M; 4 days) alone or in combination with retinoids did not result in cellular staining for oil red O in these cells. Likewise, cell surface expression of another marker of adipocyte differentiation (CD36, the receptor for oxidized LDL) did not change in PC-3 and LNCaP cells after culture with troglitazone (10^{-5} M; 4 days) either alone or in combination with one of the retinoids (data not shown).

Morphological Changes. After culture with troglitazone (10^{-5} M; 4 days), PC-3 cells showed dramatic morphological changes compared with control cells. More than 80% of these cells had prominent enlargement of the cytoplasm with development of numerous cytoplasmic vacuoles (Fig. 5). In some cells, vacuoles were finely dispersed in the cytoplasm, whereas in others, vacuoles were very large, displacing the nuclei and resembling adipocytes or signet ring cells. The vacuoles were negative for lipids as shown by negative staining with oil red O stain. Only ~10% of troglitazone-treated LNCaP cells had similar morphological changes when studied by light microscopy. Ultrastructural analysis revealed that troglitazone-treated PC-3 cells were large with macronucleoli and round, oval, or irregular nuclear outlines. Many cells revealed cytoplasmic lumens with short microvillous processes, indicating adenocarcinomatous differentiation (Fig. 6*A*). The cytoplasm was abundant and in most cells contained numerous secondary lysosomes (Fig. 6*B*) The cell surfaces also revealed short microvillous processes.

Antitumor Effect *in Vivo*. Because the *in vitro* experiments showed antitumor activity of troglitazone, we evaluated its effect *in vivo* using PC-3 tumors growing in triple-immunodeficient mice.

Table 1 *Inhibition of clonal proliferation of human prostate cancer cells*

Dose-response experiments were performed; the data were plotted, and the curves were used to calculate the concentration of the analogues achieving a 50% inhibition (ED_{50}) of clonal growth.

Compounds	Nuclear receptor selectivity	ED_{50} (M)		
		PC-3	LNCaP	DU145
Troglitazone	PPARγ	3×10^{-7}	NR[a]	NR
BRL49653	PPARγ	8×10^{-8}	NR	NR
15dPGJ$_2$	PPARγ	2×10^{-6}	6×10^{-6}	NR
Ciglitizone	PPARγ	NR	NR	NR
Indomethacin	PPARγ	NR	NR	NR
9-*cis*-RA	RAR + RXR	8×10^{-7}	5×10^{-8}	3×10^{-7}
9-*cis*-RA + troglitazone	Both	3×10^{-7}	5×10^{-8}	3×10^{-7}
SR11345	RXR	NR	2×10^{-6}	NR
SR11345 + troglitazone	Both	3×10^{-7}	2×10^{-6}	NR
LG100268	RXR	3×10^{-6}	$<1 \times 10^{-8}$	NR
LG100268 + troglitazone	Both	5×10^{-8}	$<1 \times 10^{-8}$	NR
ATRA	RAR	1×10^{-6}	2×10^{-7}	7×10^{-7}
ATRA + troglitazone	Both	3×10^{-7}	2×10^{-7}	7×10^{-7}
ALRT1550	RAR	4×10^{-6}	1×10^{-6}	NR
ALRT1550 + troglitazone	Both	3×10^{-7}	1×10^{-6}	NR

[a] NR, not reached (ED_{50} was not reached at $\leq 10^{-5}$ M of the ligand).

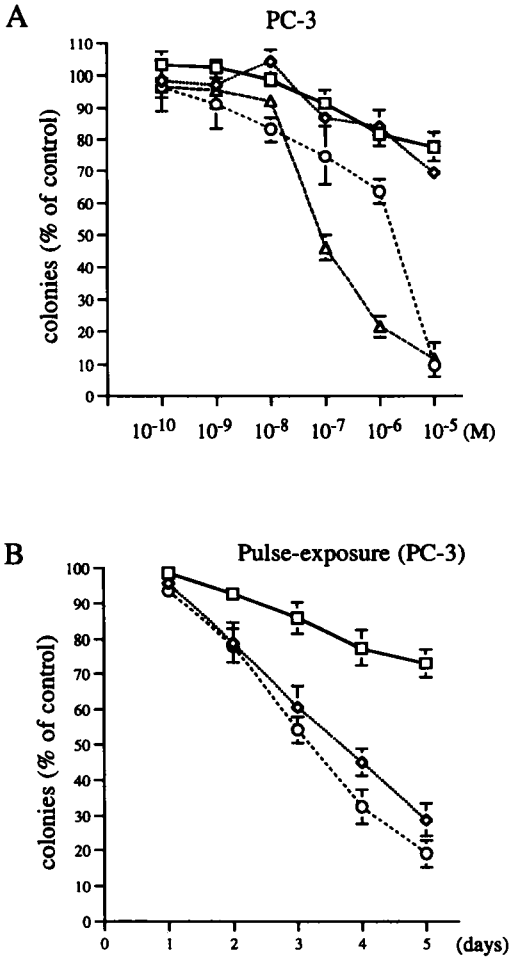

Fig. 3. Effect of other PPARγ ligands (*A*) and pulse-exposure to troglitazone (*B*) on the clonal growth of PC-3 cells. In *A*, dose-response clonogenic assays of PC-3 cells using ligands for PPARγ were performed as in Fig. 2. The results are expressed as a mean percentage of results of control plates containing no ligands. Each point represents a mean of three independent experiments with triplicate dishes; *bars*, SD. □, indomethacin; ◇, Ciglitizone; ○, 15dPGJ$_2$; △, BRL49653. In *B*, PC-3 cells were exposed to either 10^{-5} M troglitazone (◇), 10^{-7} M LG100268 (□), or a combination of both compounds (○) for 1–5 days, thoroughly washed, and plated in soft agar; colonies were enumerated after 14 days of culture. The results were expressed as a mean percentage of colonies in control plates containing cells not exposed to ligand.

Tumor volumes were measured every week (Fig. 7*A*) and tumor weights were measured at autopsy (Fig. 7*B*). Troglitazone suppressed in a statistically significantly manner ($P = 0.01$) the growth of the prostate tumor. The antitumor effect of ATRA was greater than that of troglitazone ($P < 0.0001$). No statistical significance was noted between ATRA alone and the combination of ATRA and troglitazone. The tumors were fixed, stained, and viewed by light microscopy. The histological analysis of tumors treated with either troglitazone, ATRA, or both revealed necrosis and fibrosis, as well as cytological changes of apoptosis including nuclear and cytoplasmic shrinkage and formation of nuclear fragments or apoptotic bodies (Fig. 8).

During the study, all mice were weighed once a week; the mean weights of each experimental group were statistically the same as those of the control group (data not shown and Table 2). Blood analysis was done several hours before the conclusion of the study. No difference in the mean blood chemistries and hematopoietic values were observed between treated and untreated mice except an elevation of transaminases and triglycerides and a fall in the cholesterol in the experimental groups that received either troglitazone, ATRA, or both (Table 2). The ATRA and ATRA plus troglitazone groups had a decrease in their peripheral blood white blood count. Plasma glucose levels were within the normal range in the troglitazone-treated group, as has been described previously in nondiabetic animals receiving the compound (17).

Effect of Troglitazone on Fresh Prostate Cancers. Twelve prostate cancers and their adjacent normal prostate tissues obtained at surgery were placed in FCS containing culture media plus either diluant (control) or 10^{-5} M troglitazone. Five cases were insufficient for evaluation because of either necrosis at the beginning of culture or the tumors were too small for evaluation. After 4 days of exposure, four of the seven troglitazone-treated prostate cancer samples showed prominent necrotic changes (~60% of the cells; Fig. 9). Interestingly, the normal adjacent prostate tissues treated with troglitazone remained normal in appearance, with no necrosis (data not shown).

DISCUSSION

PPARγ belongs to the nuclear steroid receptor superfamily, which includes vitamin D_3, retinoid, and thyroid hormone receptors; each is critical for cellular growth and differentiation (38). Previous studies have reported that PPARγ ligands activated PPARγ-expressing cells including adipocytes, fibroblasts, myoblasts, liposarcoma cells, and breast cancer cells and helped to induce their terminal differentiation (22, 23). The PPARγ and RXR form a heterodimeric complex that functions as a central regulator of adipocyte differentiation (21, 22). Our RT-PCR and Western blot data show that the three prostate cancer cell lines as well as the fresh prostate cancers expressed PPARγ. We tested the hypothesis that prostate cancer cells that express PPARγ and RXR may be inhibited in their proliferation and induced in their differentiation by PPARγ and RXR agonists; together, the two types of agonist may be more active. We also examined the combined effect of troglitazone and RAR agonists, because RAR agonists by themselves have an antiproliferative effect against prostate cancer cells (24), especially ATRA, which is used clinically

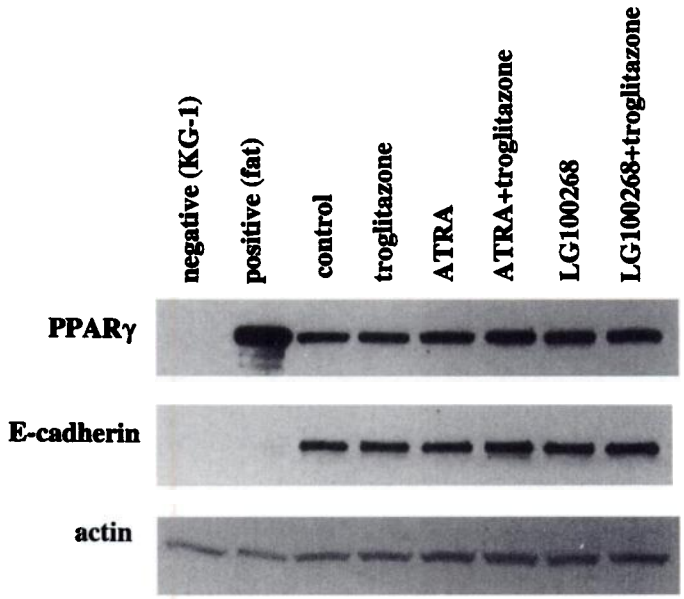

Fig. 4. Effect of troglitazone and retinoids on the expression of PPARγ and E-cadherin in PC-3 prostate cancer cells. PC-3 cells were cultured with either troglitazone (10^{-5} M; 4 days), ATRA (10^{-7} M; 4 days), LG100268 (10^{-7} M; 4 days) or a combination of both compounds. Lysates were extracted, electrophoresed, Western-blotted, and reacted with an antibody specific for PPARγ. The antibody was removed, and the same membrane was exposed to antibodies for E-cadherin and α-actin. KG-1 myeloblasts were a negative control; normal human fat cells were a positive control for PPARγ. Control lane showed untreated PC-3 cells. These compounds induced negligible differences in expression of PPARγ and E-cadherin.

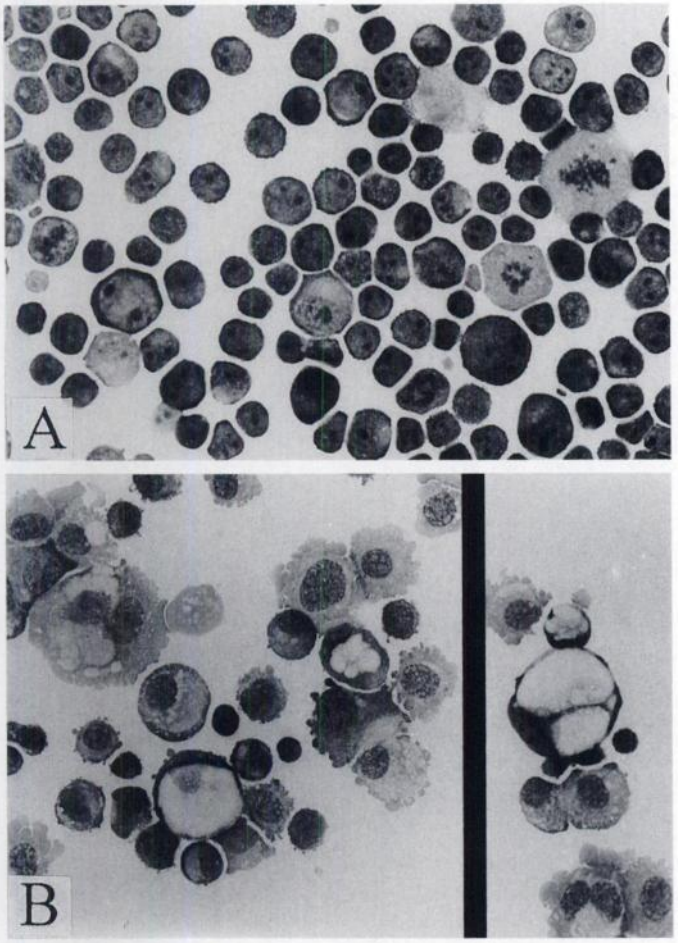

Fig. 5. Morphological changes of PC-3 cells induced by troglitazone. *A*, control; untreated cells showed viable adenocarcinoma cells with large nuclei and abundant cytoplasm. Mitoses were frequent (Giemsa stain; ×400). *B*, troglitazone-treated cells (10^{-5} M; 4 days). Treated cells had cytoplasmic vacuoles of different sizes, including large vacuoles displacing the nucleus and causing a "signet-ring" or adipocyte-like appearance (*right*; Giemsa stain; ×400).

for treatment of acute promyelocytic leukemia (3, 4). Each of the RXR and RAR ligands were effective against the three prostate cancer cell lines, particularly LNCaP, which was the most sensitive to clonal inhibition by the retinoids. In contrast, troglitazone inhibited the *in vitro* proliferation of the PC-3 cells. Mild additional inhibition occurred when troglitazone was combined with a RXR ligand, LG100268. The combination of troglitazone and ATRA is no greater than either alone against prostate cancer cells. These results might indicate that troglitazone and ATRA may be acting on prostate cancer cells by a common pathway. LNCaP was moderately resistant and DU145 was completely resistant to the antiproliferative effects of troglitazone. Nevertheless on Western blot, the troglitazone-sensitive PC-3 cells and troglitazone-resistant DU145 cells expressed an equivalent amount of PPARγ. The reason for the differences of sensitivity to troglitazone remains to be determined.

To help to determine whether the dramatic effects on PC-3 by troglitazone were mediated through PPARγ, we evaluated other PPARγ ligands such as BRL49653, $15dPGJ_2$, ciglitizone, and indomethacin on all three prostate cancer cell lines. BRL49653 was more potent than troglitazone in growth inhibition. $15dPGJ_2$, a natural ligand for PPARγ, had a similar potency as troglitazone, and the other two ligands were slightly less potent than troglitazone when treated against PC-3 cells. These results suggest that these effects were mediated through PPARγ. Interestingly, $15dPGJ_2$ achieved an ED_{50} of 6×10^{-6} M for LNCaP cells, whereas neither troglitazone nor BRL49653 (10^{-5} M) achieved an ED_{50} against these cells. $15dPGJ_2$ is a natural ligand with a K_d of 2 μM for PPARγ, while thiazolidinediones have K_ds of 30–700 nM for PPARγ (39). This surprising discordance suggests that $15dPGJ_2$ may be mediating part of its antiproliferative activity independent of PPARγ.

We investigated further the effects of troglitazone on PC-3 and LNCaP cells by examining the changes in morphology induced by the drug, as well as looking for changes in differentiation and the cell cycle. PC-3 cells treated with troglitazone showed remarkable vacuole formations that looked like lipid deposits. Electron microscopy revealed that troglitazone-treated PC-3 cells contained numerous lysosomes, and the large intracytoplasmic lumenal spaces were lined by short microvillous projections, which is characteristic of differentiated adenocarcinoma cells. Oil red O staining and analyses of the electron microscopy indicated that these cells had occasional lipid droplets, but

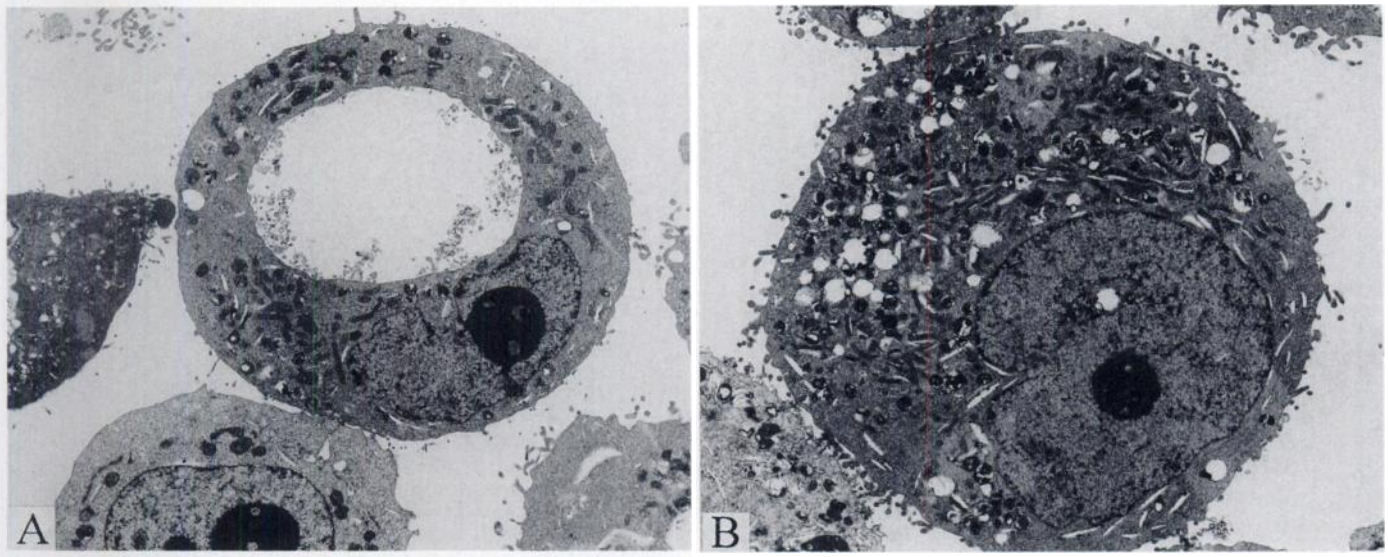

Fig. 6. Ultrastructural changes in PC-3 cells. *A*, electron micrograph showing PC-3 cells treated with troglitazone (10^{-5} M; 4 days). These cells revealed a large intracytoplasmic lumen lined with microvillous processes indicating adenocarcinomatous differentiation (uranyl acetate and lead citrate; ×3200). *B*, electron micrograph showing PC-3 cells treated with troglitazone (10^{-5} M; 4 days) with numerous cytoplasmic secondary lysosomes (uranyl acetate and lead citrate; ×3200).

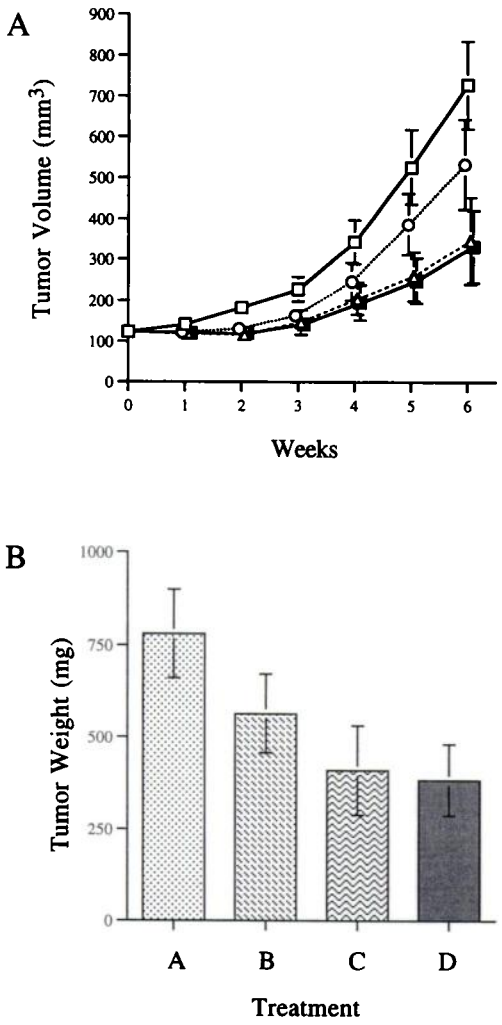

Fig. 7. Effect of troglitazone and ATRA, either alone or in combination, on growth of PC-3 tumors in BNX triple immunodeficient mice. PC-3 cells were injected bilaterally s.c. into nude mice, forming two tumors/mouse. Troglitazone (500 mg/kg/day) was administered p.o. for 5 days a week; ATRA (10 mg/kg/day) was given p.o. every other day (Monday, Wednesday, and Friday). *Panel A*, time course of tumor volumes. Tumor volumes were measured every week. Each point represents the mean volume ± SD. □, control; ○, troglitazone; △, ATRA; ◪, ATRA + troglitazone. *Bars*, SD. *Panel B*, tumor weights at autopsy. After 6 weeks of therapy, tumors were removed and weighed. Results represent means ± SD of tumor weights. *A*, control; *B*, troglitazone; *C*, ATRA; *D*, ATRA + troglitazone. Groups B, C, and D are significantly different ($P = 0.01$, $P < 0.0001$, $P = 0.0002$, respectively) from group A. Group C is not significantly different from group D ($P = 0.5$), as determined by the Mann-Whitney *U* test. *Bars*, SD.

the prominent vacuoles did not contain lipids. Furthermore, these cells did not express the cell surface antigen CD36, consist with lack of adipocyte-like characteristics. In contrast, Mueller *et al.* (23) have found that troglitazone markedly enhanced oil red O and CD36 expression in breast cancer cells (23). The CD36 antigen is the receptor of oxidized LDL. Potentially, induced expression of CD36 by troglitazone on various cells of the body might lower the levels of atherogenic LDLs.

The PC-3 cells remained negative for PSA. E-cadherin and cell cycle analyses showed no remarkable changes; nevertheless, the electron microscopic findings were impressive, suggesting further differentiation of these adenocarcinoma cells. In addition, we examined these troglitazone-treated PC-3 cells for apoptosis and surgically obtained prostate cancers by terminal deoxynucleotidyl-mediated dUTP nick end labeling assay using an *in situ* Cell Death Detection kit (Boehringer Mannheim); no significant difference was found (data not shown). LNCaP cells showed similar morphological changes as PC-3 cells when treated with troglitazone. However, the effect was weak; ~10% of the cells had enlarged cytoplasms compared with control cells. Interestingly, LNCaP cells treated with troglitazone had a 50% reduction in PSA levels in their conditioned media compared with untreated LNCaP cells. Thus, LNCaP has a slight response to ligand of PPARγ. The mechanisms of the antiproliferative activity of troglitazone remain poorly understood. The PSA data show that troglitazone can down-regulate expression of the PSA gene. The mechanism of this regulation requires further study.

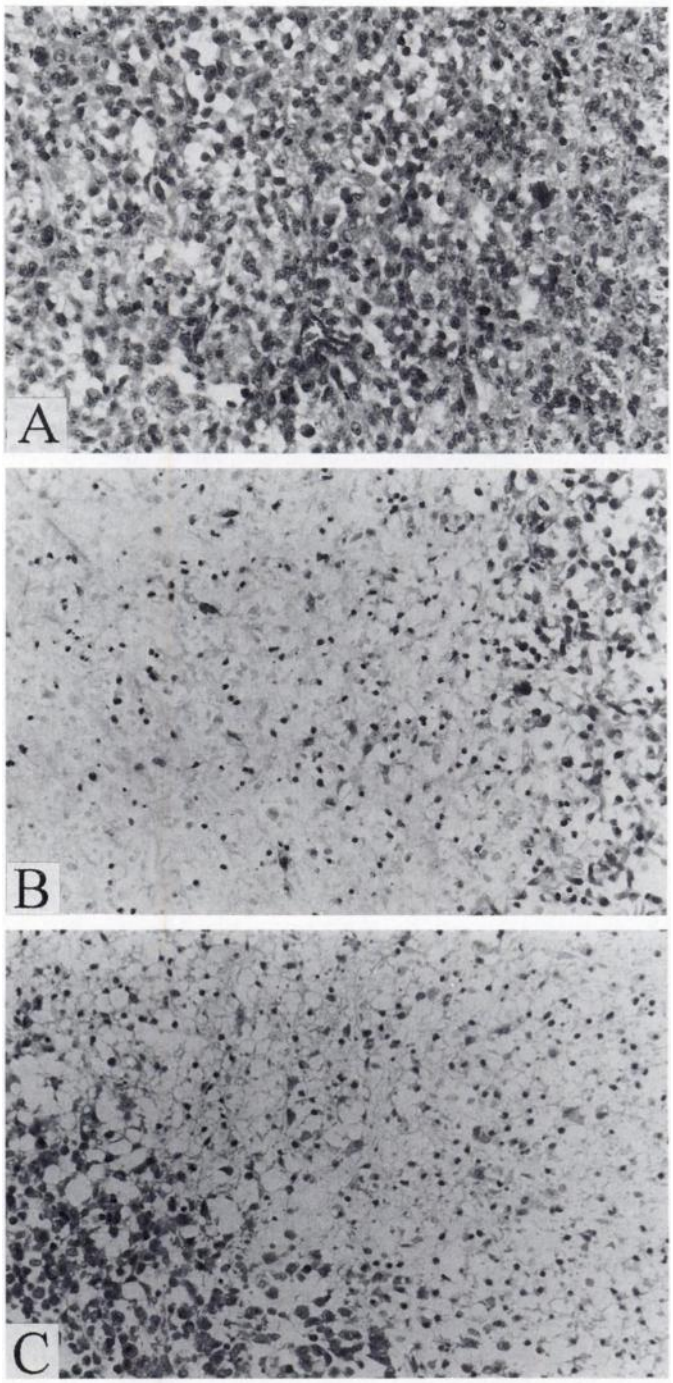

Fig. 8. Antitumor effect of troglitazone *in vivo*. After 6 weeks of growth and treatment in BNX triple immunodeficient mice, PC-3 tumors were removed, fixed, and stained (H&E, ×200). *A*, control showing poorly differentiated adenocarcinoma. *B*, tumors treated with troglitazone (500 mg/kg/day) for 5 days a week × 6 weeks revealed extensive necrosis and presence of nuclear debris or apoptotic bodies. *C*, tumors treated with ATRA (10 mg/kg/day) every other day (Monday, Wednesday, and Friday) for 6 weeks revealed predominantly necrosis.

Table 2 *Blood analyses and weights after in vivo treatment of triple immunodeficient mice carrying human prostate (PC-3) tumors*

Blood was drawn from the mice at the time of their sacrifice at completion of 6 weeks of therapy. Data represent mean value ± SD.

	Control	Troglitazone	ATRA	ATRA + troglitazone
WBC (10^3/mm^3)	6.2 ± 2.9	6.1 ± 3.9	5.7 ± 2.5	4.0 ± 2.4
RBC (10^6/mm^3)	8.7 ± 1.3	9.0 ± 0.6	9.5 ± 0.8	8.9 ± 1.5
Hgb[a] (g/dl)	13.4 ± 1.8	13.8 ± 0.7	14.0 ± 1.1	13.6 ± 1.5
Hct (%)	41.7 ± 5.7	42.9 ± 3.0	43.8 ± 3.3	41.8 ± 4.9
Plt (10^3/mm^3)	908 ± 137	1055 ± 104	1079 ± 142	1104 ± 101
Alk P (U/l)	96.4 ± 29.6	87.0 ± 6.0	95.0 ± 14.6	91.6 ± 22.3
GOT (AST)(U/l)	81.0 ± 10.8	102.2 ± 18.7	145.4 ± 30.0	109.4 ± 22.2
GPT (ALT)(U/l)	46.8 ± 19.1	72.8 ± 41.3	68.4 ± 25.7	66.6 ± 40.8
BUN (mg/dl)	15.2 ± 3.1	17.9 ± 3.7	18.3 ± 1.5	17.3 ± 1.9
Glucose (mg/dl)	176.6 ± 24.0	200.0 ± 31.7	177.8 ± 51.2	198.4 ± 24.9
Cholest (mg/dl)	91.0 ± 17.9	79.8 ± 13.0	77.4 ± 6.4	86.2 ± 18.2
Triglyc (mg/dl)	112.8 ± 14.3	120.2 ± 33.8	137.0 ± 43.1	135.4 ± 29.8
T Prot (g/dl)	5.1 ± 0.2	4.8 ± 0.4	4.8 ± 0.3	4.8 ± 0.2
Weights (g)	20.9 ± 1.2	20.7 ± 2.4	21.0 ± 2.1	20.9 ± 1.4

[a] Hgb, hemoglobin; Hct, hematocrit; Plt, platelet; Alk P, alkaline phosphatase; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; BUN, blood urea nitrogen; Cholest, cholesterol; Triglyc, triglycerides; T Prot, total protein.

Our animal studies disclosed that troglitazone is also effective *in vivo*. The growth-inhibitory action of troglitazone as well as ATRA was statistically superior compared with animals receiving diluant. Furthermore, light microscopic analyses of these tumors showed that either troglitazone or ATRA caused necrosis and fibrosis, as well as cytological changes of apoptosis. No difference occurred in the antitumor effect of ATRA alone *versus* the combination of troglitazone and ATRA. Of note, the SD of tumor sizes in the different cohorts was relatively wide. This may be due to the wide range of oral absorption of troglitazone, which can vary from 30 to 80% of the oral dose. Furthermore, accurate administration of reagents p.o. by gavage is difficult. Even so, troglitazone was found to be effective *in vivo*. Moreover, the only objective side effect was the elevation of transaminases by both the troglitazone and ATRA-treated mice; a side effect also reported previously (4) in the ATRA-treated group. Troglitazone did not affect plasma glucose levels in nondiabetic mice, as reported previously (17). Troglitazone is already being used in >770,000 insulin-resistant diabetic patients, and these individuals have experienced few side effects, suggesting that troglitazone may be nontoxic for the treatment of prostate cancer patients.

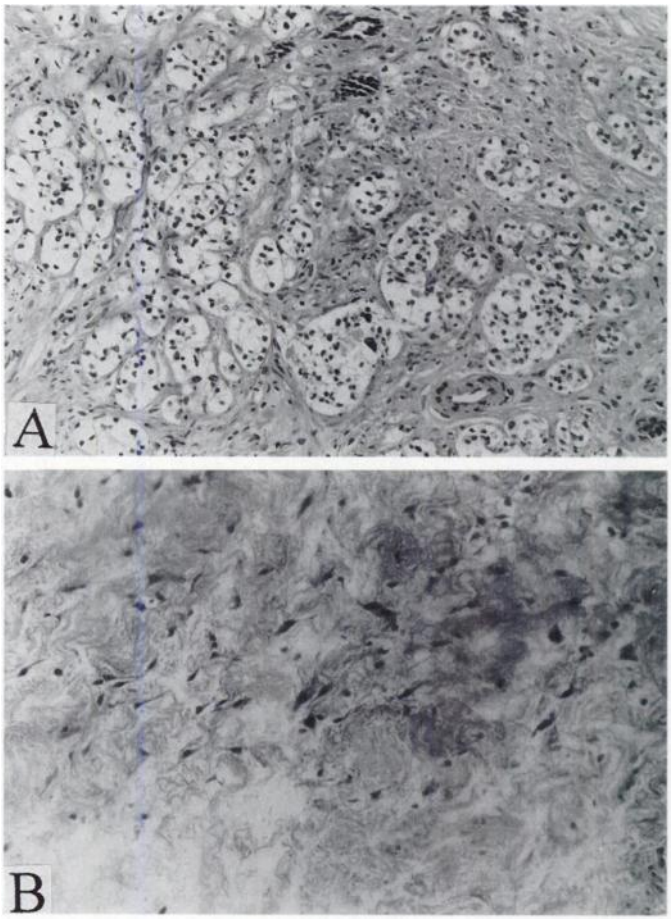

Fig. 9. Effect of troglitazone on fresh prostate cancers. Surgically obtained human prostate cancers were treated with troglitazone (10^{-5} M; 4 days). *A*, control prostate cancer revealed neoplastic glands (Gleason's grade 3 + 2 = 5) infiltrating fibrous stroma. *B*, prostate cancer treated with troglitazone revealed fibrous stroma and nuclear debris. No viable carcinoma cells were observed (H&E stain, ×200).

Surprisingly, we found that *in vitro* culture of surgically obtained human prostate cancer with troglitazone resulted in prominent necrotic changes; in contrast, normal prostate tissue did not undergo necrosis. For unknown reasons, troglitazone selectively affected the malignant prostate cells. Our immunohistochemistry data showed that normal prostate cells expressed little or negligible levels of PPARγ (data not shown). To confirm these exciting observations, more samples will need to be examined. These findings support the benefit of troglitazone for clinical use.

In conclusion, our data demonstrate that troglitazone inhibited the growth of human prostate cancer cells (PC-3) *in vitro* and *in vivo*. The underlying mechanisms of activity remain unclear and require further study. Troglitazone may mediate its potent antiproliferative effect against prostate cancer cells through differentiation, as suggested by light and electron microscopy. Troglitazone may become a useful adjuvant therapy for prostate cancer, particularly for the patients who have minimal residual disease after surgery or radiotherapy with curative intent.

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