Effects of ligand activation of peroxisome proliferator-activated receptor γ in human prostate cancer

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Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor that plays a key role in the differentiation of adipocytes. Activation of this receptor in liposarcomas and breast and colon cancer cells also induces cell growth inhibition and differentiation. In the present study, we show that PPARγ is expressed in human prostate adenocarcinomas and cell lines derived from these tumors. Activation of this receptor with specific ligands exerts an inhibitory effect on the growth of prostate cancer cell lines. Further, we show that prostate cancer and cell lines do not have intragenic mutations in the PPARγ gene, although 40% of the informative tumors have hemizygous deletions of this gene. Based on our preclinical data, we conducted a phase II clinical study in patients with advanced prostate cancer using troglitazone, a PPARγ ligand used for the treatment of type 2 diabetes. Forty-one men with histologically confirmed prostate cancer and no symptomatic metastatic disease were treated orally with troglitazone. An unexpectedly high incidence of prolonged stabilization of prostate-specific antigen was seen in patients treated with troglitazone. In addition, one patient had a dramatic decrease in serum prostate-specific antigen to nearly undetectable levels. These data suggest that PPARγ may serve as a biological modifier in human prostate cancer and its therapeutic potential in this disease should be further investigated.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that includes thyroid hormone, retinoic acid, and androgen and estrogen receptors. These receptors share common features, including a central DNA binding domain and a carboxyl-terminal domain responsible for dimerization, ligand binding, and transcriptional activation. PPARγ requires heterodimerization with the retinoid X receptor for binding to DNA at specific sites, defined as direct repeats of hormone response elements, separated by one base (DR-1).

PPARγ transcriptional activity depends on the binding of ligands. Although the identity of a true endogenous ligand for PPARγ is still unclear, both natural and synthetic ligands have been described. Natural ligands, such as certain polyunsaturated fatty acids and 15-deoxy$\Delta^{12,14}$ prostaglandin J_2, have been shown to bind to this receptor at concentrations in the micromolar range (1–3), whereas the synthetic antidiabetic thiazoledinediones are able to bind to PPARγ with a K_D of 50–700 nM (2, 4).

There are several thiazoledinedione ligands for PPARγ, including troglitazone (Rezulin), rosiglitazone (Avandia), and pioglitazone (Actos). The latter two currently are used in the treatment of type 2 diabetes mellitus. In the last 2 years, troglitazone has been prescribed for more than one million patients in the United States alone (5). These drugs improve insulin resistance, and troglitazone has been shown to be effective at doses ranging between 400 and 800 mg/day. Although the drug generally has been well tolerated at these doses, idiosyncratic liver toxicity has been noted in some patients (6). Thiazoledinediones likely work in the context of type 2 diabetes through the activation of PPARγ in adipose and/or muscle cells, but the downstream target genes that are relevant to the insulin-sensitizing effects of these drugs are still unknown.

PPARγ is expressed at highest levels in adipose tissue (7, 8). Its dominant role in the differentiation of this tissue was elucidated through experiments using exogenous expression and ligand activation in several fibroblastic cell lines. In this context, PPARγ was shown to induce the morphology and the pattern of gene expression characteristic of terminally differentiated adipocytes (9). More recently, it has been shown that PPARγ is expressed at significant levels in nonadipose cells, including epithelial cells (10–14). Its physiological function in those tissues remains unknown, although activation seems to induce certain characteristics of differentiation appropriate for those cell types.

Ligand activation of this receptor causes growth arrest in several cell types derived from tumors (10–15). Recently, we also have demonstrated that the administration of troglitazone to patients with liposarcoma dramatically enhances adipocytic differentiation, including increased expression of the genes of terminal cell differentiation and a reduction in expression of Ki-67, a histochemical marker for cell proliferation *in vivo* (16). This is a striking example of induction of differentiation of solid tumors in humans.

We recently have found naturally occurring somatic mutations in the gene encoding PPARγ in a proportion of sporadic colorectal carcinomas (17). Each of the mutations causes a profound loss of function on this receptor. One mutation causes a truncation before the ligand binding domain whereas the others result in mutant receptors that do not bind to either natural or synthetic ligands. These findings suggest that PPARγ behaves like a tumor suppressor and that loss-of-function mutations might be etiologic for colorectal carcinogenesis. More importantly, our observations might suggest that examining for the presence of PPARγ mutations before institution of ligand differentiation therapy might be useful to predict whether such therapy would result in a response.

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; PSA, prostate-specific antigen; PGJ_2, prostaglandin J_2.

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Prostate cancer is the most common malignancy in American men. In 2000, there will be approximately 180,400 new prostate cancer cases and 31,900 prostate cancer deaths (18). Despite advances in early detection/treatment and management of advanced disease, the annual number of prostate cancer deaths has been relatively stable over the past decade. Improvements in the quality and length of life for men with prostate cancer will require the identification of novel treatment strategies.

In this study we show that PPARγ is expressed in normal prostate tissue and prostatic adenocarcinomas. Ligand activation of this nuclear hormone receptor induces growth inhibition in prostate cell lines. We also report the findings of a prospective clinical study of 41 patients with advanced prostate cancer, who received the PPARγ ligand troglitazone. Furthermore, to determine whether in prostate cancers, like in colon cancers, PPARγ mutations might render the tumor unresponsive to ligands, we determined the mutation status of this gene in cell lines and in a series of 38 prostate carcinomas.

Materials and Methods

Tissue Samples and Cell Lines. The human prostate normal-tumor pairs were obtained from the Brigham and Women's Hospital, Department of Pathology. The tissues for RNA analysis were resected from patients with primary medium grade tumors and frozen in liquid nitrogen. The human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection. DU145 cells were cultured in DMEM, PC3 cells in Ham's F-12 medium, and LNCaP cells in RPMI 1640. All media were supplemented with 10% FCS (HyClone).

RNA, Protein, and Cell Growth Analysis. Cells were grown in their respective media and, when confluent, harvested for Northern or Western blot analysis. Total RNA was extracted from grinded tissues and from cell lines with Triazol (GIBCO), according to manufacturer instructions. Twenty five micrograms of total RNA was loaded on a formaldehyde gel and subsequently transferred on a nylon membrane (Biotrans, ICN). Hybridization was performed by using a human PPARγ cDNA probe. For Western blot, cells were lysed according to protocols described previously (19). One hundred fifty micrograms of protein was loaded on a 10% urea-acrylamide gel. For growth assays, 1×10^3 cells were plated per well, in 24-well plates. After 48 h, DU145 cells were treated with different doses of troglitazone, or rosiglitazone (5 μM), 15-deoxy-$\Delta^{12,\ 14}$PGJ$_2$ (5 μM), M2 (10 μM), Wyeth (10 μM), or GW0233 (5 μM). PC3 and LNCaP cells were treated with troglitazone at 10 μM. The cells were maintained in medium with 10% charcoal-stripped serum, and the medium containing the drugs was replaced every 2–3 days. Cells were counted with a Coulter Z1 counter at the times indicated.

Quantification of Prostate-Specific Antigen (PSA) Secretion. LNCaP cells at passage 25 were plated at a density of 3×10^3 cells per well, into 12-well plates and allowed to attach for 48 h. Cells were treated with 1 ml of freshly diluted drug or culture medium daily. Every 24 h, the supernatants were collected and the adherent cells were trypsinized and counted with a Coulter Z1 counter. The amount of PSA secreted was measured by using the Tandem-E PSA assay (Hybritech) according to the company's instructions.

Patients. Forty-one patients were enrolled in this study. All patients had histologically confirmed adenocarcinoma of the prostate and the disease progression, after local therapy or androgen deprivation, was defined as either: (*i*) rising PSA >1 ng/ml and >150% the nadir PSA after radical prostatectomy, (*ii*) rising PSA >4 ng/ml and 150% the nadir PSA after prostate radiation therapy, or (*iii*) rising PSA >150% nadir PSA after androgen deprivation and antiandrogen withdrawal. Exclusion criteria included symptomatic metastatic disease, radiation therapy within 28 days, antiandrogens or glucocorticoids within 4 weeks, cancer and leukemia group B performance status >2, or aspartate amino transferase (AST) >1.5 times the upper limit of normal. The protocol was approved by the Institutional Review Board and all patients provided written informed consent before study entry.

Pretreatment evaluation included history and physical examination, determination of performance status, alanine amino transferase (ALT), and PSA. Radiographic staging was not required. Troglitazone was provided as 400 mg tablets. Patients were instructed to take two tablets (800 mg/day) by mouth daily. A minimum of 12 weeks of treatment was planned, but troglitazone was continued for >12 weeks at the discretion of the treating physician. Toxicity assessment was performed every 4 weeks by using National Cancer Institute common toxicity criteria. Evaluations occurred monthly at which time PSA levels and liver function tests were obtained. All patients were asymptomatic. No patient had bidimensionally measurable disease at study entry. Treatment was discontinued in all patients in April 2000 after the Food and Drug Administration recommended withdrawal of troglitazone from the market because of liver toxicity.

Mutation Analysis. Genomic DNA was extracted from paraffin-embedded tissue sections of primary human prostate carcinomas as well as from adjacent nontumor tissue, by using standard protocols (20). The genomic DNA was used as template for PCR-based mutation analysis. Primers used for denaturant gradient gel electrophoresis span all seven coding exons of the gene, exon-intron junctions, and flanking intronic sequences. The seventh exon (exon II), which lies upstream of exon 1, represents the splice variant PPARγ2. Mutation analysis was carried out in four multiplex groups comprising a total of 14 fragments as described (21).

To determine whether PPARγ was deleted, microsatellite marker D3S1263, which is either in the gene or no more than 1 Mb from the gene, was used for loss of heterozygosity analysis, as described (22).

Results

PPARγ Is Expressed in Human Prostate Adenocarcinomas and Cell Lines. To determine whether PPARγ is expressed in human prostate tissue, we analyzed PPARγ mRNA levels in normal and prostatic adenocarcinomas obtained from five patients. As shown in Fig. 1A, PPARγ is expressed in both normal and malignant tissue obtained from the same patients. Its levels appear to be somewhat reduced in the tumors, compared with normal tissue. We subsequently analyzed PPARγ levels in the androgen-sensitive cell line LNCaP, and two androgen-independent cell lines, DU145 and PC3. As shown in Fig. 1*B*, PPARγ mRNA is expressed in all of the cell lines, albeit with a wide quantitative variation. LNCaP cells show the lowest amount of PPARγ mRNA whereas DU145 cells have an intermediate level. PC3 cells express PPARγ mRNA at levels that are even higher than those seen in the colon cancer cell line Moser, which was used as a positive control. We next examined the protein levels in all three cell lines. As shown in Fig. 1*C*, the differences observed at the gene expression level are also present at the level of the protein. Notably, despite high amounts of RNA in PC3 cells, a significant amount of the protein appears to be in the phosphorylated, inactive form that migrates more slowly (19).

PPARγ Mutation Analysis in Prostate Cancers and Cell Lines. To determine whether prostate cancers might have mutations in PPARγ, we analyzed LNCaP, DU145, and PC3 cell lines and 38 primary sporadic prostate adenocarcinomas of medium grade. The three cell lines did not harbor any mutations in this gene and

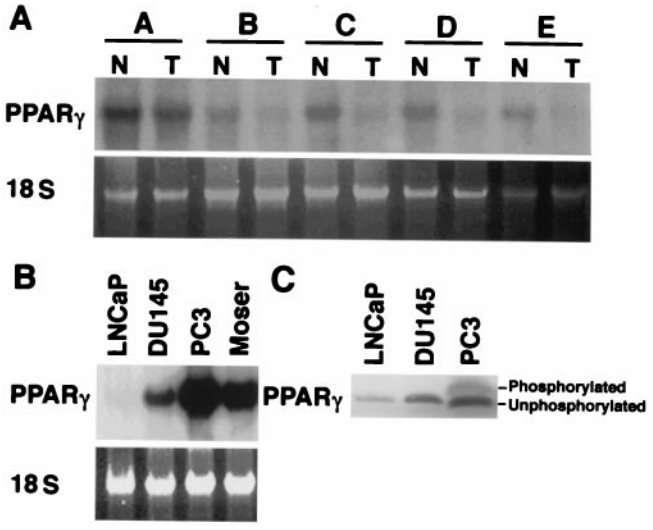

Fig. 1. PPARγ is expressed in human normal prostate, carcinomas, and cell lines. Expression of PPARγ mRNA in (*A*) samples from five patients (from A to E), obtained from normal prostate tissue (N), neighboring carcinomas (T), and (*B*) in LNCaP, DU145, and PC3 prostate cancer cell lines and colon cancer cells (Moser), used as control. (*C*) PPARγ protein levels in LNCaP, DU145, and PC3 cells.

no deletions in that chromosomal region were noted either. Further, none of the 38 primary prostate cancers were found to have intragenic PPARγ mutations. To determine whether this gene was deleted, loss of heterozygosity analysis was performed at D3S1263. As shown in Table 1, of the 38 tumors, 21 were informative at that locus and 40% were found to have hemizygous deletion of the PPARγ gene.

PPARγ Activation Inhibits the Growth of Prostate Cancer Cells. To investigate the role of PPARγ in human prostate cancer cells, we applied several ligands of different potency and selectivity to the cell line DU145 and determined the cell number after continuous treatment with these drugs. As shown in Fig. 2*A*, troglitazone caused a dose-dependent suppression of the growth of these cells, compared with vehicle-treated cells. The decrease occurred in a time- and dose-dependent manner, with a 50% inhibition detected at 7 days, at 10 μM concentration. LNCaP and PC3 cells also were treated with troglitazone at 10 μM. As shown in Fig. 2*B*, in both cell lines growth inhibition can be observed and the extent of this phenomenon appears to correlate with the levels of PPARγ protein present in these cells.

Next, DU145 cells were treated with 5 μM of other two PPARγ ligands, rosiglitazone and 15-deoxy-$\Delta^{12,\ 14}PGJ_2$, and cells were counted after 7 days of treatment. As shown in Fig. 2*C*, the maximum effects were seen with rosiglitazone, which showed a 70% inhibition of growth at 5 μM, compared with the vehicle-treated cells. The natural ligand 15-deoxy-$\Delta^{12,\ 14}PGJ_2$ gave greater than 60% growth inhibition under the same conditions. The profiles of growth responses to these agonists are consistent with their affinities to PPARγ and with their effects observed in adipocytes and other epithelial cells.

To critically determine whether cell growth inhibition is the result of PPARγ activation, we compared the growth response of DU145 to troglitazone with that elicited by M2, an inactive metabolite of troglitazone that is unable to bind and activate PPARγ. As shown in Fig. 2*C*, cells exposed to M2 did not show any decrease in number, compared with the vehicle-treated cells. We also treated these cells with ligands that activate specifically the other PPAR family members. Wy 14,464 is a specific activator of PPARα and GW233 is an activator of PPARδ, at 10 and 5 μM, respectively. The growth of the cells was not detectably affected by these ligands, suggesting that the inhibitory effects of PPARγ on cell growth do not extend to the other PPARs.

To determine whether the cells treated with these PPARγ ligands undergo programmed cell death or necrosis, we examined the propidium iodide (PI) staining of LNCaP, DU145, and PC3 cells by using cell sorting analysis. Despite the differences in cell number, 5 days of treatment with troglitazone or rosiglitazone at 10 μM did not cause a significant increase in PI positive staining (data not shown). These data suggest that changes in cell growth, but not cell death, account for the reduction in cell number observed after treatment with these drugs.

Table 1. Mutation and deletion analysis of PPARγ in prostate cancer cell lines and prostate primary carcinomas

	Mutations	Deletions
Cell lines (LNCaP, DU145, PC3)	None	None
Tumors		
Informative	0/21	8/21
Noninformative	0/17	0/17

PPARγ Activation Decreases PSA Production of LNCaP Cells. PSA is a widely used marker for the diagnosis and management of patients with prostate cancer. In general, the level of serum PSA reflects tumor volume. To determine whether the activation of PPARγ can alter PSA levels, we measured PSA in supernatants from LNCaP cells after exposure to troglitazone. As shown in Fig. 3, the amount of PSA secreted per cell decreased as a result of treatment with troglitazone. After 48-h treatment with troglitazone, PSA secretion decreased by 4.7%, 24.9%, and 66.9% at 0.4, 2, and 10 μM troglitazone, respectively. PSA secretion continued to decrease with troglitazone treatment at 10 μM over the next 48 h by 75% compared with the values of the control cells. These data suggest that activation of PPARγ can exert a modulatory effect on the PSA expression of these cells.

Effects of PPARγ Activation on PSA Levels in Prostate Cancer Patients. To evaluate the effects of PPARγ activation in patients with androgen-dependent and androgen-independent prostate cancer, we conducted a phase II clinical study using the PPARγ ligand troglitazone. The baseline patient characteristics are summarized in Table 2. Forty-one men with histologically confirmed prostate cancer, and no symptomatic metastatic disease, were treated with troglitazone at 800 mg daily. The results of the treatment are summarized in Table 3. One patient with androgen-dependent prostate cancer had a decrease in PSA greater than 50% confirmed on multiple determinations. Interestingly, this PSA response was achieved after 16 months of treatment (Fig. 4*A*). No PSA decreases >50% were observed in patients with androgen-independent prostate cancer. PSA decreases less than 50%, confirmed on two determinations at least 4 weeks apart, were observed in 3/12 (25%) patients with androgen-dependent prostate cancer and 4/29 (14%) patients with androgen-independent prostate cancer.

Treatment with troglitazone was associated with long periods of stable disease characterized by the absence of new symptoms and no new metastases. The median duration of treatment was 18 weeks (range 5.4 to 90 weeks). For men with androgen-dependent prostate cancer, the median duration of treatment was 26.8 weeks (range 13 to 90 weeks). Among men with androgen-independent prostate cancer, the median duration of treatment was 14.3 weeks (range 5.4 to 54 weeks). The PSA

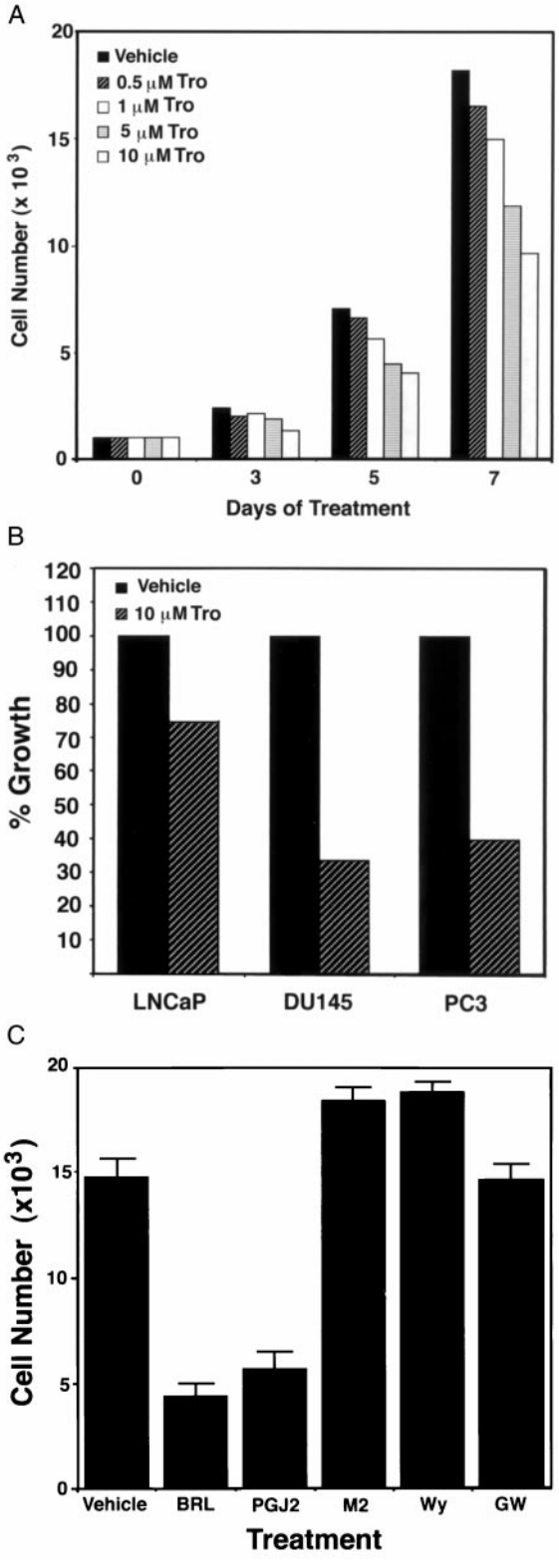

Fig. 2. PPARγ activation inhibits cell growth in prostate cancer cell lines. (*A*) Time course and dose-response inhibition of cell growth of DU145 treated with troglitazone. (*B*) Comparison of the growth inhibitory effects of troglitazone at 10 μM in LNCaP, DU145, and PC3 cells. (*C*) Growth inhibition in DU145 cells treated for 7 days with 5 μM rosiglitazone (BRL) and 5 μM 15-deoxy-$\Delta^{12,14}$PGJ$_2$ (PGJ2). DU145 treated with 10 μM M2, an inactive metabolite of troglitazone, 10 μM of Wy, or 5 μM of GW 00233, PPARα and PPARδ ligands, respectively, did not show any growth inhibition compared with troglitazone-treated cells.

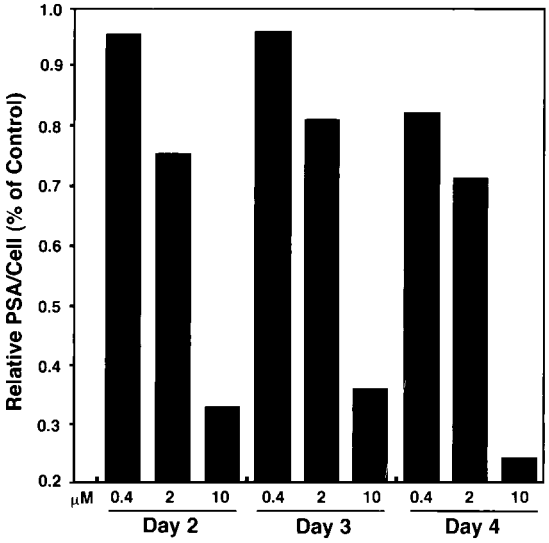

Fig. 3. PSA levels are decreased by troglitazone *in vitro*. LNCaP cells show decreased levels of PSA after treatment with different doses of troglitazone compared with vehicle-treated cells.

histories for the two patients with the longest PSA stabilizations are shown in Fig. 4. There were insufficient pretreatment data to evaluate the effect of troglitazone treatment on PSA velocity or PSA doubling time. Troglitazone treatment was tolerated well overall, except for one patient in whom a transient grade 3 elevation in transaminases was noted and another patient in whom reversible grade 3 diarrhea was observed. There were no other grade 2 or greater toxicities.

It has been shown that troglitazone treatment decreases total and free testosterone levels in women with polycystic ovary syndrome (23). To evaluate the effect of troglitazone treatment on gonadal steroids in men with androgen-dependent prostate cancer, we determined the levels of sex hormone binding globulin (SHBG), total and free testosterone, estradiol, lutenizing hormone (LH), and follicle stimulating hormone (FSH) at baseline and after 4 weeks of treatment. Treatment with troglitazone resulted in significant increases in SHBG and total testosterone levels with no significant change in the levels of free testosterone, estradiol, LH, or FSH (data not shown). These data suggest that changes in serum PSA seen after troglitazone treatment in men with androgen-dependent prostate cancer are mediated directly

Table 2. Baseline characteristics of the patients enrolled in the phase II clinical study

Age	
Median	70 years
Range	49–86 years
PSA	
Median	13.7 ng/ml
Range	1.0–919.6 ng/ml
Androgen dependent	12/41 (29%)
Androgen independent	29/41 (71%)
Metastases	16/41 (39%)
Bidimensionally measurable disease	0/41 (0%)
Gleason sum	
4–6	4/41 (11%)
7	14/41 (34%)
8–10	23/41 (55%)

Table 3. Changes in PSA levels in prostate cancer patients treated with troglitazone

Changes in PSA	Overall, $n = 41$	Androgen-dependent, $n = 12$	Androgen-independent, $n = 29$
>50% Decrease	1/41	1/12	0/29
<50% Decrease	7/41	3/12	4/29
No decrease	33/41	8/12	25/29
Median time on study	18+ weeks	20+ weeks	18+ weeks

through PPARγ rather than by an indirect effect of troglitazone on gonadal steroid levels.

Discussion

Inhibiting proliferation of cancer cells via induction of differentiation is an attractive approach to human cancer therapy. Although all-trans retinoic acid has been shown to induce differentiation and lead to clinical benefit in patients with acute promyelocytic leukemia (24), no treatment based on cell differentiation exists for human solid tumors. Given the role of PPARγ in inducing cell growth inhibition through a differentiation-like response in several cell lines and in a small clinical trial for liposarcoma (16), we attempted to extend this approach to human prostate cancer. The relatively low toxicity of PPARγ ligands adds considerable interest to this approach.

We show here that PPARγ is expressed in human prostate epithelium, both in normal tissue and carcinomas. PPARγ also is expressed in several of the common prostate cancer cell lines, albeit at varying levels. More importantly, activation of PPARγ by different classes of ligands influences the growth of prostate cancer cells. In particular, the ligand activation of this receptor inhibits cell growth in a dose-dependent manner. Importantly, we demonstrate that these effects are the specific consequence of PPARγ activation, because PPARα or PPARδ ligands do not show similar effects. To ensure that no intragenic PPARγ mutations occur in prostate cancers similar to those seen in colon cancers (17), which might interfere with ligand-related differentiation therapy, we examined the gene in 38 primary prostate cancers and the cell lines LNCaP, DU145, and PC3. None of these primary carcinomas or cell lines were found to have any intragenic mutation.

Encouraged by the results obtained *in vitro* and by the low toxicity of troglitazone, we decided to examine the effects of this drug in patients. Forty-one patients with metastatic prostate cancer were treated with 800 mg/day of troglitazone. Patients were monitored every 4 weeks by measuring serum PSA levels. Eight of 41 men experienced sustained PSA decreases after treatment. Twenty percent of patients had sustained serum PSA decline between 1% and 50%. Perhaps more impressively, a significant fraction (39%) of patients demonstrated prolonged stabilization of their serum PSA. In one patient, troglitazone treatment resulted in a dramatic fall in serum PSA (98%) below baseline levels. The drug was extremely well tolerated with no side effects related to the treatment except for transient liver function test abnormalities seen in one patient and diarrhea in another.

The interpretation of these data are complex for several reasons. The effects on serum PSA observed in a fraction of patients could reflect primary changes in the relative expression of the PSA gene per cell or changes in the rate of cell growth. The changes that occur immediately after this drug is given may reflect an alteration in PSA synthesis and secretion per unit of tumor. However, changes in gene transcription rates generally occur over hours or days at the most. The very prolonged stabilization of PSA values in a subset of patients is more likely

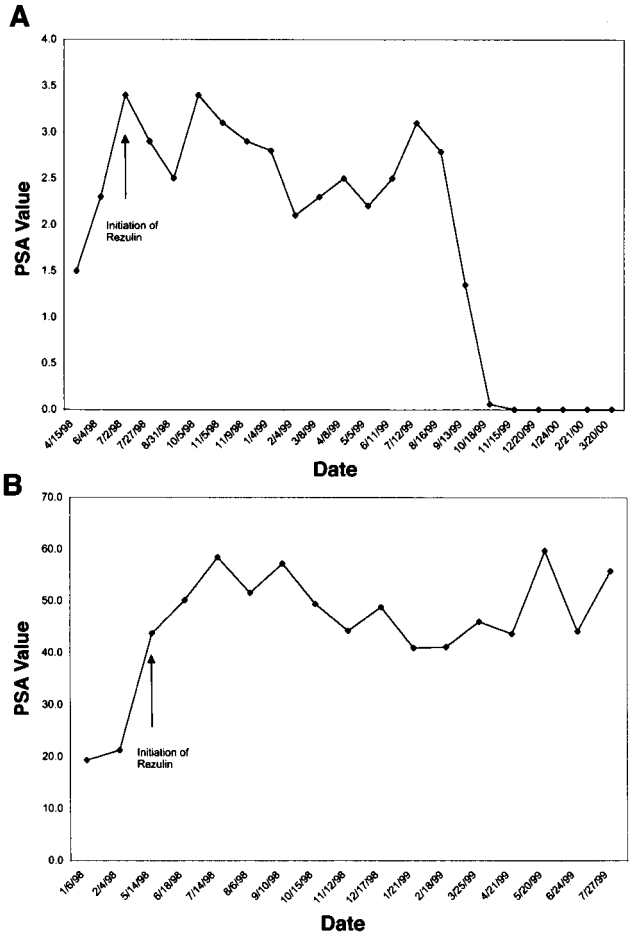

Fig. 4. PSA levels are modulated by troglitazone in prostate cancer patients. The PSA levels of prostate cancer patients, treated with 800 mg of troglitazone/day, were measured every 4 weeks. (A) The patient received radiation therapy in 11/94, nadir of 0.1. (*B*) Patient had post radiation therapy nadir of 1.9 on 6/2/94.

to reflect a prolonged change in the growth status of the tumor in these patients. This possibility is supported by the clinical status of these patients. None of the patients became symptomatic during the treatment period. Data on a control group, obtainable in a larger clinical trial, are needed to confirm this trend.

These results suggest that targeting PPARγ for the treatment of prostate cancer may be useful for at least a certain subset of patients. Markers for biologic effects are currently lacking. How the patients who had stable PSA values in response to troglitazone differ from those who didn't remains unclear. However, our previous studies with breast cancer cells (10) suggested that tumors having high levels of active mitogen-activated protein (MAP) kinase may exhibit *de novo* resistance to PPARγ ligands. MAP kinase is known to modify and suppress PPARγ function (19).

Because mitogen-activated protein kinase, a downstream target of activated ras, often is elevated also in prostate cancer (25), it is possible that it could contribute to resistance also in this context. Another biologic marker for response is the status of the gene encoding PPARγ itself. For example, in colon cancers, all mutations found compromised the ability of this receptor to respond to ligands (17). Although none of the 38 prostate cancer samples analyzed had intragenic mutations,

almost half of the informative tumors were found to carry hemizygous deletions in this region. It is possible that hemizygosity for PPARγ predicts for a poorer or less durable response. This finding also would confirm that haploinsufficiency can contribute to the reduction in the tumor suppressing effect of this receptor, as originally postulated for colon cancers (17). Both somatic and germ-line profiling of PPARγ in the context of response to ligands must be pursued in the future as an important pharmacogenomic issue.

It is possible that treatment with any effective activator of PPARγ could provide an additional useful agent in the treatment of prostate cancer. This approach is especially attractive because of the mild side effect profile associated with PPARγ ligands. Larger phase II and phase III studies with clinical endpoints are needed to confirm the biologic activity of PPARγ ligands and to delineate subsets of patients who may benefit from such treatments.

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