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Role of Peroxisome Proliferator-Activated Receptor γ and Its Ligands in Non-Neoplastic and Neoplastic Human Urothelial Cells

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Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors and is expressed in several types of tissue. Although PPARγ reportedly is expressed in normal urothelium, its function is unknown. We examined the expression of PPARγ in normal urothelium and bladder cancer in an attempt to assess its functional role. Immunohistochemical staining revealed normal urothelium to express PPARγ uniformly. All low-grade carcinomas were positive either diffusely or focally, whereas staining was primarily focal or absent in high-grade carcinomas. A nonneoplastic urothelial cell line (1T-1), a low-grade (RT4) carcinoma cell line, and two high-grade (T24 and 253J) carcinoma cell lines in culture expressed PPARγ mRNA and protein. Luciferase assay indicated that PPARγ was functional. PPARγ ligands (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, troglitazone and pioglitazone) suppressed the growth of nonneoplastic and neoplastic urothelial cells in a dose-dependent manner. However, neoplastic cells were more resistant than were nonneoplastic cells. Failure to express PPARγ or ineffective transcriptional activity may be some of the mechanisms responsible for resistance to the inhibitory action of PPARγ ligands. ***(Am J Pathol 2001, 159:591–597)***

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors and functions as a regulator of adipocyte differentiation and lipid metabolism.[1,2] PPARγ is expressed in several types of tissue including the kidney, spleen, colon, breast, and urinary tract as well as adipose tissue.[3–6] Recent studies indicate that this receptor can induce differentiation in liposarcoma cells,[7] monocytes/macrophages,[8] and breast cancer cells *in vitro*.[9] Furthermore, it has been shown that the growth of colon cancer cells and androgen-independent prostate cancer cells is inhibited by treatment with PPARγ agonists *in vitro* and *in vivo*.[10,11] Although these studies suggest that PPARγ may be a potential target for cancer treatment, Lefebvre and colleagues[12] and Saez and colleagues[13] demonstrated that PPARγ agonists promoted the development of colorectal tumors in mice.

We are interested in elucidating the role of PPARγ and its ligands in bladder cancer. Despite its rich expression in normal urothelium, the role of PPARγ in the urothelium is unknown.[5,6] One of the natural ligands for PPARγ, prostaglandin D metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2),[14,15] is present in urine abundantly.[16] In this study, we examined the expression of PPARγ protein in normal human urothelium and bladder carcinoma tissue by immunohistochemistry, and tested the effect of two classes of ligands for PPARγ, 15d-PGJ_2, and troglitazone (TRO) and pioglitazone (PIO) (thiazolidinedione derivatives)[10] on the growth of nonneoplastic and neoplastic human urothelial cells *in vitro*.

Materials and Methods

Immunohistochemistry

Portions of normal ureter derived from 3 nephrectomy specimens removed for renal cell carcinoma, 2 normal bladder mucosal biopsies, and 48 bladder carcinoma specimens removed transurethrally were used. Excised specimens were fixed immediately in cold 4% paraformaldehyde (EM Science, Gibbstown, NJ) solution. After overnight fixation in the refrigerator, they were processed by the routine procedure and embedded in paraffin. Before staining, sections mounted on poly-L-lysine-coated slides were deparaffinized with xylene, and rehydrated in graded ethanol. For the purpose of antigen retrieval, sections were incubated in Target retrieval solution (DAKO, Carpinteria, CA) at 95°C for 20 minutes and cooled at room temperature for 20 minutes. After blocking with 3% horse serum in phosphate-buffered saline,

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samples were incubated at room temperature for 3 hours with the monoclonal mouse anti-PPARγ antibody (E-8, lot no.H218; Santa Cruz Biotechnology, Santa Cruz, CA) or the polyclonal rabbit anti-PPARγ1, 2 antibody (Calbiochem, San Diego, CA) diluted to 1:50 or 1:2000 with the blocking solution, and for the subsequent steps the avidin-biotin-peroxidase complex method with a Vectastain ABC kit (Vector, Burlingame, CA) was used. Carcinomas were graded according to the World Health Organization classification.[17]

Cells and Cell Culture

The three human bladder carcinoma cell lines used were RT4, T24, and 253J.[18] The cells were grown in Ham's F12 (RT4 and T24) or RPMI 1640 (253J) medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

An immortalized nonneoplastic human urothelial cell line (1T-1) was established in our laboratory.[19] It originated from Hu1 cells[20] that were derived from the ureter of a 71-year-old male undergoing nephrectomy for renal cell carcinoma. The benign nature of the starting material was confirmed by histological examination of the remaining portion of the ureter. Seven days after plating when epithelial outgrowth from explants reached ~2 cm in diameter, infection with an amphotrophic retrovirus vector LXSN16E6E7, containing E6 and E7 genes of human papilloma virus type 16 (kindly provided by Dr. D. Galloway, Fred Hutchinson Cancer Center, Seattle, WA) was performed overnight in 4 ml of keratinocyte serum-free medium (K-SFM; Life Technologies) in the presence of 4 μg/ml of polybrene (Sigma, St. Louis, MO). G418 (Life Technologies) at 200 μg/ml was added at 48 hours for selection of immortalized cells. Cells at passage 16 were subjected to soft agar assay[19] and several colonies were picked up, expanded, and designated as 1T-1, 1T-2, and 1T-3. One of the clones (1T-1) was used. 1T-1 cells were maintained in K-SFM supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Isolation of RNA for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cytoplasmic RNA from cultured cells was extracted as follows. Cells grown in monolayers were harvested at an early confluency. RNA was prepared by lysing cells in hypotonic buffer containing Nonidet P-40 (Sigma), followed by removal of nuclei. Cytoplasmic RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies) at 42°C for 60 minutes with the use of random primers (5 μmol/L; Life Technologies). Subsequently, 1 μl of the product was subjected to PCR amplification. PCR was performed as follows. The final concentration of deoxynucleotide triphosphates and primers in the reaction mixture was 200 μmol/L and 1 μmol/L, respectively. *Taq* DNA polymerase (Cetus Perkin-Elmer, Norwalk, CT) was added to the mixture at a final concentration of 0.05 U/ml, and the reaction was performed in a DNA thermal cycler (Cetus Perkin-Elmer). To amplify PPARγ1 (790 bp) and PPARγ2 (877 bp), the nucleotide bases used were 5′-CCG CTC GAG CGG GCC GCC GTG GCC GCA GAA-3′ as an upstream primer for human PPARγ1, 5′-CCG CTC GAG CGG AAA CCC CTA TTC CAT GC-3′ as an upstream primer for human PPARγ2, and 5′-AGG AAT TCA TGT CAT AGA TAA CG-3′ as a downstream primer for both PPARγ1 and 2 and 5′-GAA ATC CCA TCA CCA TCT TCC AGG-3′ as an upstream primer and 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ as a downstream primer for glyceraldehyde-3-phosphate dehydrogenase.[21]

Western Blotting

Cells grown in monolayers were harvested at subconfluency and lysed with a lysing buffer [62.5 mmol/L Tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, and 7 mol/L urea] (Sigma). The sample was boiled for 10 minutes and was then forcefully passaged five times through a 25-gauge needle. The samples were centrifuged at 12,000 × *g* for 10 minutes and the precipitates were discarded. Fifty-μg protein samples of the supernatant were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel. Protein was transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and the membrane was incubated with an anti-PPARγ antibody (E-8; Santa Cruz Biotechnology or Calbiochem), anti-PPAR binding protein (PBP) antibody,[22] or anti-retinoid X receptor α (RXRα) antibody (D-20; Santa Cruz Biotechnology) and treated with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Densitometric analysis was done with an NIH Image 1.59.

Cell Growth Assay

Cells were seeded on a flat-bottom 96-well plate (Falcon, Becton Dickinson, Franklin Lakes, NJ) at the density of 2×10^3 (1T-1, T24, and 253J) or 5×10^3 (RT4) cells per well in the respective appropriate growth medium. Twenty-four hours later, cells were grown in the same medium containing 15d-PGJ_2 (0 to 10 μmol/L; Cayman Chemical, Ann Arbor, MI), TRO (0 to 50 μmol/L; a gift from Parke-Davis, Ann Arbor, MI), or PIO (0 to 50 μmol/L; a gift from Takeda Chemical Industries, Ltd., Tokyo, Japan). After 24 hours of incubation, cell proliferation was assessed by Cell proliferation enzyme-linked immunosorbent assay, BrdU (Roche Molecular Biochemicals, Mannheim, Germany). We also assessed cell number by manual counting; cells were seeded on a flat-bottom 6-well plate (Falcon, Becton Dickinson) at the density of 5×10^4 cells per well. Twenty-four hours later, cells were treated with above PPARγ ligands. After 3 days, cells were recovered by treatment with 0.05% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid (Life Technologies) and counted with a hemocytometer.

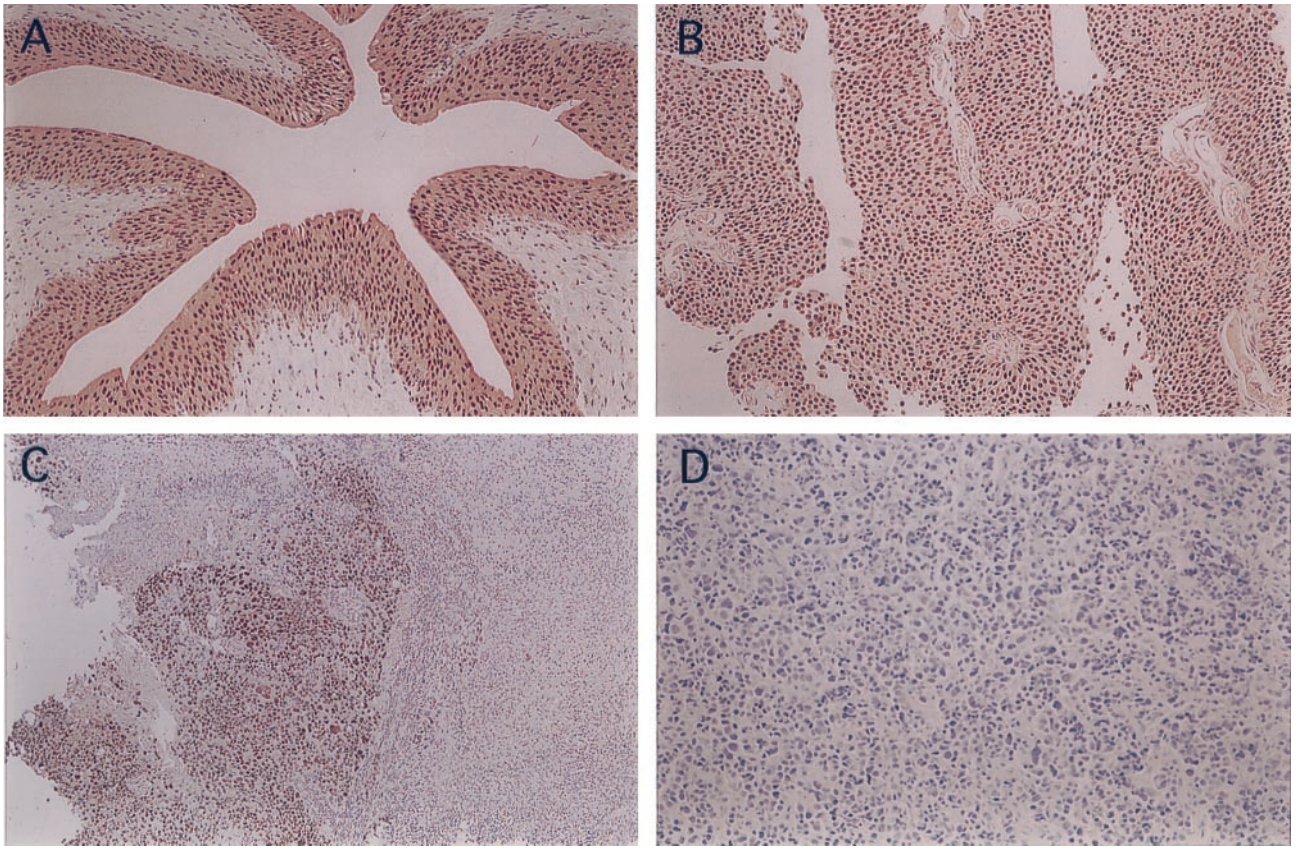

Figure 1. Immunohistochemical staining for PPARγ protein in normal human ureter (**A**) and low-grade (**B**) and high-grade (**C** and **D**) bladder carcinomas. All nuclei are uniformly stained in ureteral urothelium [original magnification, ×100 (**A**)] and grade 1 transitional cell carcinoma [original magnification, ×100 (**B**)]. In grade 3 transitional cell carcinoma, nuclear staining is focal [original magnification, ×40 (**C**)] or completely absent [original magnification, ×100 (**D**)].

Transfections and Luciferase Assay

Cells were seeded on a flat-bottom 6-well plate (Falcon, Becton Dickinson) at the density of 3×10^5 cells per well in the respective appropriate growth medium. Twenty-four hours later, transfection was done by using the Effectene transfection reagent (Qiagen, Valencia, CA) mix with a reporter plasmid PPRE (PPAR response elements)-TK-LUC produced in our laboratory.[23] The PPRE-TK-LUC was constructed by inserting three copies of PPRE (*AGGACAAAGGTCA*) into *Hin*dIII/*Sal*I site of TK-LUC. The transfection mix was replaced with the complete medium with or without PPARγ ligands (15d-PGJ_2 and TRO) and was further incubated for 24 hours. The cells were lysed with cell culture lysis reagent (Promega, Madison, WI). Luciferase activity was measured with the use of a luciferase assay reagent (Promega) in a scintillation counter (Aloka, Tokyo, Japan).

Results

Immunohistochemical Demonstration of PPARγ Protein in Normal Urothelium and Bladder Cancer Tissue

PPARγ protein was uniformly expressed in the urothelium of three normal ureters and two normal bladder mucosal biopsies and was localized to nuclei (Figure 1A). The staining reaction was more intense in the superficial and intermediate cells than in the basal cells. Localization and staining intensity were similar when the results with the two antibodies were compared. Expression of PPARγ protein in bladder carcinoma cells is summarized in Table 1. All cases of transitional cell carcinomas of low grades (grade 1 and 2) demonstrated an intense nuclear staining either in all cells or focally up to 90% of tumor cells (Figure 1B). In contrast, staining was focal to absent in high-grade carcinomas (Figure 1, C and D). Statistically significant loss of PPARγ expression was evident in grade 3 carcinomas as compared to the expression in

Table 1. Immunohistochemical Expression of PPARγ in Bladder Carcinoma

		Cases expressing PPARγ, *n*		
Grade	*n*, Total	Diffuse*	Focal*	None
1	18	17	1	0
2	14	11	3	0
3	16	3	7	6†

*Diffuse staining: all tumor cell nuclei stained. Focal staining: 75% of tumor cell nuclei stained in grade 1 carcinomas whereas stained nuclei ranged from 30 to 90% in grade 2 carcinomas, and 10 to 95% in grade 3 carcinomas.

†$P = 0.0007$ when compared to that of grade 1 and 2 carcinomas combined (Fisher's exact test).

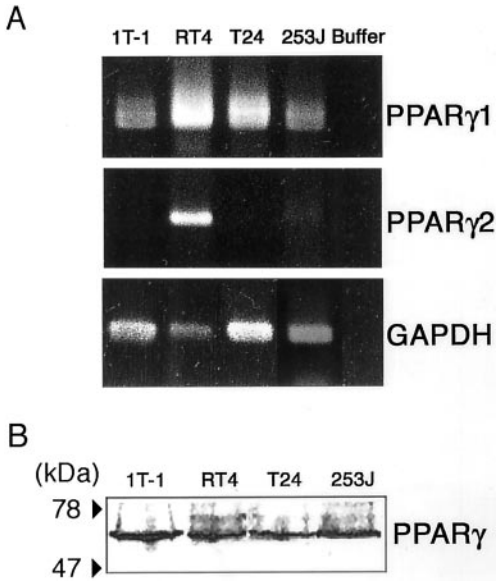

Figure 2. Expression of PPARγ by nonneoplastic and neoplastic human urothelial cells. **A:** RT-PCR for expression of PPARγ1 and -2 mRNA. All samples demonstrate PPARγ1 message whereas PPARγ2 was expressed only in RT4 cells. Primers specific for PPARγ1 and -2 and glyceraldehyde-3-phosphate dehydrogenase cDNAs generated fragments of 790 bp, 877 bp, and 782 bp, respectively. **B:** Western blot analysis for PPARγ protein. Cell lysates were isolated and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (50 μg of protein/lane). Proteins from gels were transferred to polyvinylidene difluoride membrane, and PPARγ was detected with mouse monoclonal anti-PPARγ antibody and an enhanced chemiluminescence kit. All samples demonstrate PPARγ protein (~58 kd).

combined grade 1 and 2 carcinomas ($P = 0.0007$, Fisher's exact test).

Expression in Vitro *of PPARγ in Nonneoplastic and Neoplastic Urothelial Cells*

First, we examined the expression of PPARγ mRNA in immortalized nonneoplastic and neoplastic urothelial cells. PPARγ1 mRNA was detected by RT-PCR in all cell types whereas PPARγ2 mRNA was expressed only in RT4 cells (Figure 2A). By Western blotting, PPARγ (~58 kd) was detected at varying levels in all cell types (Figure 2B). Expression of PPARγ protein was reduced by 49, 63, and 68%, respectively, in RT4, T24, and 253J cells as compared to the expression in 1T-1 cells. By immunocytochemical staining, the protein was uniformly localized to nuclei in all cells in all cell lines (data not shown).

Effect of 15d-PGJ$_2$, TRO, and PIO on Growth in Vitro *of Nonneoplastic and Neoplastic Urothelial Cells*

We next tested the effect of PPARγ ligands on the growth of immortalized nonneoplastic and neoplastic cells. Treatment with PPARγ ligands suppressed the growth of all tested cells in a dose-dependent manner (Figure 3). Low-dose 15d-PGJ$_2$ (0.5 μmol/L) almost completely inhibited the growth of 1T-1 cells. In contrast, all carcinoma cell lines were resistant to its suppressive effect at concentrations up to 1 to 5 μmol/L (Figure 3A).

The response to TRO was similar to that to 15d-PGJ$_2$; low-dose TRO (2 to 8 μmol/L) strikingly suppressed the growth of 1T-1 cells. Cancer cells were more resistant; complete suppression required TRO at a much higher concentration (20 μmol/L for RT4 and 50 μmol/L for T24). 253J cells were more resistant and 50% of cells survived at 50 μmol/L (Figure 3B). Treatment with another synthetic PPARγ ligand, PIO, also showed the similar inhibitory effect on nonneoplastic and neoplastic human urothelial cell lines except 253J cells (Figure 3C).

Ligand-Induced Transcriptional Activity of PPARγ

We examined the transcriptional activity of PPARγ using a luciferase reporter plasmid containing a PPARγ response element. TRO ranging from 0.1 μmol/L to 50 μmol/L and 15d-PGJ$_2$ from 0.1 to 10 μmol/L were tested. The concentrations of TRO shown in Figure 4 are those that resulted in the best transcriptional activity. We could not examine the activity of RT4 cells because of low transfection efficiency. Treatment of 1T-1 cells with TRO (0.1 μmol/L) strikingly increased the transcription of luciferase gene by 5.2-fold. In T24 and 253J cells TRO (10 or 20 μmol/L) activated transcription by twofold (Figure 4). Treatment with 15d-PGJ$_2$ (0.1 μmol/L) increased luciferase activity by twofold in 1T-1 cells. However, luciferase activity by 15d-PGJ$_2$ (up to 10 μmol/L) was not detected in T24 and 253J cells (data not shown).

Expression of PBP and RXRα Protein in Nonneoplastic and Neoplastic Urothelial Cells

We examined the expression of PBP, a PPARγ co-activator, and RXRα, a PPARγ heterodimeric partner, by Western blotting. PBP protein (~165 kd) was detected only in RT4 cells (Figure 5). RXRα protein (~55 kd) was expressed in all cell lines. 1T-1 and RT4 cells expressed RXRα protein at a much higher level than did T24 and 253J cells (Figure 5).

Then we tested synergistic effect of a RXRα ligand, 9-*cis*-retinoic acid (9-*cis*-RA) on a PPARγ ligand. In RT4, T24, and 253J cells, 9-*cis*-RA (5 μmol/L) enhanced the inhibitory effect of TRO by 1.1-fold, 1.5-fold, and twofold, respectively. The growth of 1T-1 cells was inhibited completely by treatment with 9-*cis*-RA (5 μmol/L) alone whereas it had no effect on the growth of all carcinoma cell lines (data not shown).

Discussion

Previous studies have reported that PPARγ ligands mostly inhibit the growth of breast,[9] prostate,[10] and colon cancer[11] cells *in vitro* and *in vivo*. We demonstrated here that PPARγ was expressed in nonneoplastic and neoplastic urothelial cell lines at both mRNA and protein levels. However, the sensitivity to the inhibitory effects by

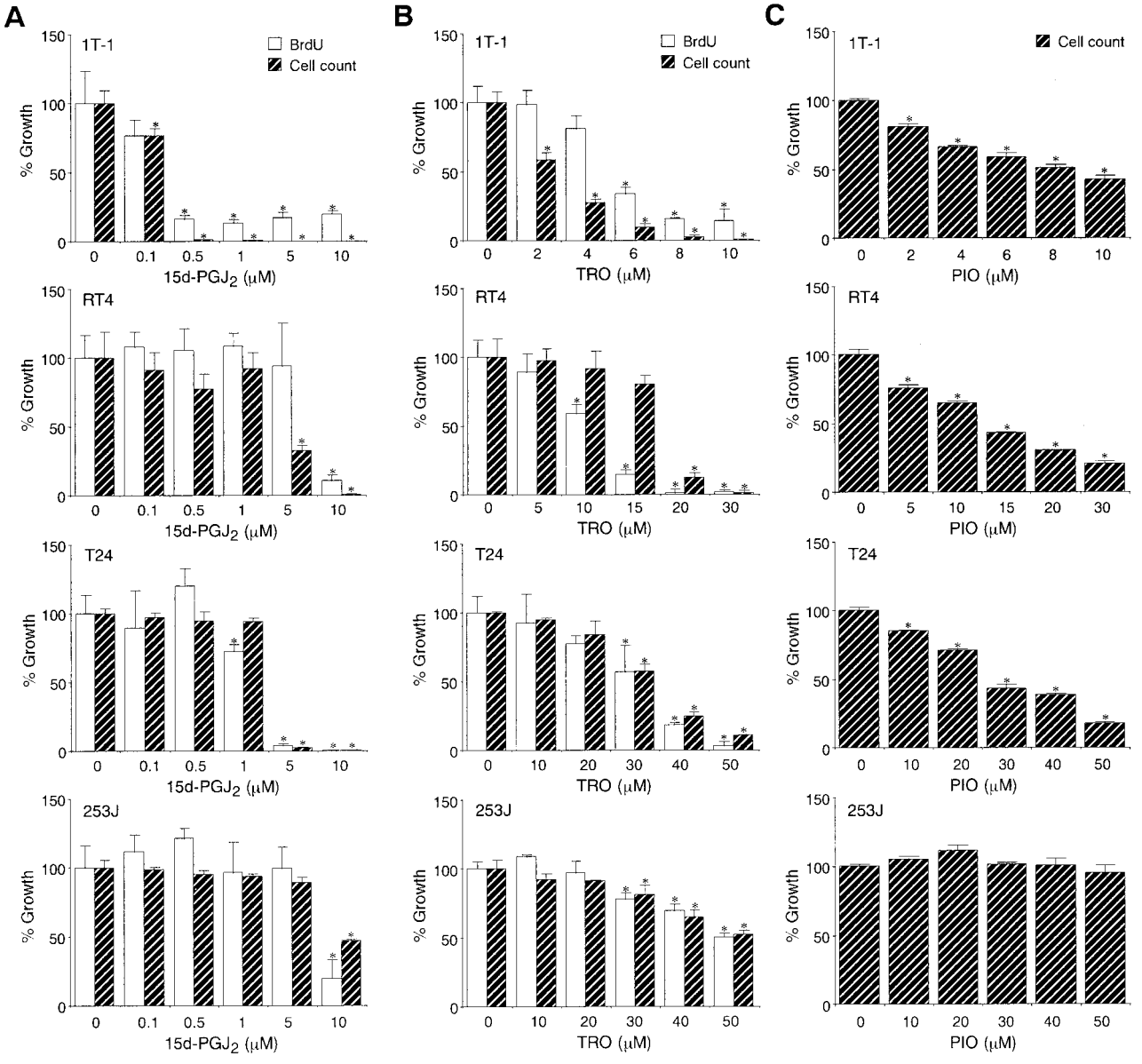

Figure 3. Effect of 15d-PGJ_2 (**A**), TRO (**B**), and PIO (**C**) on growth of nonneoplastic and neoplastic urothelial cells. Cells were seeded on a 96-well or 6-well plate in the medium with 5% fetal bovine serum appropriate for each cell type. Twenty-four hours later, medium was changed to the same medium containing 15d-PGJ_2 (0 to 10 μmol/L), TRO (0 to 50 μmol/L), or PIO (0 to 50 μmol/L). After incubation for 24 hours, cell proliferation was assessed by cell proliferation enzyme-linked immunosorbent assay, BrdU kit. After 3 days, the number of cells was counted with a hemocytometer after cells were recovered by trypsinization. Results are expressed as ratio to the respective control culture. Bars denote SD of triplicate samples. The ligand concentrations in the abscissa are on an arbitrary scale. *, $P < 0.0001$ compared with the respective control culture.

PPARγ ligands (15d-PGJ_2, TRO, and PIO) was variable. Nonneoplastic urothelial cell line 1T-1 was sensitive to the inhibitory effects of PPARγ ligands. In contrast, carcinoma cell lines were resistant.

Studies by others using various carcinoma cell lines all indicate that PPARγ ligands reduce growth rate.[10,11,24,25] Thus PPARγ ligands have been suggested to be a useful therapeutic agent for breast, prostate, or colon carcinomas.[9–11] The rationale behind this approach is that whereas normal cells either do not express PPARγ or, if they express, only at a very low level, whereas carcinoma cells express the receptor abundantly.[10] This allows selective action of PPARγ ligands on neoplastic cells and induces cell death or differentiation. However, there have been recent reports indicating that treatment with a PPARγ ligand promotes the development of colon tumor in Min+/− mice that lack one functional copy of the *APC* tumor suppresser gene.[12,13] Normal human ureter reportedly expressed PPARγ protein.[5] In the present study, we confirmed this observation. However, in bladder carcinoma tissues as well as in carcinoma cell lines, its expression seemed affected by the degree of malignancy: in low-grade (grades 1 and 2) carcinomas, PPARγ protein was uniformly or diffusely demonstrated by immunohistochemistry, whereas in high-grade (grade 3) carcinomas the expression of PPARγ protein was mostly heterogeneous or absent.

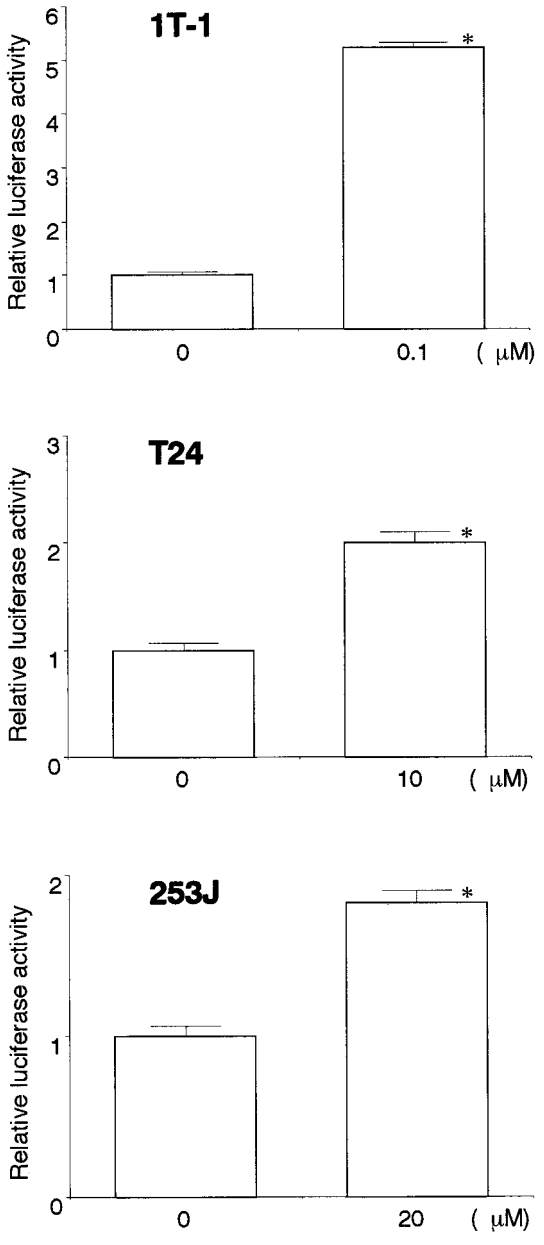

Figure 4. Ligand-induced transcriptional activation of PPARγ. Cells (1T1, T24, and 253J, 3×10^5 per well) were seeded on a 6-well plate in the medium appropriate for each cell type. Twenty-four hours later, transient transfection was done by using Effectene transfection reagent with PPRE-TK-LUC. The transfection mix was replaced by complete medium with or without TRO (0.1, 10, or 20 μmol/L) and incubated for an additional 24 hours. The cells were lysed with cell culture lysis reagent. Luciferase activity was measured by using luciferase assay reagent in a scintillation counter. Results are expressed as ratio to the respective control culture. Bars denote SD of triplicate samples. *, $P < 0.0001$ compared with the respective control culture.

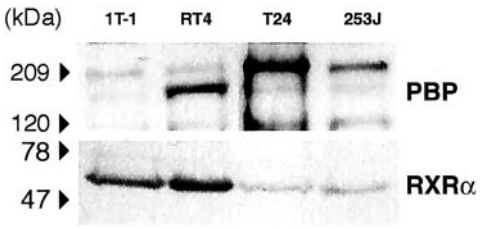

Figure 5. Expression of PBP and RXRα protein by nonneoplastic and neoplastic human urothelial cells. Cell lysates were isolated and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (50 μg of protein/lane). Proteins from gels were transferred to polyvinylidene difluoride membrane, and PBP and RXRα were detected with rabbit anti-PBP and -RXRα antibody and an enhanced chemiluminescence kit. Only RT4 cells demonstrate PBP protein. RXRα expression is at much higher levels in 1T-1 and low-grade carcinoma cell line RT4 than in high-grade carcinoma cell lines T24 and 253J.

In the *in vitro* study we demonstrated that high-grade carcinoma cell lines T24 and 253J expressed RXRα at a lower level than did the nonneoplastic cell line 1T-1 and a low-grade carcinoma cell line RT4. Expression of cofactor PBP was almost exclusive to RT4 cells. The luciferase reporter assay indicates that ligand-induced transcriptional activity of PPARγ is most active in 1T-1 in which a fivefold to sixfold increase was observed at the TRO concentration as low as 0.1 μmol/L. On the other hand, transcriptional activity of carcinoma cells was low (up to twofold) requiring ligand concentrations as high as 10 to 20 μmol/L. Treatment with 15d-PGJ_2 did not induce transcriptional activity. It is possible that mutations in PPARγ gene may affect ligand-dependent transcriptional activity.[26,27] In human colon cancer,[26] two missense mutations were detected in the ligand-binding domain and impaired the function of the protein. One of mutations showed a normal response to synthetic ligands but decreased transcription when exposed to natural ligands. Though 15d-PGJ_2 significantly inhibited the growth of neoplastic urothelial cells, it induced no PPRE luciferase activity. The result suggests that the growth-inhibitory action of 15d-PGJ_2 may depend on some other mechanisms. For example, according to a recent report,[28] 15d-PGJ_2 is a direct inhibitor of IkB kinase (IKKβ) and prevents nuclear factor-kB activation.

Based on these observations we suggest several mechanisms to account for the differential response of urothelial cells to PPARγ ligands: nonneoplastic cells are highly sensitive to their cytocidal effects because of their efficient transcriptional activity whereas carcinoma cells are resistant because of low transcriptional activity or because of their failure to express PPARγ. Our data suggest that intravesical administration of PPARγ ligand in an attempt to treat bladder cancer may result in severe cytotoxic effects on normal urothelial cells before therapeutic effects on cancer cells can be demonstrated. Furthermore some cancer may be totally refractory because of their mutations or failure to express PPARγ. Additional studies are needed before therapy with PPARγ ligands is attempted.

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