Peroxisome Proliferator-Activated Receptor γ Inhibition Prevents Adhesion to the Extracellular Matrix and Induces Anoikis in Hepatocellular Carcinoma Cells

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Abstract

Activation of the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ) inhibits growth and survival of hepatocellular carcinoma (HCC) cell lines. To further investigate the function of PPARγ in HCC, PPARγ expression patterns in primary tumors were examined, and the responses of two HCC cell lines to PPARγ activation and inhibition were compared. PPARγ expression was increased in HCC and benign-appearing peritumoral hepatocytes compared with remote benign hepatocytes. Both compound PPARγ inhibitors and PPARγ small interfering RNAs prevented HCC cell lines from adhering to the extracellular matrix. Loss of adhesion was followed by caspase-dependent apoptosis (anoikis). PPARγ inhibitors had no effect on initial β1 integrin-mediated adhesion, or on total focal adhesion kinase levels but did reduce focal adhesion kinase phosphorylation. The PPARγ inhibitor T0070907 was significantly more efficient at causing cancer cell death than the activators troglitazone and rosiglitazone. T0070907 caused cell death by reducing adhesion and inducing anoikis, whereas the activators had no direct effect on adhesion and caused cell death at much higher concentrations. In conclusion, PPARγ overexpression is present in HCC. Inhibition of PPARγ function causes HCC cell death by preventing adhesion and inducing anoikis-mediated apoptosis. PPARγ inhibitors represent a potential novel treatment approach to HCC. (Cancer Res 2005; 65(6): 2251-9)

Introduction

Primary hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide and is on the rise in the United States (1). Currently, unresectable HCC has an extremely poor prognosis. The nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ) has recently become a putative therapeutic cancer target in a variety of epithelial cell tumors. PPARγ activation may inhibit neoplastic processes by suppressing tumor cell replication and decreasing tumor cell survival (2). In particular, PPARγ ligands, such as the thiazolidenediones and 15-β-prostaglandin J2 (15β-PGJ2), cause both growth arrest and/or apoptosis in a variety of tumor cell lines (3–9). Despite these suggestions that PPARγ stimulation might favor cancer remission, clinical trials with thiazolidenediones have currently shown modest to little efficacy (6, 10, 11).

Whereas PPARγ activation is associated with tumor cell death in some cases, several observations suggest that the inhibition of PPARγ function may also be beneficial in treating certain cancers. PPARγ is overexpressed in many epithelial tumor cells, including those of the stomach, breast, and lung (3, 5, 12) and may represent a prosurvival factor. In one genetic murine model of colon cancer, stimulation of PPARγ with thiazolidenediones actually led to an increase in tumor formation (13). In addition, PPARγ has been shown to regulate production of hepatocyte growth factor, which favors tumor growth (14). Finally, it has been noted that the greatest clinical benefit shown to date with thiazolidenediones involved prostate tumors, a type with reduced PPARγ levels and possible loss of function (6). In contrast, thiazolidenedione treatment of cancers with normal or higher levels of PPARγ, such as colorectal cancer and liposarcoma, has shown little to no clinical benefit in human trials. Taken together, these results suggest that there may not actually be a loss of PPARγ function in some malignancies, and that exogenous stimulation with PPARγ activators will therefore not have any significant effects on the tumors.

In the present study, the expression of PPARγ in HCC and the effects of PPARγ activation and inhibition on HCC cell lines were assessed. PPARγ levels are greatly increased both within and immediately adjacent to HCC tumors. In addition, the PPARγ inhibitors T0070907 and GW9662, but not the thiazolidenediones troglitazone and rosiglitazone, were able to prevent reattachment of HepG2 and Hep3B cells to extracellular matrix (ECM) proteins and result in apoptotic cell death subsequent to cell detachment (anoikis). Notably, the PPARγ inhibitor T0070907 was at least 5-fold more effective at causing eventual cell death than the thiazolidenediones.

Materials and Methods

Tissue Specimens. Twenty samples of HCC were obtained from surgical resection tissues at the University of Tokyo, Tokyo, Japan. Paraffin-embedded sections were made from each tumor site and from an area at least 5 cm away from the tumor. Subjects included those who developed HCC in the setting of Hepatitis B virus (seven patients), Hepatitis C virus (11 patients), and neither virus (two patients). The degree of tumor differentiation was determined by review by two independent pathologists and ranged from poor to well defined. Serum α-fetoprotein levels ranged from 3 to 39,315 units/mL and did not associate with tumor differentiation status, or with their underlying liver disease process.

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Immunohistochemistry. PPARγ expression was detected using a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by the Vectastain avidin-biotin complex kit and 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA). Staining specificity was verified with a no primary antibody control and by use of the relevant blocking peptide (Santa Cruz Biotechnology).

Cell Culture and Treatment with PPARγ Activators and Inhibitors. HepG2 and Hep3b cells (American Type Culture Collection, Manassas, VA) bind to tissue culture plates via ECM proteins from serum. Unless otherwise noted, PPARγ activators and inhibitors (Cayman Chemical, Ann Arbor, MI) or matched concentrations of vehicle (DMSO) were added to the cells while in the process of readhering to tissue culture plates following trypsinization and cell replating. Polyhydroxyethylmethacrylate (Sigma-Aldrich Chemicals, St. Louis, MO) was coated onto tissue culture plates where applicable. In the process of readhering to tissue culture plates following trypsinization, adherent cells (obtained by trypsinization) and nonadherent cells (obtained by aspiration) and adherent cells (obtained by trypsinization) were determined by counting with a hemocytometer.

Detection of Cell Death and Apoptosis. Cell death was determined by trypan blue exclusion (Sigma-Aldrich Chemicals). Apoptosis was detected using the BD Biosciences Apoptosis Detection Kit (BD Biosciences, San Jose, CA) and the DeadEnd fluorometric terminal deoxynucleotidyl transferase–mediated nick labeling system (Promega, Madison, WI). DNA degradation was measured using the Nicotelli method (15). Where applicable, 200 μmol/L zVAD-FMK (BD Biosciences) were added to the cells directly after plating, 90 minutes before addition of other inhibitors.

Inhibition of PPARγ Function Using Small Interfering RNA. PPARγ expression reduction was achieved using 200 nmol/L small interfering RNA (siRNA; Dharmacon, Lafayette, CO) transfected into 30% confluent HepG2 cells using Oligofectamine (Invitrogen, Carlsbad, CA). siRNA sequences were lamin A/C (control; 5′-CTGGACTTCCAGAAGAACA-3′) and PPARγ (5′-GCCCTTCACTACTGTTGAC-3′). Reduction of PPARγ levels was verified using Western blotting as described in Signaling Experiments using a polyclonal antibody specific for PPARγ that recognizes PPARγ1 (molecular weight, 56 kDa) and PPARγ2 (molecular weight, 60 kDa; ref. 16). Relevant bands were identified by comparison with protein molecular weight markers (Bio-Rad, Hercules, CA).

Measurement of the Effect of T0070907 on PPARγ-Dependent Transcription. The pH(D)x(3)PPRE-luc plasmid has been described previously (17). As Renilla luciferase control plasmids are sensitive to steriod/thyroid/retinoid nuclear receptor stimulation (18), transfection variability was assessed in parallel experiments, using the pRL-TK plasmid (Promega). Transfection efficiency varied <20% from sample to sample.

Determination of the Role of β1 Integrins in Cell Adhesion. Flow cytometric analysis of β1 integrin expression was done using the anti-β1 integrin antibody MAR4 (BD Biosciences). Surface expression of β1 integrin was not affected by trypsinization (data not shown). Identical results were obtained with the anti-β1 integrin antibody TS2/16.2.1 (American Type Culture Collection), which binds β1 integrin regardless of activation state or ligand binding (19, 20). Integrin conformation was determined using the anti-β1 integrin antibody B4 (Chemicon, Temecula, CA) that recognizes the β1 integrin only when it is in the high-affinity ligand-binding form (21).

Competition experiments were done with the antibody TS2/16.2.1. Upon binding, this antibody induces a conformational shift in the integrin that favors ligand binding (19, 20). Experiments were done either with soluble antibody (10 μg/mL TS2/16 antibody for 30 minutes followed by washing and subsequent addition of inhibitors) or plate-bound antibody (1μg/mL in PBS overnight, followed by washing). Identical results were also obtained (data not shown) with the anti-β1 integrin antibody B4, which has been used in soluble form to induce β1-mediated adhesion (22).

Signaling Experiments. At 17 hours after addition of T0070907, cell lysates (adherent and nonadherent together) were made without prior trypsinization using Tris/SDS lysis buffer (Promega). Primary antibodies specific for phospho-FAK (Tyr 925; rabbit polyclonal, Cell Signaling Technologies, Beverly MA), focal adhesion kinase (FAK; rabbit polyclonal, BD Biosciences), and actin (mouse polyclonal, Santa Cruz Biotechnology), appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies), and the Enhanced Chemiluminescence Plus reagent (Amersham Biosciences, Buckinghamshire, United Kingdom) were used. Equivalent results were obtained when blotting for FAK and pFAK on separate membranes, and when using one single membrane subjected to chemical stripping.

Determination of ED50 for PPARγ Activators and Inhibitors. SE3 HepG2 cells were incubated with the indicated concentrations of inhibitor or activator. After 72 hours, cell number was assessed using the CellTiter96 Nonradioactive Proliferation Assay Kit (Promega).

Statistical Analysis. Differences in averages were evaluated using the Student’s t test. Values were considered to be significant when \( P < 0.05 \).

Results

Immunostaining Reveals PPARγ Overexpression. PPARγ expression in HCC from 20 hepatic resection tissues was examined. All of the HCC tumor cells stained positively for PPARγ (Fig. 1A and B) without any noticeable difference between samples. Additionally, within the surrounding tumor capsule, intense positive staining for PPARγ was detected in histologically normal-appearing hepatocytes.

Figure 1. HCC tumors overexpress PPARγ. A. low-power view of PPARγ staining. Diffusely staining tumor (T) and peritumor hepatocytes (arrows). B. higher magnification demonstrating the PPARγ immunostaining of normal liver (i and iv), HCC tumor (ii and v), and benign peritumoral hepatocytes (iii and vi). Top, H&E staining (i–iii); bottom, corresponding PPARγ stain (iv–vi). C. Western blot of lysates from HCC cell lines Hep3B and HepG2 showing PPARγ expression.
Hepatocytes in uninvolved benign-appearing parenchyma, remote from the tumor site, were consistently negative for PPARγ expression (Fig. 1 A and B). This PPARγ staining pattern (i.e., positive tumor and more intense peritumoral capsular staining with negative remote hepatocyte staining) was observed in all 20 subjects. This pattern did not change regardless of the presence or type of viral hepatitis (hepatitis B or C), serum α-fetoprotein levels, or grade of tumor differentiation (poor, moderate, or well; data not shown).

**Treatment with PPARγ Inhibitors Interferes with Adhesion.** To determine whether inhibiting PPARγ function affects HCC cell growth or survival, two transformed PPARγ-expressing HCC cell lines, HepG2 and Hep3B, (Fig. 1C) were incubated with the PPARγ specific inhibitors T0070907 and GW9662. When T0070907 or GW9662 were added to resuspended cells in the process of adhering (see Materials and Methods), they prevented the cells from properly reattaching to a tissue culture plate (Fig. 2). Similar results were obtained with both the HepG2 (shown) and Hep3B (data not shown) cells.

**PPARγ Inhibitors Prevent Cell Flattening but not Initial Adhesion.** HepG2 adhesion follows a defined pattern, which is altered by PPARγ inhibitors. Four hours after plating, 78% of the HepG2 cells are resistant to aspiration; 93% are adherent by 12 hours and remain so for at least 48 hours (Fig. 2 A). The inhibitor T0070907 does not prevent the rapid adherence between 4 and 12 hours, with 78% and 92% adherent at 4 and 12 hours, respectively. However, by 16 hours, a small but statistically significant loss of adhesion is observed, with only 84% of the T0070907-treated cells still adherent, whereas 95% of the control cells are adherent. Over time, T0070907-treated cells continue to lose adhesion, with 50% of the cells nonadherent by 24 hours and 90% nonadherent by 48 hours. These nonadherent cells were completely round.

This reduction in adherence caused by T0070907 is concomitant with alterations in the morphologic changes that normally occur...
Loss of Adhesion (Anoikis).

Cell detachment from the plate by aspiration led to complete cell death, as measured by trypan blue staining (Table 1). The same results were obtained at 24 hours, when T0070907-treated cells had detached from the plate (data not shown). Similarly, there was no increase in degradation of DNA, as measured by amount of DNA in the subdiploid peak (Table 1; 3.9% versus 3.3%, respectively), or by terminal deoxynucleotidyl transferase–mediated nick labeling staining (data not shown) at the 17-hour time point. By 40 hours, when the cells had been detached for 16 hours, Annexin V staining was not increased (11% versus 10%, respectively), although there was an increase in degraded DNA (12% for T0070907 versus 2% for control). By 66 hours, clear increases in Annexin V staining (24% with T0070907 versus 7% in control) and DNA degradation (10% versus 3%, respectively) were seen. Similarly, cells treated with GW9662 did not start to show significant increases in number of cells staining for Annexin V or in degradation of DNA until 64 hours. This onset of apoptotic markers was preceded by complete detachment from the plate by at least 16 hours. Importantly, loss of adhesion led to complete cell death, as measured by trypan blue staining (Table 1). The pan-caspase inhibitor z-VAD-FMK did not prevent PPARγ inhibition of apoptosis (Table 1).

PPARγ Inhibitors Cause Apoptotic Cell Death That Follows Loss of Adhesion (Anoikis). Cell detachment from the plate preceded acquisition of apoptotic markers by at least 16 hours (Fig. 2; Table 1). After treatment with T0070907 for 17 hours, when cells were rounded and adherent, the same percentage of cells was positive for early apoptosis (Annexin V+ and propidium iodide (PI+)) as in the control (9% with T0070907 versus 7% in control). The pan-caspase inhibitor z-VAD-FMK did not prevent PPARγ inhibitors' negative effects on adhesion but did prevent apoptosis (Table 1). In addition, cells that had become nonadherent after T0070907 exposure were able to readhere to the plate after inhibitor was removed (Fig. 3A).

Table 1. Comparison of the timing of PPARγ inhibitor effects on adhesion and on measurements of apoptosis and cell death.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Morphology</th>
<th>% DNA in subdiploid peak %</th>
<th>Cells early apoptosis %</th>
<th>Cells Annexin V+ %</th>
<th>Cells dead</th>
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<td>17</td>
<td>Mixed rounded and flattened 3.3 ± 0.6 6.9 ± 0.3 18 ± 2 12 ± 6</td>
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<td></td>
<td>Poly-HEMA Not adherent 13 ± 5 15 ± 2 38 ± 3 10 ± 4</td>
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<td></td>
<td>T0070907 Rounded 3.9 ± 0.4 8.5 ± 0.3 20 ± 1 10 ± 5</td>
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<td></td>
<td>GW9662 Mixed rounded and flattened 3.5 ± 0.5 7.9 ± 0.9 21 ± 2 13 ± 5</td>
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<td>40</td>
<td>Control Flattened 2.4 ± 0.4 10 ± 3 26 ± 7 20 ± 10</td>
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<td></td>
<td>Poly-HEMA Not adherent 9 ± 3 12 ± 3 28 ± 6 57 ± 9</td>
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<td>T0070907 Not adherent 12 ± 1 11 ± 2 24 ± 6 22 ± 4</td>
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<td></td>
<td>GW9662 Rounded 3 ± 1 18 ± 2 34 ± 3 19 ± 6</td>
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<tr>
<td>67</td>
<td>Control Flattened 3 ± 3 6.7 ± 0.5 10 ± 1 4 ± 1</td>
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<td></td>
<td>Poly-HEMA Not adherent 7 ± 4 9 ± 3 20 ± 5 76 ± 6</td>
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<td></td>
<td>T0070907 Not adherent 9.7 ± 0.1 24 ± 2 28 ± 2 27 ± 4</td>
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<tr>
<td></td>
<td>T0070907 +Z Not adherent 1.3 ± 0.4 5.1 ± 0.5 7 ± 1 6 ± 3</td>
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<td></td>
<td>GW9662 Not adherent 12 ± 4 27 ± 3 33 ± 2 4 ± 1</td>
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<td>88</td>
<td>Control Flattened 5 ± 2</td>
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<td>Control flattened 7 ± 9</td>
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<td>GW9662 Not adherent 89 ± 16</td>
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NOTE: Data correspond to images shown in Fig. 2, with the percentage of cells in early apoptosis (Annexin V+ PI−) and in both early and late apoptosis (total Annexin V+ = Annexin V+ PI− and Annexin V+ PI+) determined using the quadrants as shown. Poly-HEMA denotes control cells placed on plates coated with polyhydroxyethylmethacrylate, which prevents all cell adhesion to the plate. Cells pretreated with the pan-caspase inhibitor z-VAD-FMK for 2 hours before addition of T0070907 (Z). Cell death was assessed using trypan blue exclusion. Replicate standard deviations (n = 3). Representative of at least three independent experiments.

during HepG2 adhesion. Normal adherence involves resistance to aspiration, followed by changes in cell morphology. By 12 hours post-trypsin, all control cells were small, rounded, and resistant to removal from the plate by aspiration. These cells were defined as "rounded cells." None showed any signs of extending processes (data not shown). By 17 hours post-trypsin, most control cells (data not shown) still had the same rounded morphology; however, a few were starting to extend processes and seemed larger and flatter, and were defined as "flattened cells" (thin arrow). By 40 hours, all control cells (Fig. 2B, middle) were flattened. In contrast, cells treated with the inhibitor T0070907 showed defects in flattening by 17 hours. Although 80% of the cells were resistant to removal by aspiration (Fig. 2A), none of them showed any signs of flattening or extending processes (Fig. 2B, left). By 24 hours, 30% of the T0070907-treated cells were not only unable to extend processes, but were fully nonadherent and freely floating in the tissue culture dish (Fig. 2A and B, left inset). The longer the time after treatment, the smaller the number of the T0070907-treated cells that were able to adhere. GW9662 also reduced adhesion and eventually resulted in complete detachment of the cells. However, the effect took significantly longer, with half of the cells rounded but not detached at 40 hours, rather than 17 hours (data not shown).
PPARγ Inhibitors Affect the Process of Adhering and the Maintenance of Stable Adhesion in Rapidly Proliferating Cells.

The antiadhesive effects of the PPARγ inhibitor T0070907 were observed both in cells that were in the process of adhering and in already adherent cells (Fig. 3B). However, the effect took significantly longer in already adherent cells, and did not occur with slowly proliferating cells. Whereas 30% of the adhering cells were fully detached from the plate after 24 hours of exposure to T0070907 (Fig. 2), rapidly proliferating adherent cells were all still adherent to the plate at 24 hours (Fig. 3B), although they had lost morphologic flattening. By 48 hours, 48% of these cells were nonadherent; by 72 hours, 63% were nonadherent. In contrast, all slowly proliferating cells remained firmly adherent and flattened for at least 72 hours after addition of T0070907.

Loss of Adhesion Requires almost Complete Loss of PPARγ Activation. PPARγ levels in HepG2 cells were reduced using siRNA (Fig. 4). By 24 hours after siRNA transfection, PPARγ levels were ~5-fold lower than control; after another 24 hours, levels were further reduced. The fraction of residual PPARγ protein remaining was proportional to the magnitude of the effect on adhesion. At 24 hours, the number of nonadherent cells was approximately the same in the lamin control and PPARγ siRNA–treated cells (<10%). However, by 48 hours, when virtually all of the lamin-treated cells were adherent, more than half of the PPARγ siRNA–treated cells were no longer adherent to the plate, although cell-cell adhesion was unaffected. As with the PPARγ inhibitors, the loss of adhesion occurred before cell death, as 95% of the PPARγ siRNA–treated cells excluded trypan blue at 48 hours.

To further assess what fraction of the normal PPARγ activation levels must be inhibited for adhesion to be affected, the effect of the inhibitor T0070907 on PPARγ–response element (PPRE)–dependent transcription was measured (Fig. 4C). PPRE-dependent transcription was half-maximally inhibited around 0.1 μmol/L T0070907. However, a 5-fold reduction was not achieved until greater than 10 μmol/L, and even 50 μmol/L was associated with detectable levels of PPRE-dependent transcription (12% of that in the absence of T0070907).

Decreasing the amount of PPARγ in the cells with siRNA reduces the amount of the PPARγ inhibitor required to affect adhesion (Fig. 4D). T0070907 (10 μmol/L) is not able by itself to reduce

Figure 3. Loss of cell adhesion by PPARγ inhibitors is reversible and occurs in already adherent and adhering cells. A, HepG2 cells were trypsinized and treated with T0070907 (50 μmol/L) for 24 hours. Half of the cells were then diluted 10-fold into each of the indicated mediums, and morphology assessed at the indicated times (hours). Representative of at least two independent experiments. B, DMSO control or T0070907 (50 μmol/L) was added to single cell suspensions of HepG2 cells (adhering cells) or to cells that had been previously plated and were firmly adherent to the plate (adherent cells). C, % cells that were nonadherent as a function of time (hours). Representative of at least two independent experiments. Control adhering cells (*), T0070907-treated adhering cells (○), control rapidly proliferating cells (▲), T0070907-treated rapidly proliferating cells (●), control slowly proliferating cells (□), and T0070907-treated rapidly proliferating cells (+). Bars, SDs (n = 3).
adhesion, either in normal cells (Fig. 5D) or in control siRNA–treated cells (Fig. 4D). However, in combination with PPARγ siRNA, 10 μmol/L T0070907 results in 11% nonadherent cells, compared with 3% nonadherent in control siRNA–treated cells. Similarly, at 50 μmol/L T0070907, 7% of the control siRNA–treated cells are nonadherent, whereas 22% of the PPARγ-treated cells are nonadherent.

**Cells in the Presence of PPARγ Inhibitors Show Normal β1 Integrin Expression and Binding Affinity, but Reduced FAK Activation.** PPARγ inhibitors affected adhesion on several ECM substrates, including laminin, collagen, and fibronectin (data not shown). To determine if PPARγ inhibitors reduce binding by decreasing the cell surface expression of the β1 integrin ECM binding partner common to all of these substrates, surface expression was measured by flow cytometry. β1 integrin levels were virtually unaffected by the presence of T0070907 after 40 hours of incubation (Fig. 5A). The small downshift observed in the presence of T0070907 reflected a small difference in size of the cells rather than an actual down-regulation of integrin (data not shown).

The effects of T0070907 on β1 integrin conformation were also measured. Binding of the anti-β1 integrin antibody B44, which recognizes the integrin only when it is capable of binding, was not affected by T0070907 (Fig. 5A). In addition, the activating TS2/16 antibody was unable to overcome T0070907’s effects, regardless of whether the antibody was added to the cells (soluble) or cross-linked to the plate (bound; Fig. 5B), although TS2/16 did tether the cells to the plate when in plate-bound form.

The effects of the PPARγ inhibitor T0070907 on FAK tyrosine 925 phosphorylation were also evaluated. At 17 hours, when cells were adherent but not flattened, T0070907 clearly selectively reduced the phosphorylated form of FAK (Fig. 5C). This effect was also evident at 24 hours, when half of the cells were not adherent (Fig. 5D).

**PPARγ Inhibitors Kill Cells at Concentrations Less than those Needed with Activator Ligands.** The effects of two thiazolidenediones, rosiglitazone and troglitazone, and the PPARγ inhibitor T0070907 on cell adhesion, apoptosis, and eventual cell growth/cell survival (total cell number) were compared. T0070907 has a much greater effect on adhesion than either rosiglitazone or troglitazone. Half-maximal reductions in adhesion occur at 25 μmol/L T0070907, whereas at least 100 μmol/L troglitazone and <250 μmol/L rosiglitazone are needed for this effect (Fig. 5D). The loss of adhesion seen with PPARγ activators and inhibitors is a result of different processes. T0070907’s effects on adhesion are not, at 24 hours, associated with apoptosis. Whereas 50 μmol/L T0070907 causes 70% of the cells to lose adherence at 24 hours, only 3% of them are showing signs of apoptosis (DNA

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**Figure 4.** Reduction of PPARγ levels interferes with adhesion. A, HepG2 cells were transfected with no siRNA (control), lamin A/C control siRNA (lamin), or PPARγ-specific (PPARγ) siRNA mixes at t = 0. B, PPARγ levels in the transfected cells were examined at the indicated times by Western blot. C, HepG2 cells were transfected with a PPARγ-response element-luciferase reporter construct, and stimulated with 1 μmol/L rosiglitazone in the presence of varied concentrations of T0070907. Luciferase activity was measured at 16 hours. A similar curve was obtained using cells with only endogenous PPARγ activation (data not shown). Representative of at least two independent experiments. Bars, SDs (n = 3). D, HepG2 cells were transfected with lamin A/C control siRNA (solid columns) or PPARγ-specific (PPARγ) siRNA mixes (hatched columns). Cells were subsequently trypsinized and plated in the presence of the indicated concentration of T0070907. Twenty-four hours later, the percentage of adherent cells was determined. Differences between control and PPARγ siRNA were significant (P < 0.01) for both the 10 and 50 μmol/L concentrations.
degradation). In contrast, to achieve the same loss of adhesion with troglitazone, >200 μmol/L compound is needed, and these cells are clearly apoptotic (Fig. 5D). In addition, the primary loss of adhesion seen with T0070907 is associated with reductions in phosphorylated FAK (pFAK), whereas rosiglitazone and troglitazone do not reduce pFAK levels relative to total FAK levels at any concentrations.

The number of cells in a proliferating cell sample is a function of both apoptosis and of possible inhibitions of cell growth (cell cycle arrest). To assess the overall ability of PPARγ activators and inhibitors to reduce cell numbers by a combination of both factors, the number of cells 72 hours after addition of compounds was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. T0070907 (50 μmol/L) reduces the number of cells by half, whereas roughly 250 μmol/L of either activator were required for the same effect (Fig. 5D).

Discussion

The results of these experiments reveal new insights into the specific expression patterns of PPARγ within HCC tumors and suggest novel functions for PPARγ. PPARγ is highly expressed in, and adjacent to, the primary tumor cells, and PPARγ specific inhibitors induce cell death in HCC-derived cell lines by preventing attachment to the ECM.

The finding of increased levels of PPARγ staining within HCC tissue is consistent with observations in many epithelial-related tumors (3, 4, 23–25) but has not been previously reported for HCC. In one study of five HCC specimens, there was no difference in PPARγ protein levels in HCC samples and paired adjacent normal appearing tissue as measured by Western blot analysis (26). This observation contrasts with the results of this study, which clearly shows increased expression of PPARγ in and around the HCC tumors. This apparent difference may be a reflection of whether the...
peritumoral region was included in the normal samples in the previous study, but tissue staining and examination was not done in that study.

PPARγ inhibitors cause HCC cell lines to undergo anoikis, or apoptotic cell death caused by loss of adherence to the ECM (27). PPARγ-mediated cell death bears the hallmarks of an apoptotic process, shown both by Annexin V binding and DNA degradation, as well as caspase dependence. However, indicators of apoptosis do not appear until after the cell has become nonadherent, indicating that the loss of adhesion is the cause and not the result of cell death. In addition, the apoptotic effects of the inhibitors are initially reversible, a finding characteristically associated with anoikis (28, 29).

Treatment of HepG2 cells with PPARγ-specific siRNAs also causes a loss of adhesion ability. As with the compound PPARγ inhibitors, the inability to adhere precedes cell death. Interestingly, the loss of adhesion caused by interfering with PPARγ-dependent signaling requires that almost all PPARγ activation be eliminated. At a time point when at least 80% of the PPARγ is reduced by the siRNA treatment, cells are still able to adhere; loss of adhesion does not occur until over 90% of the PPARγ is eliminated. This result parallels the dose dependence of the effect of the inhibitor T0070907 on adhesion. Whereas 50 μmol/L T0070907 cause 70% of the cells to be unable to adhere at 24 hours, 10 μmol/L have little or no effect on adhesion. T0070907 (10 μmol/L) reduces PPRE-dependent transcription to 25% of the control value, whereas 50 μmol/L reduce it further to 12%. Taken together, the siRNA and inhibitor results suggest that PPARγ-dependent transcription must be reduced by ~90% to affect adhesion.

The effects of abrogating PPARγ function on adhesion most likely reflect signaling downstream of integrin binding, rather than an inability of the integrin to bind ECM. Initial adhesion was able to take place in the presence of the inhibitors, as cells were resistant to aspiration from the plate at the 12- and 17-hour time points. In addition, surface expression and regulated affinity of the β1 integrin was unaffected by T0070907. Because integrin-ECM binding requires an αβ heterodimer, it is formally possible that α integrin expression or affinity, rather than that of β, is affected. However, T0070907 had similar effects on adhesion to three different ECM substrates, all of which require a different α subunit for binding, making this possibility unlikely. Whereas cells in the presence of T0070907 were able to form integrin-ECM contacts, these contacts did not lead to normal integrin-dependent signaling or cytoskeletal rearrangement, as cells adhering in the presence of PPARγ inhibitors had reduced levels of FAK phosphorylation and did not flatten.

The fact that PPARγ inhibitors and PPARγ-specific siRNAs both reduce adhesion, along with the fact that PPARγ-specific siRNAs reduce the amount of PPARγ-inhibiting compound required to reduce adhesion strongly suggests that PPARγ is integral to normal adhesive functions. However, T0070907 does not start to have a detectable effect on adhesion until at least 16 hours after addition. This result suggests that PPARγ may be required for transcription of some factor necessary for adhesion. Further experiments will be necessary to delineate the nature of the requirement for PPARγ in adhesion.

The fact that PPARγ inhibitors interfere with adhesion and inhibit FAK phosphorylation suggests several adjuvant therapeutic uses. Tumor cell adhesion to the ECM plays an important role in counteracting the efficacy of other antitumor chemotherapies (30–33). In addition, the ability of the inhibitors to selectively prevent readherence might make them ideal for preventing metastases. The results of this study complement recent work showing that reductions in expression levels of proteins involved in cell adhesion, including FAK and CEACAM6, in pancreatic cancer cells increased anoikis in cell lines and prevented their establishment in nude mouse models of metastasis (34, 35).

As targets for use as adjuvant cancer chemotherapeutic agents, the PPARγ activators, such as the thiazolidinediones, have been of primary focus due to a recognized importance of PPARγ in cell growth and differentiation. In this report, it is shown that PPARγ inhibitors can also have antitumor effects, through a totally different mechanism. T0070907 was at least 5-fold more efficacious in reducing cell number than either of the PPARγ activators in HepG2 cells, when added to the cells whereas they were in the process of adhering. These results strongly indicate that inducing cell death by anoikis may be more effective in vivo than causing cell death by cell cycle arrest, especially if metastatic growth is involved.

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