Activation of Peroxisome Proliferator-Activated Receptor-γ Decreases Pancreatic Cancer Cell Invasion through Modulation of the Plasminogen Activator System

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Abstract
Cancer cell invasion and metastasis require the concerted action of several proteases that degrade extracellular matrix proteins and basement membranes. Recent reports suggest the plasminogen activator system plays a critical role in pancreatic cancer biology. In the present study, we determined the contribution of the plasminogen activator system to pancreatic cancer cell invasion in vitro. Moreover, the effect of peroxisome proliferator-activated receptor (PPAR)-γ ligands, which are currently in clinical use as anti-diabetic drugs and interestingly seem to display antitumor activities, on pancreatic cancer cell invasion and the plasminogen activator system was assessed. Expression of components of the plasminogen activator system [i.e., urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1, and uPA receptor] was detected in six human pancreatic cancer cell lines. Inhibition of urokinase activity by specific synthetic compounds reduced baseline pancreatic cancer cell invasion. The PPAR-γ ligands 15-deoxy-Δ12,14-prostaglandin J2 and ciglitazone also attenuated pancreatic cancer cell invasion. This effect was abrogated by dominant-negative PPAR-γ receptors and pharmacologic PPAR-γ inhibitors. Moreover, activation of PPAR-γ by ligands increased plasminogen activator inhibitor-1 and decreased uPA levels in pancreatic cancer cells, and this was accompanied by a reduction in total urokinase activity. The present study shows that the plasminogen activator system plays an integral role in pancreatic cancer cell invasion in vitro. Activation of the nuclear receptor PPAR-γ by ligands reduced pancreatic cancer cell invasion, which was largely mediated by modulation of the plasminogen activator system. These findings further underscore the potential role of PPAR-γ ligands as therapeutic agents in pancreatic cancer. (Mol Cancer Res 2006;4(3):159–67)

Introduction
Tumor progression and metastasis involve degradation of extracellular matrix, a process that is governed by an intricate balance of proteases, their activators, and their inhibitors, allowing malignant cells to infiltrate adjacent structures and to gain access to lymph and blood vessels. These proteases can be categorized broadly into three families: matrix metalloproteases, serine proteinases, and cysteine proteinases. Human pancreatic cancer, a highly aggressive tumor, is characterized by up-regulation of several proteases from each family. Recent studies using microarray technology indicate an important role of the serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) in pancreatic cancer (1, 2). In addition, concomitant overexpression of uPA and uPAR in patients with pancreatic cancer was associated with a shorter postoperative survival (3). uPA, a 53-kDa serine protease, is secreted by tumor cells in an enzymatically inactive single-chain form (sc-uPA or pro-uPA) and binds to uPAR (CD87), a glycoprotein linked to the cell membrane by a glycosyl phosphatidylinositol anchor (4). Although the physiologic activator is unknown, different proteases, including plasin and cathepsin B, have been shown to activate uPA in vitro (5). The enzymatically active two-chain uPA catalyzes the conversion of plasminogen to plasmin, another serine protease with broad substrate specificity. The enzymatic activity of uPA is counter-balanced by plasminogen activator inhibitor (PAI)-1 and PAI-2, which belong to the serine protease inhibitor family of protease inhibitors (6). The 43-kDa PAI-1 is thought to be the primary physiologic inhibitor of uPA. It reacts rapidly with uPA, forming a stable complex with a 1:1 stoichiometry (6). In addition, PAI-1 binds to the extracellular matrix protein vitronectin, thereby potentially modulating cell adhesion and migration, and induces the internalization and degradation of uPAR-bound uPA (5). Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors, which belong to the nuclear hormone receptor superfamily (7). On ligand binding, PPARs form functional heterodimers with retinoid X receptors. The PPAR/retinoid X receptor complex binds to specific peroxisome proliferator response elements (PPRE) controlling
the expression of a large array of genes (8). Three major subtypes have been described (PPAR-α, PPAR-β/δ, and PPAR-γ), which are linked to hyperlipidemia, type II diabetes, adipocyte differentiation, and atherosclerosis (9). More recently, a possible role of PPAR-γ in human malignancies has been implicated (10). PPAR-γ is overexpressed in various tumors, including pancreatic cancers, and affects many facets of cancer biology, including differentiation, proliferation, invasion, and angiogenesis (10). In pancreatic cancer cells, activation of PPAR-γ induces cell cycle arrest with terminal differentiation and apoptosis (11-13) and decreases invasion (14, 15). PPAR ligands include the fotate class of hypolipidemic drugs, the thiazolidinedione class of antidiabetic drugs, and derivatives of fatty acid metabolism (16). Thiazolidinediones are clinically used in the treatment of type II diabetes and have been shown to increase sensitivity of insulin receptors in patients with type II diabetes and to attenuate atherosclerosis by improving endothelial cell function and inhibiting inflammation (17). Regulation of the plasminogen system by PPAR-γ ligands may contribute to the antiatherogenic properties of PPAR-γ (18-20).

In this study, we describe the importance of the plasminogen activator system for the invasion of pancreatic cancer cells. We found that PPAR-γ ligands decreased pancreatic cancer cell invasion by specific PPAR-γ-mediated regulation of uPA and PAI-1. To our knowledge, this is the first comprehensive report of a regulatory function of PPAR-γ on both uPA and PAI-1 in human cancers.

Results

Inhibitors of uPA Decrease Baseline Pancreatic Cancer Cell Invasion

The expression of various components of the urokinase activator system by pancreatic cancer cells was first evaluated using semiquantitative PCR. Using specific intron-spanning primers to rule out amplification of genomic DNA, uPA, PAI-1, and uPAR mRNA transcripts were detected in six human pancreatic cancer cell lines (Fig. 1A). Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were added together with the uPA, PAI-1, and uPAR primers to the same reaction tube for semiquantitative analysis. ELISA analysis showed that MIA PaCa-2 and PANC-1 cells had the highest concentration of uPA and PAI-1 protein in the culture medium, whereas PAI-1 levels in Capan-2 cells were virtually undetectable (Fig. 1B and C). Notably, PAI-1 levels were generally 5 to 10 times higher than uPA levels. Using a chromogenic substrate for uPA, cell culture medium of Capan-2 cells showed the highest urokinase activity (Fig. 1D). To determine whether the plasminogen activator system contributes to baseline invasion of pancreatic cancer cells, we used two small-molecule uPA inhibitors, WX-582 and WX-UK1. WX-UK1, a derivative of 3-aminophenylalanine, has been shown to selectively inhibit tumor-specific proteases, including uPA, and to reduce growth and metastasis in a rat breast cancer model (21). WX-582, a phenylguanidine derivative, is a novel, highly selective, and active uPA inhibitor. Both inhibitors dose-dependently decreased urokinase activity in Capan-2 conditioned culture medium, with WX-582 being more effective at lower concentrations (IC_{50} < 1 μmol/L; Fig. 2A).

In this context, we describe the importance of the plasminogen activator system for the invasion of pancreatic cancer cells.
invasion assay, both inhibitors at 1 and 10 μmol/L significantly decreased invasion of MIA PaCa-2, PANC-1, and Capan-2 cells (Fig. 2B). WX-582 was slightly more effective in reducing invasion than WX-UK1, which corresponded to the stronger inhibitory effect of WX-582 on urokinase activity at these concentrations. Moreover, the anti-invasive effect of both uPA inhibitors was significantly more pronounced in Capan-2 cells, which displayed the highest urokinase activity in vitro.

**PPAR-γ Ligands Reduce Pancreatic Cancer Cell Invasion**

We have reported previously that PPAR-γ ligands decrease pancreatic cancer cell growth by induction of apoptosis (11). In addition, there is accumulating evidence that PPAR-γ ligands also affect cancer cell invasion and metastasis (22). Because some of the antiatherogenic properties of PPAR-γ ligands in patients with type II diabetes may be related to modulation of plasminogen system (17), we investigated the potential effect of PPAR-γ ligands on pancreatic cancer cell invasion and assessed the contribution of the plasminogen activator system to this process. Two established PPAR-γ ligands, 15-deoxy-Δ12,14-prostaglandin J2 (15-PGJ2), a naturally occurring, nonenzymatically formed dehydration product of prostaglandin D2, and ciglitazone, a thiazolidinedione, dose-dependently decreased invasion of MIA PaCa-2, PANC-1, and Capan-2 cells (Fig. 2A-C). At a concentration of 10 μmol/L, both ligands decreased invasion of all three cell lines by ~70% to 80%. Adding the uPA inhibitors WX-582 and WX-UK1 (at concentrations that inhibited urokinase activity by 70-90%) to 15-PGJ2 and ciglitazone had no additional effect on pancreatic cancer cell invasion. The anti-invasive effects of both PPAR-γ ligands at 10 μmol/L with or without the uPA inhibitors, however, were slightly more pronounced than of the uPA inhibitors alone (Fig. 2C and D).

**PPAR-γ Ligands Decrease uPA and Increase PAI-1 Levels in Pancreatic Cancer Cells**

Having shown that PPAR-γ ligands and uPA inhibitors reduced pancreatic cancer cell invasion in vitro, the effect
of PPAR-γ ligands on expression and activity of the plasminogen activator system was assessed. 15-PGJ2 and ciglitazone dose-dependently decreased uPA protein levels in the culture medium of MIA PaCa-2, PANC-1, and Capan-2 cells (Fig. 3A). Conversely, both ligands dose-dependently increased PAI-1 levels in all three cell lines (Fig. 3B). Significant changes were seen at concentrations as low as 1 μmol/L of both ligands. Because the uPA and PAI-1 ELISA kits detect both inactive and active forms of uPA and PAI-1 together with uPA/PAI-1 complexes, the PPAR-γ ligand-induced changes in uPA and PAI-1 protein levels in the culture medium reflect changes in total protein levels rather than fluctuations between proform and mature form of both proteins. In addition, 15-PGJ2 and ciglitazone had no effect on uPAR expression (data not shown). PPAR-γ ligand-induced changes in uPA and PAI-1 levels correlated with a reduced overall urokinase activity in MIA PaCa-2, PANC-1, and Capan-2 cells (Fig. 3C). To determine whether the effects of PPAR-γ ligands on uPA and PAI-1 protein expression are regulated on the transcriptional levels, RNase protection assays were done. Multiplex RNase protection assay with GAPDH as an internal control showed a dose-dependent decrease in uPA transcripts in MIA PaCa-2 cells treated with 15-PGJ2 (Fig. 3D). In contrast, 15-PGJ2 dose-dependently increased PAI-1 mRNA levels (Fig. 3D).

Anti-invasive Effects of 15-PGJ2 Are Mediated by PPAR-γ

There is increasing evidence that some effects of PPAR-γ ligands are mediated by PPAR-γ-independent mechanisms (23). To determine whether the effects of 15-PGJ2 on pancreatic cancer cell invasion were indeed mediated by activation of PPAR-γ, a dominant-negative PPAR-γ receptor and a pharmacologic PPAR-γ inhibitor were assessed. Two highly conserved hydrophobic and charged amino acid residues (Leu468 and Glu471) in helix 12 of the ligand-binding domain of PPAR-γ were mutated into alanine. This compound PPAR-γ mutant (LEA) retains ligand and DNA binding but exhibits markedly

![Figure 3](https://example.com/figure3.png)

**Figure 3.** PPAR-γ ligands modulate uPA/PAI-1 expression and urokinase activity in pancreatic cancer cells. **A** and **B.** MIA PaCa-2, PANC-1, and Capan-2 cells were incubated with 15-PGJ2 (top) or ciglitazone (bottom) for 24 hours in serum-free medium, and uPA (**A**) and PAI-1 (**B**) levels in the culture medium were determined by ELISA and normalized to cell number. Columns, mean of three independent experiments; bars, SD. *, *P < 0.01 versus 15-PGJ2 (0 μmol/L) or ciglitazone (0 μmol/L). **C.** Pancreatic cancer cells were incubated with 15-PGJ2 for 24 hours in serum-free medium and total urokinase activity in the conditioned cell culture medium was determined by a chromogenic assay using a specific uPA substrate. Absorbance of the free chromophore was measured at 405 nm. Columns, mean percentage compared with control of at least three independent experiments; bars, SD. *, *P < 0.01 versus 15-PGJ2 (0 μmol/L) or ciglitazone (0 μmol/L). **D.** RNase protection assay was used to evaluate the effect of 15-PGJ2 (0-10 μmol/L) on uPA (top left) and PAI-1 (bottom left) mRNA transcripts in MIA PaCa-2 cells. Yeast RNA treated with RNase confirmed sufficient digestion of unprotected fragments. Yeast RNA without RNase confirmed integrity of riboprobe. Representative samples from three independent experiments. Band density was determined by laser densitometry (right). Columns, mean of three independent experiments; bars, SD. *, *P < 0.01 versus 15-PGJ2 (0 μmol/L).
**FIGURE 4.** Anti-invasive effects of PPAR-γ ligands are mediated by PPAR-γ. 

**A.** MIA PaCa-2 cells were either infected with adenoviruses encoding for the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control or pretreated with GW9662 (10 μM) for 30 minutes before adding 15-PGJ2 for 24 hours in serum-free medium. The inhibitory effect of the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control before adding 15-PGJ2 for 24 hours in the presence or absence of WX-582 (10 μM) in serum-free medium. The inhibitory effect of the dominant-negative PPAR-γ receptor in MIA PaCa-2 cells infected with adenoviruses encoding for the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control before adding 15-PGJ2 for 24 hours in serum-free medium. Columns, number of invading cells per high-power field of three independent experiments; bars, SD. *, P < 0.01 versus control; #, P < 0.01 versus 10 μM 15-PGJ2 alone (column 2). 

**B.** MIA PaCa-2 cells were infected with adenoviruses encoding for the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control before adding 15-PGJ2 for 24 hours in the presence or absence of WX-582 (10 μM) in serum-free medium. The inhibitory effect of the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control before adding 15-PGJ2 for 24 hours in serum-free medium. The inhibitory effect of the dominant-negative PPAR-γ receptor in MIA PaCa-2 cells infected with adenoviruses encoding for the dominant-negative PPAR-γ receptor (LEA) or LacZ as a control before adding 15-PGJ2 for 24 hours in serum-free medium. Columns, number of invading cells per high-power field of three independent experiments; bars, SD. *, P < 0.01 versus control; #, P < 0.01 versus 10 μM 15-PGJ2 alone (column 2). 

**C.** PPAR-γ expression in MIA PaCa-2 cells infected with adenoviruses encoding for the dominant-negative PPAR-γ receptor (Adx-D/N-PPAR-γ) or LacZ (Adx-LacZ) was determined by Western blot. A β-actin Western blot served as a loading control. 

**D.** PPAR-γ expression in MIA PaCa-2 cells infected with adenoviruses encoding for PPAR-γ (Fig. 4B). WX-582 had no additional effect in control virus-transfected cells. The contributions of the serine protease uPA to baseline pancreatic cancer cell invasion were assessed using two small-molecule inhibitors of uPA. At concentrations of both inhibitors that nearly abolished uPA activity in conditioned cell culture medium, invasion of three pancreatic cancer cell lines was almost completely reversed the 15-PGJ2-induced decrease in cell invasion, which was not different compared with control virus-transfected cells (data not shown). The inhibitory effect of the dominant-negative PPAR-γ receptor on the 15-PGJ2-induced decrease in cell invasion was significantly attenuated by adding the specific uPA inhibitor WX-582 (Fig. 4B). WX-582 had no additional effect in control virus-infected cells. To confirm whether dominant-negative PPAR-γ and GW9662 decreased PPAR-γ transcriptional activity, reporter assays were done. 15-PGJ2 at 10 μM markedly increased PPAR-γ activity, which was almost completely inhibited by the double-mutant PPAR-γ and the pharmacologic inhibitor GW9662 (Fig. 4C). When compared with control virus-infected cells, infection of MIA PaCa-2 cells with PPAR-γ-encoding adenoviruses lead to a robust overexpression of PPAR-γ (Fig. 4D). In addition, the dominant-negative PPAR-γ completely blocked the effects of 15-PGJ2 on uPA and PAI-1 levels in the culture medium of MIA PaCa-2 cells, whereas the control virus had no effect (Fig. 4E and F).

**Discussion**

There is growing evidence that the plasminogen system represents a critical regulatory system in pancreatic cancer invasion. Components of the plasminogen system, including plasminogen activators (particularly the urokinase-type), uPAR, and PAI, are overexpressed in human pancreatic cancers and correlate to poor survival (3, 25–27). In our study, uPA, uPAR, and PAI-1 expression was detected in six human pancreatic cancer cell lines. Interestingly, although well-differentiated Capan-2 cells secreted only moderate amounts of uPA into the culture medium, the virtual absence of secreted PAI-1 translated into the highest overall enzymatic uPA activity in this cell line. The contribution of the serine protease uPA to baseline pancreatic cancer cell invasion was assessed using two small-molecule inhibitors of uPA. At concentrations of both inhibitors that nearly abolished uPA activity in conditioned cell culture medium, invasion of three pancreatic cancer cell lines was...
reduced by 60% to 75%, indicating a pivotal role of uPA in baseline pancreatic cancer cell invasion in vitro. This is supported by the recent finding that targeting the uPAR inhibited the invasion and metastatic spread in an orthotopic mouse model of pancreatic cancer (28).

We have shown previously that ligands of the nuclear receptor PPAR-γ attenuated pancreatic cancer cell growth in vitro by induction of apoptosis (11). In the present study, the endogenous and exogenous PPAR-γ ligands 15-PGJ2 and ciglitazone, respectively, dose-dependently inhibited pancreatic cancer cell invasion, further supporting the notion of PPAR-γ ligands as being potentially therapeutic agents in pancreatic cancer. There is now accumulating evidence that PPAR-γ ligands possess potent anti-invasive properties in human cancers (29-31). Our findings that PPAR-γ ligands decreased uPA and increased PAI-1 levels in pancreatic cancer cells, thereby substantially reducing overall urokinase activity, strongly suggested that the anti-invasive properties of PPAR-γ ligands were mediated by modulating the plasminogen activator system. However, both PPAR-γ ligands reduced cell invasion to a slightly greater extent than both uPA inhibitors. The combination of uPA inhibitors and PPAR-γ ligands had no additional anti-invasive effect compared with the PPAR-γ ligands alone. These findings suggest that PPAR-γ ligands decrease pancreatic cancer cell invasion largely by reducing urokinase activity but also affect other protease systems. Members of the matrix metalloproteinase family are candidate proteases, which have been shown to be regulated by PPAR-γ agonists (32-34).

Our data suggest that PPAR-γ ligands regulate uPA and PAI-1 levels on the transcriptional level. The precise mechanisms by which PPAR-γ ligands modulate PAI-1 and uPA transcription remain to be defined. The PAI-1 promoter, which lacks a consensus PPAR response element (PPRE), contains cis-acting elements for CAAT/enhancer-binding protein and signal transducers and activators of transcription (35, 36). It is conceivable that activation of PPAR-γ induces the expression of other transcription factors, which in turn regulate PAI-1 gene transcription (37, 38). This hypothesis is supported by a recent study (39) showing slow kinetics of PAI-1 mRNA induction with pioglitazone (maximal induction observed after 72 hours), which is consistent with de novo protein synthesis (e.g., for new transcription factors). In our study, however, we found a significant increase in PAI-1 mRNA and protein within 24 hours of incubation with 15-PGJ2 and ciglitazone, which is in accordance to a study by Xin et al. showing increased PAI-1 mRNA expression after 24-hour treatment of human umbilical vein endothelial cells with 15-PGJ2 (40). This suggests rather a direct regulatory effect of PPAR-γ on PAI-1 gene transcription. A putative, atypical PPRE (TCCCCCATGCCCT) can be found in the 5′ flanking region of the PAI-1 gene, which shares great similarity to another published nonconsensus PPRE (TGCCCCATGCCCT) in the lipoprotein lipase promoter (41). However, whether PPAR-γ binds to this putative PPRE, thereby regulating PAI-1 gene expression, or the effect of PPAR-γ is mediated by other transcription factors remains to be determined. In addition to changes in PAI-1 expression, we found that 15-PGJ2 and ciglitazone dose-dependently decreased uPA mRNA levels. The nuclear factor-κB, which is constitutively active in pancreatic cancer cells and functions as a survival factor, seems to regulate uPA expression in pancreatic cancers (42). Furthermore, the transforming growth factor-β, which is over-expressed in pancreatic cancers, has been shown to increase uPA levels in pancreatic cancer cells (43, 44). Recent studies suggest that PPAR-γ can inhibit gene transcription by transrepression of other transcription factors (45). The observations that PPARs directly interact with nuclear factor-κB subunits and transforming growth factor-β/Smad signaling support the hypothesis that PPAR-γ ligands may regulate uPA expression in pancreatic cancer cells by transrepression of nuclear factor-κB and/or transforming growth factor-β signaling (46, 47).

There is now substantial evidence that PPAR-γ ligands exert some of their biological effects by PPAR-γ-independent mechanisms (23). Using a dominant-negative PPAR-γ mutant and a pharmacologic inhibitor, our data showed that the anti-invasive properties of 15-PGJ2 were indeed mediated by PPAR-γ. Interestingly, the inhibitory effect of the dominant-negative mutant on the 15-PGJ2-induced decrease in pancreatic cancer cell invasion was reversed by the specific uPA inhibitor, further indicating that PPAR-γ regulates pancreatic cancer cell invasion by modulation of the urokinase activator system. The efficacy of both genetic and pharmacologic approaches to inhibit PPAR-γ transcriptional activity was confirmed by reporter assays. These results are in contrast to a recent study describing that thiazolidinediones inhibit pancreatic cancer cell invasion by PPAR-γ-independent mechanisms (48). In this study, the thiazolidinediones rosiglitazone and pioglitazone reduced pancreatic cancer cell invasion, which was not attenuated by the synthetic PPAR-γ inhibitors biphenol A diglycidyl ether and GW9662. The exact reasons for this discrepancy are unclear, but the use of different PPAR-γ ligands and differences in the invasion assay protocol may account for that observation. In contrast to our study, the PPAR-γ ligands and inhibitors were added simultaneously to the cells and invasion was determined after only 6 hours. It is conceivable although speculative that a putative effect of the inhibitors may have been detectable after 24 hours. Furthermore, the authors did not determine whether both inhibitors actually blocked PPAR-γ transcriptional activity. Our study, however, is in accordance to a recent report in which GW9662 inhibited the rosiglitazone-induced reduction in pancreatic cancer invasion (14).

In summary, we found that the urokinase activator system plays a major role in baseline invasion of pancreatic cancer cells. Ligands of the nuclear receptor PPAR-γ attenuated pancreatic cancer cell invasion, thereby confirming previous studies. The anti-invasive properties of PPAR-γ ligands were largely mediated by specific PPAR-γ-dependent changes in the plasminogen activator system. Our study showed for the first time a regulatory role of PPAR-γ in uPA and PAI-1 expression in pancreatic cancer, thereby attenuating cell invasion. These findings further support the notion of PPAR-γ ligands as being novel therapeutic agents in pancreatic cancer.

**Materials and Methods**

**Reagents**

The PPAR-γ ligands 15-PGJ2 and ciglitazone, the polyclonal PPAR-γ antibody, and the pharmacologic PPAR-γ inhibitor GW9662 were purchased from Cayman Chemical...
(Ann Arbor, MI). The mouse monoclonal anti-α-actin antibody was purchased from Sigma (St. Louis, MO). The full-length PPAR-γ1 expression vector was obtained from Alex Elbrecht (Merck Research Laboratories, Rahway, NJ; ref. 49). The plasmids pCMV-Luc and pRACO, pneo were kindly provided by Syngenta CTL Cell Biology Group (Macclesfield, Cheshire, United Kingdom). The uPA inhibitors WXUK1 and WX-582 were a generous gift from Wilex AG (Munich, Germany).

Cell Culture

The human pancreatic cancer cell lines AsPC-1 (well to poorly differentiated), BxPC-3 (well to poorly differentiated), Capan-2 (well differentiated), HPAF-II (moderately differentiated), Mia PaCa-2 (undifferentiated), and PANC-1 (poorly differentiated) were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described previously (50).

Reverse Transcription-PCR Amplification of uPA, PAI-1, and uPAR

Total RNA of human pancreatic cancer cell lines was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Roche Diagnostics Corp., Indianapolis, IN). Intron-spanning PCR primers for human uPA, PAI-1, uPAR, and GAPDH (Genbank accession nos. XM_055906, X_04429, NM_002659, and NM_002046, respectively) have been designed as follows: uPA fragment forward (5'-GCCTTGTGGA-GATCCGTTCAAGGAGGCC-3'; 858-887), uPA fragment reverse (5'-GCAGATTCTGTGACCTG-3'; 1,316-1,287), PAI-1 fragment forward (5'-GCCTAATTCTGGAGGC-3'; 49-68), PAI-1 fragment reverse (5'-CTGGCCACCTCTGT-3'; 270-251), uPAR fragment forward (5'-CAGG-TGCAGTCACTGACAG-3'; 496-515), uPAR fragment reverse (5'-CAGGAATTGGAAGTGCTG-3'; 996-977), GAPDH fragment forward (5'-AAGTACCACCTGCT-3'; 730-749), and GAPDH fragment reverse (5'-CCTTGGT-3'; 1,093-1,112), PAI-1 fragment reverse (5'-GGGAGAGGCTCTTGGTCTG-3'; 1,092-1,073), GAPDH fragment forward (5'-AGGGTCTGAA-GCTGAA-3'; 730-749), and GAPDH fragment reverse (5'-CCCTCCTGAAGGTTAC-3'; 1,224-1,205). The size of the riboprobes was designed to allow for simultaneous multiplex analysis of samples. A T7 RNA polymerase promoter was ligated to the cDNA using the Lig'N'Scribe Kit (Ambion, Inc., Austin, TX). Antisense radiolabeled riboprobes were generated using an in vitro transcription kit (MAXscript; Ambion) with 1 µg uPA, PAI-1, or GAPDH fragment, T7 RNA polymerase, and [α-32P]UTP. Probes were run on a 5% acrylamide/8 mol/L urea gel and full-length riboprobes were gel purified.

RNase Protection Assay

Cells were incubated with 15-PGJ2 (0-10 µM/L) for 6 hours. Total RNA was reverse transcribed with avian myeloblastosis virus (Roche). Using the RNase Protection Assay III (Ambion), 10 µg total mRNA was hybridized with 80,000 cpm uPA and PAI-1 and 40,000 cpm GAPDH radiolabeled antisense riboprobe, which protected 275-, 264-, and 547-bp fragments, respectively. Free probe and single-stranded mRNA were digested with RNase A and T1. Hybridized samples were precipitated and electrophoresed on a 5% acrylamide/8 mol/L urea gel. The gel was exposed to Kodak BioMax MS film (Eastman Kodak Co., Rochester, NY). Yeast RNA incubated with uPA, PAI-1, or GAPDH riboprobes with or without RNase treatment served as RNase efficiency and full-length riboprobe integrity controls.

Dominant-Negative PPAR-γ

A dominant-negative form of PPAR-γ was constructed by mutating the Leu668 and Glu671 of the full-length PPAR-γ1 into Ala using the QuickChange Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA). Mutations at these sites create a dominant-negative form of PPAR-γ (24). The dominant-negative PPAR-γ was subcloned into recombinant type 5 adenovirus using the Adeno-X Expression System (Clontech) and designated as Adx-D/N-PPAR-γ. Recombinant type 5 adenovirus expressing the LacZ gene was generated similarly and used as a control vector (Adx-LacZ). Cells were infected with 75 plaque forming units/cell in DMEM containing 0.4% fetal bovine serum for 24 hours.

uPA Activity Assay

Total uPA activity in cell culture supernatants was determined by the uPA Activity Assay kit (Chemicon International, Temecula, CA) using a chromogenic substrate. uPA activity was determined by measuring the increase in absorbance of the free chromophore pNA at λ₄05.

Preparation of Riboprobes

Templates for riboprobes were generated using the following primers for uPA, PAI-1, and GAPDH (Genbank accession nos. XM_044354.6, M16006, and NM_002046, respectively): uPA fragment forward (5'-TCACCACAAAAATGCTGTG-3'; 1,093-1,112), uPA fragment reverse (5'-AGGCGATTCTCTT-CCTTGTT-3'; 1,315-1,296), PAI-1 fragment forward (5'-CTCTCTCTGCCCTCAACCAAC-3'; 881-900), PAI-1 fragment reverse (5'-GGGAGAGGCTCTTGGTCTG-3'; 1,092-1,073), GAPDH fragment forward (5'-AAGGGTCTGAA-GCTGAA-3'; 730-749), and GAPDH fragment reverse (5'-CCCTCCTGAAAGTTAC-3'; 1,224-1,205). The size of the riboprobes was designed to allow for simultaneous multiplex analysis of samples. A T7 RNA polymerase promoter was ligated to the cDNA using the Lig'N'Scribe Kit (Ambion, Inc., Austin, TX). Antisense radiolabeled riboprobes were generated using an in vitro transcription kit (MAXscript; Ambion) with 1 µg uPA, PAI-1, or GAPDH fragment, T7 RNA polymerase, and [α-32P]UTP. Probes were run on a 5% acrylamide/8 mol/L urea gel and full-length riboprobes were gel purified.

PPAR-γ and Pancreatic Cancer Cell Invasion

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**PPAR-γ Transcriptional Activity**

Cells were transfected with the luciferase vector pCMV-Luc and the reporter plasmid prACO-βneo, which contains two functional PPREs of the acyl-CoA oxidase promoter (length: −1273 to +20 of the promoter). In some experiments, transfected cells were additionally infected with either Adx-D/N-PPAR-γ or Adx-LacZ for 24 hours or preincubated with GW9662 for 30 minutes before challenged with the indicated ligands for 4 hours. Cell lysates were prepared with the Reporter Lysis Buffer (Promega Corp., Madison, WI). β-Galactosidase activity was measured using the β-Galactosidase Enzyme Assay System (Promega) and normalized to luciferase activity, which was determined by the Luciferase Assay System (Promega).

**Western Blot**

PPAR-γ protein expression was determined as described previously (11). Briefly, total cellular lysates were separated on SDS-PAGE and transferred to nitrocellulose. Membranes were probed with a rabbit polyclonal PPAR-γ antibody and an anti-rabbit immunoglobulin. Protein-antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). To ensure equal protein loading, membranes were directly reprobed with an β-actin antibody.

**Invagination Assay**

Invagination assays were done using a Matrigel invasion chamber (BD Biosciences Discovery Labware, Bedford, MA) as described previously (51). Briefly, pancreatic cancer cells (2 x 10⁵) were seeded in the upper chamber in serum-free medium in the presence or absence of the indicated ligands and inhibitors. In some experiments, cells were infected with either Adx-D/N-PPAR-γ or Adx-LacZ before being seeded into the upper chamber. Complete medium containing 20% fetal bovine serum served as a chemoattractant in the lower chamber. After 24 hours, invading cells on the lower membrane surface were stained and counted. Control inserts without overlying Matrigel matrix were used to normalize the effect of the compounds on invasion to their effect on cell growth. All invasion experiments were carried out at least in triplicate using separate cultures and the same lot of Matrigel chambers.

**Statistical Analysis**

Data are mean ± SD. Differences in the mean of two samples were analyzed by an unpaired *t* test. Comparisons of more than two groups were made by a one-way ANOVA with *post hoc* Holm-Sidak analysis for pairwise comparisons and comparisons versus control. An *α* value of 0.05 was used to determine significant differences. All statistics were done in SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA).

**References**


Molecular Cancer Research

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