IL-6 and PPARγ Signalling in Human PC-3 Prostate Cancer Cells

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Abstract. Background: Peroxisome proliferator-activated receptor gamma (PPARγ) ligands and interleukin (IL)-6 are key factors for controlling prostate cancer cell proliferation and survival. Materials and Methods: Herein we used the natural PPARγ ligand, 15deoxy Δ12-14 PGJ2 (15dPGJ2), and IL-6 to define their interactions on proliferation and signal transduction in PC-3 cells. Cytotoxic and trypan blue exclusion assays, Western blot analysis of mitogen-activated protein kinases (MAPK) and Janus kinase/Signal transducer and activator of transcription (JAK/Stat) and real-time polymerase chain reaction (PCR) were methods employed as investigation tools. Results: 15dPGJ2 reduced PC-3 cell proliferation, while IL-6 increased it. IL-6 induced PPARγ expression but did not affect the PPARγ ligand-mediated effects on the proliferation of PC-3 cells. However, 15dPGJ2 inhibited the IL-6-mediated increase of PC-3 cell proliferation. 15dPGJ2 activated Erk1/2 phosphorylation without affecting Akt phosphorylation and reduced phosphorylated and unphosphorylated Stat3 in PC-3 cells. IL-6 suppressed endogenous activation of Stat3 without affecting Erk1/2 and Akt phosphorylation and suppressed the 15dPGJ2-mediated activation of Erk1/2 phosphorylation in PC-3 cells. Conclusion: The interplay between PPARγ ligands and IL-6 signalling could be important in controlling the growth of androgen independent prostate cancer cells as exemplified by PC-3 cells.

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kinase/signal transducer and activators of transcription (JAK-STAT) pathway is one, with SOCS (suppressor of cytokine signalling) acting as JAK kinase inhibitor. The second is the mitogen-activated protein kinase (MAPK) cascade (18, 19). There is controversy about Stat3 presence and activity in PC-3 cells. Several studies did not detect phosphorylated or unphosphorylated Stat3 protein (20-25), while several others confirmed that Stat3 is active in PC-3 cells (26-28).

Another key regulator of prostate cancer is peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor with a role still to be defined in cancer biology. Indeed, several malignant tumours express PPARγ, whereas exogenous administration of PPARγ ligands produces an anticancer action, which is mediated by both genomic and non-genomic actions (29-33).

A naturally occurring PPARγ ligand, the terminal derivative of prostaglandin J2 metabolism, 15deoxy Δ12-14 PGJ2 (15dPGJ2), does inhibit prostate cancer cell proliferation through an S-phase arrest (34). 15dPGJ2 affects the IL-6-induced Jak-Stat signalling pathway in several cell lines, astrocytes, microglia, endothelial cells, primary human lymphocytes, Jurkat cells and immortalized rheumatoid arthritis B cells via a PPARγ-independent mechanism (35-38).

In this article, we investigated the interplay between 15dPGJ2 and IL-6 in PC-3 androgen independent prostate cancer cells.

Materials and Methods

Cell culture. PC-3 cells are an androgen-insensitive, p53-negative, and K-Ras-mutated human prostate cancer cell line and were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (DMEM; Cambrex, Walkerville, MD USA) supplemented with 10% foetal bovine serum (FBS) (Biochrom, Berlin Germany), 100 U/ml penicillin/streptomycin (Cambrex) at 37°C in a humidified atmosphere of 5% CO2. Treatments were made in 0.5% FBS, except where stated differently. Cells were cultured in 0.5% FBS medium for twenty-four hours prior to treatment. 15dPGJ2 was purchased from Cayman Chemicals, Ann Arbor, MI USA and IL-6 was from R&D Systems, Minneapolis, MN USA.

Cytotoxic assay. Twenty-four hours prior to treatment, PC-3 cells were plated at cell density of 750 cells/well in 96-well plates with DMEM/F-12 containing 10% FBS. Cells were treated for 96 hours with the appropriate drug or drug combination in dose dependent manner. IL-6 concentration ranged from 1.56 to 50 ng/ml and 15dPGJ2 from 0.31 to 10 mM. After the treatments cells were incubated with 10% MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St. Louis, MO, USA) as described elsewhere (4) and the percentage of viable cells was measured by VectraMax plate reader.

Trypan blue exclusion assay. PC-3 cells were plated at a cell density of 3.5x10⁴ cells/well in 6-well plates and treated in DMEM/F12 containing 0.5% FBS for forty-eight hours. PC-3 cells were exposed to IL-6 (1 and 25 ng/ml), 15dPGJ2 (2 μM) or drug combination (25 ng/ml IL-6 plus 2 μM 15dPGJ2) and the cell number was counted as described elsewhere (4).

Western blot analysis. PC-3 cells were plated in 6-well plates. Twenty-four hours prior to treatment, the growth medium was changed to 0.5% FBS. The cell protein content was extracted using RIPA buffer [50 mM Tris-HCl; 150 mM NaCl, 1% Igepal CA360, 0.5% NaDOC; containing protease and phosphatase inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodiumorthovanadate and 1 mM NaF; all chemicals from Sigma]. Protein concentrations were determined by Bradford protein assay (BIO-RAD Laboratories) followed by electrophoresis on 10% SDS–PAGE under denaturing conditions, and transferred onto nitrocellulose membrane (BIO-RAD Laboratories, Hercules, CA USA). The blots were blocked with TBS-T (20 mmol/l Tris–HCl, pH 7.6, 137 mmol/l NaCl, and 0.1% Tween 20) containing 5% non-fat dried milk at room temperature for 1 hour. Membranes were probed overnight with primary antibodies against phosphorylated (on residue Tyr705) Stat3 and unphosphorylated Stat3, phosphorylated Erk 1/2 and phosphorylated Akt plus GAPDH as control reference for protein loading (all 1:1,000 dilution, except GAPDH 1:2,000) in TBS/T containing 5% bovine serum albumin (BSA) (all primary antibodies purchased from Cell Signalling, Beverly, MA, USA). The blots were washed and incubated with the appropriate secondary antibodies for 1 hour at room temperature (Sc2004, Sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:2000 dilution). The bands were visualized by exposure to x-ray film after incubation of the blots with SuperSignal ECL substrate (Pierce Biotechnology, Rockford, IL, USA). Quantification was performed using Kodak imaging software (EDAS Kodak software).

Real-time quantitative polymerase chain reaction (PCR). PC-3 cells were treated with IL-6 (25 ng/ml) for forty-eight hours and total RNA was extracted by homogenization of 5x10⁶ cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by standard chloroform extraction and isopropanol precipitation. Ten mg/ml of RNA were used for the reverse transcription reaction; the reaction was completed by the addition of RNase A (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by column purification (PCR Purification Kit, Qiagen Hilden, Germany) and elution using RNase and DNase-free double distilled water. Reactions were carried out in a 20 μl volume with 0.5 μl primers and MgCl2 concentration optimized between 2-5 mM. The assay was performed with the Light Cycler DNA Fast Start SYBR Green I kit (Roche Diagnostics, IN, USA) according to the manufacturer’s instructions. Oligonucleotides used to detect PPARγ mRNA expression were derived from the published sequence of human PPARγ (Genbank Accession Number: NM_138712). Sequence alignment was performed by the GeneTool 3.0 software, while a specific set of primers was designed by the DNA Synthesizer 3900 (Perkin-Elmer Life Sciences, Boston, MA, USA).

The pair of resulting primers used was: 5'-GTC-GGA-TCC-ACA-AAA-AAA-GTA-GAA-3' (forward primer) and 5'-AGC-GGG-AAG-GAC-TCT-ATG-TAT-GA-3' (reverse primer) which gave rise to a 229-bp fragment. As a reference, transcripts of ATPase (chosen, among other housekeeping genes, as one that varies less among the treatments) for each sample were simultaneously quantified during...
each real-time-PCR run and negative controls (containing no template cDNA) were included. The cycle profile was 95˚C for 10 min and 40 cycles at 95˚C for 10 s, 58˚C for 5 s and 72˚C for 13 s. Data were collected during the extension stage of each cycle and analyzed using the Light Cycler Data Analysis Administrator.

The real-time-PCR reaction ‘end-point’ for each sample was defined as the PCR cycle at which the fluorescence threshold was crossed. The intensity of fluorescence signals generated during PCR reactions was increased as cycles progressed. The melting curve was constructed by increasing the temperature to 95˚C and by plotting the first negative derivative (–dF/dT) of the fluorescence vs. temperature thereby determining the melting temperature of the PCR products.

Overall, real-time-PCR analysis revealed a stratification pattern between the samples which was similar to that observed in relative quantitative-PCR analysis, where different RNA extraction methods and different set of primers were used.

**Statistical analysis.** All experiments were performed in triplicate. Values are means±standard deviation (SD). Statistical analysis was performed by Student’s t-test. The level of statistical significance was set at \( p<0.05 \).

**Results**

In our experiments, IL-6 did not affect Erk1/2 or Akt phosphorylation in PC-3 cells (Figure 1). On the contrary, IL-6 inhibited baseline Stat3 phosphorylation (Figure 1), whereas 15dPGJ2 inhibited both phosphorylated and unphosphorylated Stat3 (Figure 2). However, 15dPGJ2 activated Erk1/2 phosphorylation in PC-3 cells and this activation was inhibited by IL-6. Akt phosphorylation was not affected by 15dPGJ2 with or without IL-6, (Figure 3).
IL-6 increased (by almost 2-fold) the PPARγ mRNA expression as determined by real-time PCR analysis in PC-3 cells treated with 15dPGJ2 (2 μM) alone and in combination with IL-6 (25 ng/ml) for 15, 30, 60 minutes and 12 hours. Note that 15dPGJ2 did activate pErk in PC-3 cells, suggesting a non-genomic action on PC-3 cells. However, IL-6 abated the 15dPGJ2-induced phosphorylation of Erk 1/2 without affecting Akt phosphorylation.

15dPGJ2 inhibited PC-3 cell proliferation in a dose dependent manner whereas, IL-6 (1 and 25 ng/ml) stimulated the PC-3 cell growth (Figure 5). Combinational treatments using IL-6 (25 ng/ml) with increasing doses of
15dPGJ2 did not alter the 15dPGJ2 effect on PC-3 cells. However, 15dPGJ2 did block the IL-6-related increase of PC-3 cell growth. These data were documented using trypan blue exclusion and MTT assays (Figures 5 and 6).

**Discussion**

Previous findings have shown that troglitazone, a synthetic PPARγ ligand, has antiproliferative effect with anti-inflammatory and antidiabetic actions, both in LNCaP androgen-sensitive cells and in advanced stage prostate cancer patients (39). Furthermore, PPARγ ligands down-regulate prostate-specific antigen (PSA) levels in LNCaP cells (33, 39, 40).

Recent findings indicate that 15dPGJ2, a natural PPARγ ligand, acts through a PPARγ-independent mechanism in PC-3 cells that promotes p42/p44 (Erk1/2) phosphorylation but has no effect on Akt phosphorylation. Rosiglitazone, a synthetic PPARγ ligand, blocks the IGF-1-induced Akt activation in PC-3 cells (41). This specific ligand has potent anti-inflammatory activity by inhibiting cytokine production (tumour necrosis factor (TNF)-α, IL-1β, and IL-6) (38).

Researchers that share the experimental context of using 10% FBS in growth medium did not detect Stat3 activity in PC-3 cells (20-28). As described by Pu et al. under these conditions, PC-3 cells constitutively secrete IL-6 and this secretion is inhibited when low serum conditions are used (17). Thus our findings for the detection of both the phosphorylated and unphosphorylated Stat3 protein in PC-3 cells grown in 0.5% FBS are here presented. In addition, we showed that IL-6 affects pStat3 protein expression by decreasing its expression to undetectable levels after 12 hours of treatment. The latter may explain why Stat3 activity is inhibited when the growth medium contains serum, which is able to stimulate IL-6 production in PC-3 and in turn down-regulates phosphorylated Stat3.

Furthermore, IL-6 does not phosphorylate Erk1/2 or Akt and, as mentioned, down-regulates pStat3 in a time-dependent manner. Apparently, PC-3 cells expressing IL-6 use uStat3 as a transcription factor. The latter has recently been described by Yang et al. to form a complex with unphosphorylated nuclear factor (NF)κB (42). This complex is a transcription factor that activates a subset of κB-dependent genes. Furthermore, Stat3 has the capacity of entering the nucleus in the unphosphorylated form, thus activating gene transcription without tyrosine phosphorylation (43).

An IL-6-mediated effect is the increase of PPARγ expression. However, the PPARγ ligand, 15dPGJ2, induces the arrest of PC-3 cells, an effect which is not modified by IL-6, whereas IL-6 blocks both the 15dPGJ2-induced phosphorylation of pErk and the down-regulation of uStat3. Finally, 15dPGJ2 inhibited the IL-6-mediated increase of the
PC-3 cell growth, as assessed in MTT assays. These data provide information on the complex interaction between this PPARγ ligand, 15dPGJ2, and IL-6 in human PC-3 prostate cancer cells and delineate the importance for further research on this subject.

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