Peroxisome proliferator-activated receptor γ agonist rosiglitazone inhibits migration and invasion of prostate cancer cells through inhibition of the CXCR4/CXCL12 axis

LIANG QIN¹, CHEN GONG², AN-MIN CHEN¹, FENG-JING GUO¹, FEI XU¹, YE REN¹ and HUI LIAO¹

Departments of ¹Orthopedics and ²Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

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Abstract. It has been indicated that the C-X-C chemokine receptor type 4/C-X-C chemokine ligand 12 (CXCR4/CXCL12) axis is involved in promoting invasion and metastasis in tumors. Therefore, novel drugs capable of downregulating the CXCR4/CXCL12 axis may demonstrate potential for the treatment of metastatic prostate cancer (PCa). Rosiglitazone (RSG), a thiazolidinedione ligand of the peroxisome proliferator-activated receptor (PPAR) γ, has been found to inhibit proliferation, induce apoptosis, suppress angiogenesis and inhibit metastasis. However, the precise mechanisms by which RSG regulates CXCR4 gene expression and the consequent effects on prostate cell migration and invasion are not fully understood. In this study, it was observed that RSG is capable of downregulating the expression of CXCR4 in PCa cells in a dose-, time- and PPARγ-dependent manner. Furthermore, it was observed that the downregulation of CXCR4 expression occurred at a transcriptional level, as indicated by a reduction in CXCR4 mRNA expression. Suppression of CXCR4 expression by RSG further correlated with the inhibition of CXCL12-induced migration and invasion in PCa cells. Analysis of the predominant intracellular signaling pathways that act downstream of the activated CXCR4/CXCL12 axis, namely the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) cascades, revealed that RSG rapidly interferes with the phosphorylation/activation of Akt, which mediates CXCL12-stimulated migration and invasion. Overall, the findings of this study suggest that RSG represents a novel inhibitor of CXCR4 expression and, thus, has significant potential as a powerful therapeutic agent for the treatment of metastatic PCas.

Introduction

Prostate cancer (PCa) is the second leading cause of increased cancer incidence and cancer-associated mortality among males in the United States (1). In 2010, the incidence of new prostate cancer cases was estimated to be 217,730, resulting in 32,050 mortalities (2). The majority of patients with advanced PCa develop bone metastases and suffer from long-term skeletal morbidity, involving pain, pathological fractures and spinal cord compression, which has a significant impact on the quality of life of the patient. Therefore, studying the molecular mechanisms underlying PCa bone metastasis is considered essential for the prevention and treatment of PCa.

Peroxisome proliferator-activated receptor (PPAR) γ regulates the expression of genes involved in the control of lipid metabolism and insulin sensitivity via ligand-activated transcriptional activity (3). PPARγ ligands include naturally occurring fatty acids, 15-deoxy-delta12,14-prostaglandin J2 (PGJ2) and thiazolidinediones (TZDs), such as troglitazone and rosiglitazone (RSG) (4). The activation of PPARγ by TZDs and other ligands has been shown to inhibit proliferation and invasion, as well as induce apoptosis and cell cycle arrest, in prostate and other cancer cells through PPARγ-dependent and -independent pathways (5-9). Therefore, PPARγ is recognized as a relevant target for cancer therapy.

The present study aimed to investigate the effect of PPARγ activation by ligands on tumor cell migration and invasion. C-X-C chemokine receptor type 4 (CXCR4) has been reported to mediate proliferation, invasion and metastasis of tumor cells; therefore, it was hypothesized that RSG may modulate the expression of CXCR4 and inhibit the migration and invasion of prostate cancer cells.

Materials and methods

Cell culture. PC-3 human prostate carcinoma cells were initially stored frozen in our laboratory (Department of Orthopedics, Tongji Medical College, Wuhan, China) prior to...
cultivation in RPMI-1640 medium (Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 1x10^5 U/l penicillin and streptomycin (Hyclone Laboratories, Inc.) in an incubator at 37°C with 5% CO₂.

**Cell migration assay.** Cell motility was assessed as an in vitro wound healing assay. PC-3 cells were plated in a six-well plate and grown until confluent. Monolayers of confluent PC-3 cells were then scarred, and the repair was monitored using an inverted microscope (Nikon TE2000-S; Nikon Corporation, Tokyo, Japan), following 12 h pretreatment with 10 µM RSG (Sigma Aldrich, St. Louis, MO, USA) then 24 h exposure to 100 ng/ml CXCL12 (R&D Systems Inc., Minneapolis, MN, USA). Wound width was measured at 0 and 24 h following the start of incubation with or without RSG and in the absence or presence of CXCL12. Experiments were performed in triplicate and data are presented as the mean ± standard deviation.

**Cell invasion assay.** The invasive potential of PC-3 cells was quantified using a Matrigel®-coated Transwell system, as described previously (10). The chamber (Corning Inc., Corning, NY, USA) contained a polycarbonate membrane filter with a pore size of 8 µm, which was coated with Matrigel and inserted into a 24-well culture plate. PC-3 cells were then seeded in the top chamber of the Matrigel. Following pre-incubation with or without RSG (10 µM) for 12 h, Transwell chambers were then placed into the wells of a 24 well plate, in which either basal medium or basal medium supplemented with 100 ng/ml CXCL12 was added for 24 h. After 48 h incubation, PC-3 cells on the upper surface of the filters were removed using cotton swabs. Cells that had invaded the lower surface of the membrane were fixed using methanol and stained with crystal violet. Each experiment was performed in triplicate and cells were counted in five fields in each well using light microscopy. The invasive ability of PC-3 cells was assessed relative to the invasive ability of the untreated control cells.

**Western blot analysis.** Cells were grown on six-well culture plates, washed with 1X phosphate-buffered saline (PBS) and harvested in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (Cell Signaling Technology Inc., Beverly, MA, USA). The cell extractions were collected and centrifuged at 10,000 x g for 10 min at 4°C before the supernatants were collected as cell lysates. Equal concentrations of total cell lysate were resolved using 10% SDS-PAGE (Boster, Wuhan, China) and harvested in lysis buffer containing 20 mM Tris- HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (Cell Signaling Technology Inc., Beverly, MA, USA). The cell extractions were collected and centrifuged at 10,000 x g for 10 min at 4°C before the supernatants were collected as cell lysates. Equal concentrations of total cell lysate were resolved using 10% SDS-PAGE (Boster, Wuhan, China) and transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Merck Millipore, Billerica, MA, USA). Non-specific binding sites were blocked with 5% non-fat dry milk in 1X Tris-buffered saline containing 0.1% Tween 20 (TBST; Boster), followed by incubation with primary antibodies against the proteins of interest in 3% bovine serum albumin (BSA; Boster)-TBST [phosphorylated Akt (p-Akt), CXCR4] or 5% non-fat dry milk-TBST (Akt, β-actin). Subsequently, the membranes were incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G; Wuhan Boster Biological Technology Ltd, Wuhan, China). Immunoblots were visualized using SuperSignal™ West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA). The antibodies raised against human Akt, p-Akt (Ser473) and β-actin (rabbit monoclonal IgG) were purchased from Cell Signaling Technology. The antibody raised against human CXCR4 (rabbit polyclonal IgG) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Quantitative polymerase chain reaction (qPCR) analysis.** PC-3 cells were plated in six-well plates for all experiments and allowed to grow for 48 h prior to any treatment. GW9662 (Santa Cruz Biotechnology, Inc.) was added 2 h prior to any other treatment. Total RNA was extracted using TRIzol® reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). A total of 1 µg RNA was reverse-transcribed according to the kit's instructions (Invitrogen Life Technologies). qPCR was performed using TransStart Green qPCR SuperMix (TransGen Biotech Co., Beijing, China) using an iQ5 Sequence Detection System (Bio-Rad Laboratories Inc., Berkley, CA, USA). Results were normalized to those obtained for GAPDH. Each sample was analyzed in triplicate and the experiment was repeated twice. The threshold cycle (CT) value (the cycle number at which the fluorescence crosses the threshold) was measured and 2^−ΔCT (ΔCT = CT - CT_GAPDH) was defined as the quantity of the amplified fragment. The primer sequences are listed in Table 1.

**Statistical analysis.** Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 11.0 statistics software (SPSS, Inc., Chicago, IL, USA). Statistical significance was analyzed using one-way analysis of variance. Where significance was observed, a Dunnett’s post-hoc test was used to determine the statistical significance of the differences between the treated- and untreated-groups, with a value of P<0.05 considered to indicate a statistically significant difference.

**Results**

RSG suppresses CXCR4 mRNA and protein levels in prostate cancer cells. A previous study has shown that CXCR4 expression is significantly higher in human PCA tissue than in hyperplastic prostate tissues (11). This finding suggests that CXCL12 may exhibit an autocrine regulatory role via its receptor, CXCR4, in the regulation of PCA cell migration, invasion and metastasis (12). Therefore, the initial investigations in the present study focused on the effect of RSG on the expression of CXCR4 in PC-3 cells. It has been reported that TZDs activate PPARα and PPARδ receptors at concentrations >10 µM (13); therefore, concentrations of ≤10 µM were utilized in the present investigations. It was observed that when PC-3 cells were incubated with various RSG concentrations for 24 h, or for various time periods with 10 µM RSG, the expression of CXCR4 was suppressed in a dose- and time- dependent manner, respectively (Fig. 1A and B). This downregulation was not due to a decrease in cell viability, as ~90% of PC-3 cells were viable under these conditions (data not shown).
It was hypothesized that the suppression of CXCR4 expression may occur at the transcriptional level. Therefore, following PC-3 cell treatment with RSG for various time-periods, the mRNA was extracted for qPCR analysis. As shown in Fig. 1D, RSG was found to downregulate CXCR4 mRNA expression in a time-dependent manner, with a significant reduction observed from 6 h following exposure. CXCR4 mRNA expression was inhibited at all RSG doses tested, with 22, 27 and 36% inhibition observed following 12 h exposure to 0.1, 1 and 10 µM RSG, respectively (Fig. 1C).

Although RSG was found to affect CXCR4 expression in PC-3 cells, the mechanism by which this was achieved was unclear. To determine whether the reduction in expression of CXCR4 by RSG was dependent upon the activation of PPARγ, PC-3 cells were treated with the PPARγ antagonist, GW9662. The downregulation of CXCR4 mRNA and protein expression induced by RSG in PC-3 cells was attenuated upon addition of GW9662 (Fig. 1). These results indicate that RSG is capable of downregulating CXCR4 expression in a manner dependent upon PPARγ activation. In combination, these data suggest that RSG may inhibit CXCR4 expression in PC-3 cells, in a PPARγ-dependent manner.

**RSG inhibits CXCL12-induced migration in PC-3 cells.**

This study further investigated a potential correlation between RSG-induced downregulation of CXCR4 and PCa cell migration. An *in vitro* wound healing assay revealed that PC-3 cells migrated more rapidly upon treatment with CXCL12 and that this effect was abolished upon treatment with RSG (Fig. 2).

**RSG inhibits CXCL12-induced invasion in PC-3 cells.**

To elucidate the inhibitory effect of RSG upon CXCL12-induced invasion in PC-3 cells, chamber invasion assays were performed. RSG was observed to suppress the invasion of PC-3 cells across the Matrigel-coated filter in a dose-dependent manner.

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**Table I. Primer sequences for qPCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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<tr>
<td>GAPDH</td>
<td>CCATGAGAAGTATGACAACAGCC</td>
<td>GGGTGCTAAGCAGTTGTTG</td>
</tr>
<tr>
<td>CXCR4</td>
<td>TGCCCAACATCTACTCCATCA</td>
<td>AGGATGACCAATCCATTGCC</td>
</tr>
<tr>
<td>VEGF</td>
<td>TTACGGTCTGTGTCAGTGTGA</td>
<td>TTCTCTGTATGTGGCCAGCC</td>
</tr>
<tr>
<td>MMP2</td>
<td>GATACCCCTTGGACGGTAAAGGA</td>
<td>CCTTCTCCCAAAGTCCATAAGC</td>
</tr>
<tr>
<td>MMP9</td>
<td>TGTACCCTATGGTTACACTCG</td>
<td>GGCAGGGACAGTTGCCTCT</td>
</tr>
</tbody>
</table>

CXCR4, C-X-C chemokine receptor type 4; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase.
manner, with 10 μM RSG found to inhibit 52% of cell invasion. RSG was also observed to suppress the CXCL12-induced invasion of the PC-3 cells (Fig. 3). These results indicate that RSG markedly inhibits the CXCL12-induced invasion of PC-3 cells.

**RSG inhibits CXCL12-induced Akt activation in prostate cancer cells.** It has previously been indicated that the signaling proteins, PI3K and Akt, may be associated with the expression of matrix-metalloproteinases (MMPs) and metastasis induction (14). To further elucidate the signal transduction pathways responsible for CXCR4 expression and PC-3 cell migration and invasion, the activation of Akt, a signaling component of pathways coupled to G-protein-coupled chemoattractant receptors, was examined. Upon rapid stimulation (30 min) of PC-3 cells with 100 ng/ml CXCL12, an increase in Akt phosphorylation at Ser473 was observed, resulting in the activation of the enzyme. Furthermore, it was observed that concomitant addition of 10 μM RSG for 30 min downregulated the CXCL12-induced phosphorylation of Akt in a dose-dependent manner in PC-3 cells (Fig. 4A). This finding suggests that the inhibition of migration and invasion associated with RSG treatment in PC-3 cells may partly occur through the suppression of PI3K pathways.
In the present study, it was observed that RSG was capable of downregulating CXCL12 expression in a PPARγ-dependent manner, which was also time- and dose-dependent (16). Furthermore, pre-clinical studies have shown that the parent compound TZD is capable of reducing metastasis of HT-29 cells implanted in the rectums of mice (17). However, in a Phase II study of patients with advanced metastatic colorectal cancer that had not responded to chemotherapy, troglitazone failed to produce an objective tumor response (18). Whether treatment with a more potent and selective PPARγ agonist, such as RSG, is also associated with a lack of tumor response is yet to be elucidated. The present data suggest that such an agent may be capable of reducing CXCR4 expression to a greater extent, and may therefore be anticipated to demonstrate greater potential for reducing metastasis.

Metastasis is the spread of a disease from one organ or tissue to another non-adjacent organ or tissue, and is regulated by numerous signaling pathways in cancer cells and the microenvironment. The CXCR4/CXCL12 axis has a role in cancer cell metastasis and proliferation, the importance of which may vary between different types of cancer cells, due to differences in expression. For example, overexpression of CXCR4 in PCa cells has been shown to accelerate prostate tumor metastasis and vascularization, as well as tumor growth in vivo (19,20). Furthermore, CXCL12 stimulates chemotaxis of metastatic PCa cells expressing high levels of CXCR4, and accelerates their migration (20). Conversely, blockade of CXCR4/CXCL12 interaction in prostate cancer cells via CXCR4 knockdown has been found to significantly inhibit bone metastasis in vivo (21).

Another important signaling pathway in PCa cells is the PI3K/Akt pathway (22,23). Akt is a serine-threonine kinase whose phosphorylation is associated with mitogenic signals. In addition to its role in survival, Akt has been found to participate in numerous intracellular signaling pathways, including the integration of proliferation and differentiation signals, such as migration and angiogenesis. Previous studies have demonstrated that the PI3K/Akt pathway may also have a role in CXCL12/CXCR4-mediated PCa cell migration and angiogenesis (24,25). In this study, it was observed that concomitant addition of RSG downregulated CXCL12-induced phosphorylation of Akt in a dose-dependent manner in PC-3 cells, indicating that the inhibition of migration and invasion by RSG may partly occur through suppression of PI3K pathways. In accordance with the present study, it has previously been reported that PPARγ agonists may be capable of decreasing Akt phosphorylation (26,27). RSG was also observed to demonstrate a similar affect on insulin growth factor 1-induced phosphorylation of Akt and extracellular signal-regulated kinases in adenocortical cancer cells (28).

In conclusion, the present data indicate that RSG is capable of inhibiting CXCL12-induced invasion and migration of PC-3 cells in vitro, through inhibition of CXCR4 expression and Akt phosphorylation. This study may provide preliminary evidence for further research into the mechanisms underlying the inhibition of metastasis by TZDs. In the present study, RSG was used only as a proof-of-principle to determine the
efficacy of the PPARγ agonist class of drugs to inhibit CXCR4 expression. Based on the unclear association between RSG and risk of myocardial infarction, as well as the apparently higher risk of long-bone fracture in females that is associated with RSG, detailed safety, pharmacokinetic and pharmacodynamic studies of this class of drugs are necessary prior to clinical use in patients.

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References