**Regular Article**

**15-Deoxy-Δ^{12,14}-prostaglandin J₂ Inhibits Cell Migration on Renal Cell Carcinoma via Down-Regulation of Focal Adhesion Kinase Signaling**

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Renal cell carcinoma (RCC) is one of the chemoresistant cancers. There is a pressing need to establish therapeutic approaches to prevent RCC proliferation and metastasis. The electrophilic 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) is an endogenous anti-cancerous agent. Treatment with high concentrations of 15d-PGJ₂ is known to induce apoptosis of RCC cells, independent of the nuclear receptor, peroxisome proliferator-activated receptor-γ (PPARγ). In this study, we investigated the effects of 15d-PGJ₂ on the metastatic properties of RCC Caki-2 cells. The metastatic potential of RCC was evaluated by measuring the migratory ability of Caki-2 cells. Although treatment with low concentrations of 15d-PGJ₂ did not cause apoptosis, it did decrease the migration of Caki-2 cells in a concentration-dependent manner. PPARγ did not mediate the inhibitory effect of 15d-PGJ₂ on the migration of Caki-2 cells. Treatment with a low concentration of 15d-PGJ₂ resulted in disassembled focal adhesions and extensive filamentous actin reorganization. Furthermore, 15d-PGJ₂ significantly reduced phosphorylation of focal adhesion kinase (FAK). In conclusion, 15d-PGJ₂ attenuated the migratory ability of RCC, independent of PPARγ. Further, 15d-PGJ₂ appeared to suppress cell migration via inactivation of FAK and subsequent disassembly of focal adhesion. Our present study highlights the therapeutic potential of 15d-PGJ₂ for prevention of RCC metastasis.

**Key words** 15-deoxy-Δ^{12,14}-prostaglandin J₂; renal cell carcinoma; metastasis; focal adhesion kinase; cell migration

**INTRODUCTION**

Renal cell carcinoma (RCC) accounts for 2–3% of all malignant tumors.² RCC is stratified into multiple distinct subtypes, including clear cell (approximately 70–75%), papillary (approximately 10–15%), and chromophobe neoplasms.² Surgical operation remains the primary treatment option for patients with localized tumors. Even with suitable oncologic removal, about 40% of patients will develop metastases after surgical resection. RCC can spread to other organs, including the lung, bone, liver, and brain.³ The five-year survival probability of patients with metastatic renal cell carcinoma (mRCC) is less than 10% because of the cancer’s resistance to chemotherapy and radiotherapy.⁴ Thus, there is an urgent need to establish novel therapeutic approaches for mRCC treatment.

The metastatic cascade has been reported to be modulated by an endogenous carnosinatic 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂).⁵ A nuclear receptor of 15d-PGJ₂ is peroxisome-proliferator activated receptor γ (PPARγ),⁵,⁷ and its membrane receptor is a D-prostanoid 2 (DP2) receptor, another named for chemoattractant receptor-homologous molecule expressed on T-helper 2 (Th2) cells (CRTH2).⁸,⁹ PPARγ agonists (pioglitazone, troglitazone and 15d-PGJ₂) inhibit growth of human RCC.¹⁰ However, a synthetic PPARγ agonist, troglitazone, induces apoptosis independently of PPARγ in RCC.¹¹ Furthermore, the cytotoxic effect of an endogenous PPARγ agonist, 15d-PGJ₂, on RCC is independent of PPARγ.¹²

Previously, we reported that 15d-PGJ₂ down-regulated the phosphorylated Akt in RCC.¹² In addition, a phosphoinositide 3-kinase (PI3K) inhibitor mimicked the anti-proliferative effect of 15d-PGJ₂ on RCC.¹³ Furthermore, we reported that 15d-PGJ₂ induced apoptosis via suppressing the PI3K-Akt pathway.¹⁴ PPARγ agonists enhanced antimetabolite (5-FU)-, platinum (CDDP)- or topoisomerase-II inhibitor-induced apoptotic cell death in other solid cancers.¹⁵–¹⁸ We also confirmed the synergistic effects of 15d-PGJ₂ on the cytotoxicity with topoisomerase-I inhibitor¹⁹ and topoisomerase-II inhibitors.¹⁴,²⁰

15d-PGJ₂ has also been reported to reduce cell migration, stimulate focal adhesion disaggregation, and induce filamentous actin (F-actin) realignment at low concentrations, independent of PPARγ.¹³ Interestingly, the p38 mitogen-activated protein (MAP) kinase inhibitor prevented both 15d-PGJ₂-induced F-actin realignment and focal adhesion disaggregation. However, it has not yet been ascertained whether 15d-PGJ₂ is involved in the metastasis of RCC. In the present study, we evaluated the effects of 15d-PGJ₂ on the migration of Caki-2 RCC cells. We provided novel evidence that 15d-PGJ₂ inhibits RCC migration via suppressing the focal-adhesion kinase (FAK) signaling.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture** We used Caki-2 the human renal cell carcinoma cell line as previously reported.¹³ Caki-2 was obtained from Summit Pharmaceuticals International (Tokyo, Japan). Caki-2 cell line were routinely cultured at 37°C and 5% CO₂-95% room air in RPMI 1640 medium (Invitrogen, Japan) with 10% fetal bovine serum (Invitrogen), 50 mg/L penicillin G and 50 mg/L streptomycin (Invitrogen). All experiments were performed in RPMI 1640 medium with 0.5% fetal bovine serum.

**Reagents** 15d-PGJ₂ was obtained from Cayman Chemicals (Ann Arbor, MI; Cabru, Milan, Italy). GW9662 was...
obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo Laboratories (Kumamoto, Japan).

**Cell Viability Analysis** To evaluate the effect of 15d-PGJ2 on Caki-2 cell growth, cell viability was determined by MTT assay. The cells were seeded on a 24-well tissue culture plate at 15000 cells/cm² and incubated for 24 h prior to drug exposure. The cells were incubated with 15d-PGJ2 at several concentrations for 24 h. After 24 h incubation, the cells were incubated with MTT solution (0.1 mg/mL in phosphate buffered saline (PBS)) for an additional 3 h at 37°C. MTT solution was aspirated off. To dissolve the formazan crystal formed in viable cells, dimethyl sulfoxide was added to each well. Absorbance was measured at a wavelength of 570 nm using a spectrophotometer.

**Measurement of Cellular Migration (Scratch Assay)** Caki-2 cells were grown to 100% confluence in 6-well tissue culture plate, and then scratched with 200 µL yellow pipette tip. Medium was immediately changed to remove floating cells and was replaced with medium containing 15d-PGJ2 and/or GW9662. The width of the wounding scratch was measured at four points in each well after initial wounding, and Caki-2 cells were incubated for several hours at 37°C in a CO2 incubator. After incubation, the same scratch width was measured again. To evaluate the cell migration ability, the width of the cell empty zone at initial wounding was compared with the width after 8 h incubation.

**Immunofluorescence Microscopy** Immunofluorescence microscopy was employed to visualize phosphorylated FAK (p-FAK) and F-actin as previously reported.21) Caki-2 cells were plated on to glass base dish, treated and fixed using paraformaldehyde (4%) for 15 min. The cells were then rinsed twice with PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated in anti-p-FAK (Tyr397) (Cell Signaling (3283)), rabbit polyclonal at 1:200 dilution for 1 h at room temperature. Alexa Fluor 488 F(ab’2) fragment of goat anti-rabbit immunoglobulin G (IgG) (H + L), (Thermo Fisher Scientific Inc. (A1070)), diluted 1:200, was used as a secondary antibody. F-Actin was stained with Alexa Fluor 555 Phalloidin (Thermo Fisher Scientific Inc. (A34055)). In p-FAK and F-actin co-staining experiments, Alexa Fluor 555 Phalloidin were co-incubated with secondary antibody. p-FAK foci were visualized using confocal fluorescence microscopy on Carl Zeiss, LSM510 laser-scanning confocal microscope with excitation from a 488 nm laser line and emission detection suitable for fluorescein. F-Actin imaging was performed with 633 nm laser line for excitation and emission detection suitable for Cy5 (indodicarbocyanine).

**Statistical Analysis** Data are given as means ± standard error (S.E.) (n = number of observations). At least, we performed three independent experiments on different days and confirmed their reproducibility. Data were analyzed statistically by use of Student’s t-test for comparison with the control group. Data on various drugs were analyzed statistically by use of one-way ANOVA followed by Scheffe’s F-test for comparison with another group.

**RESULTS**

**The Cytotoxic Effect of 15d-PGJ2 on Caki-2 Cells** 15d-PGJ2 induces apoptosis in a several of cancer cell lines.20) To confirm non-cytotoxic concentrations of 15d-PGJ2, cytotoxicity was assessed by MTT assay in the present study. The cytotoxic effect of 15d-PGJ2 was examined on human RCC cell line, Caki-2. After incubation of Caki-2 cells with 15d-PGJ2 for 8 h, their viability was not significantly changed at the concentration ranging from 1 to 5 μM (Fig. 1A). When Caki-2 cells were expose to 15d-PGJ2 at the concentration higher than 20 μM, cell viability was decreased reciprocally to apoptotic cell populations.13)

**15d-PGJ2 Attenuated Caki-2 Cell Migration** To examine the cell motility, migration was evaluated by a scratch assay. After scratch, the scratch width was narrowed in a time-dependent manner and disappeared within 12 h (Fig. 1B). Although migration was not affected by vehicle (methyl acetate) as well as control, it was significantly reduced by 5 μM 15d-PGJ2. Treatment with 3 μM 15d-PGJ2 inhibited the migration of Caki-2 cells (Fig. 2A). In comparison with control, the migratory ability was not significantly altered by vehicle (methyl acetate). On the other hand, 15d-PGJ2 significantly suppressed the migration of Caki-2 cells in a concentration-dependent manner (Fig. 2B). These results indicated that 15d-PGJ2 attenuated Caki-2 cells migration at the non-toxic...
concentration.

PPARγ Did Not Mediate the Anti-migratory Effect of 15d-PGJ

15d-PGJ has been reported to prime the eotaxin-

induced chemotaxis on human eosinophils through its nuclear receptor, PPARγ. 15d-PGJ and a specific PPARγ antagonist, troglitazone, primed eotaxin-induced shape change and actin polymerization. These priming effects were completely reversed by a specific PPARγ antagonist. To examine whether 15d-PGJ attenuated the scratch-induced cell migration via PPARγ or not, Caki-2 cells were simultaneously treated with 15d-PGJ (3 µM) and PPARγ inhibitor GW9662 (10 µM) (Fig. 3). The anti-migration effect of 15d-PGJ was not reduced by PPARγ inhibition, indicating that 15d-PGJ attenuated Caki-2 cell migration independently of PPARγ. Another receptor of 15d-PGJ is DP2, which is the type 2 membrane receptor for PGD2. DP2 is termed as CRTH2, which mediates the PGD2-induced chemotaxis in T Helper type 2 Cells, eosinophils, and basophils. 8) A DP2/CRTH2 antagonist, did not block the inhibitory effect of 15d-PGJ on the Caki-2 cell migration (data not shown). Thus, neither PPARγ nor CRTH2 mediated the anti-migratory effects of 15d-PGJ in Caki-2 cells.

15d-PGJ Reduced FAK Signaling FAK is a focal adhesion-associated protein tyrosine kinase related to cellular adhesion and spreading processes. 23) To investigate the potential role of FAK signaling in the 15d-PGJ-attenuated cell migration, we treated Caki-2 cells with 15d-PGJ for 30 min and examined phosphorylated-FAK (p-FAK) expression levels. 15d-PGJ reduced the level of p-FAK markedly at 3 µM in cell densely zone (Fig. 4) and cell free border zone (Supplementary Fig. 1). Next, we performed Western blot-
tous morphology of the F-actin cytoskeleton (Fig. 5, control column). 15d-PGJ 2 induced striking reorganization of the F-actin cytoskeleton, resulting in the peripheral localization. (Fig. 5, 15d-PGJ2 column).

DISCUSSION

In the present study, 15d-PGJ 2 attenuated Caki-2 cell migration in a concentration-dependent manner. 15d-PGJ 2 inhibited the cell migration at the concentration lower than 5 µM, whereas it induced apoptosis at the concentration higher than 20 µM. The inhibitory potency of 15d-PGJ 2 on cell migration was distinguished from its apoptosis-inducing potency. A PPARγ antagonist did not eliminate the inhibitory effect of 15d-PGJ 2 on the migration of Caki-2 cell.

Previously, we have reported that 15d-PGJ 2 exhibits cytotoxicity via down-regulating of PI3K–Akt signaling. The down-regulation of PI3K–Akt signaling has been reported to mediate the inhibition of metastasis in RCC, suggesting that 15d-PGJ 2 might inhibit the metastasis of Caki-2 cells via the suppression of PI3K–Akt signaling. In addition, topoisomerase inhibitors such as camptothecin, etoposide and doxorubicin enhanced the 15d-PGJ 2 -induced apoptosis. Although their enhancements were also independent of PPARγ, they did not enhance the inhibitory effect of 15d-PGJ 2 on the migration of Caki-2 cell (data not shown).

FAK is a protein tyrosine kinase that is reported its abnormal expression in several cancers. 15d-PGJ 2 did not alter total FAK protein levels at the concentration lower than 5 µM. In breast cancer, p-FAK distribution was markedly altered. We confirmed that p-FAK was also localized to the lamellipodium of F-actin filaments in Caki-2 cells. The treatment of Caki-2 cells with 15d-PGJ 2 resulted in reducing and spreading of p-FAK protein expression. On the other hand, it had been reported that Western blot analysis showed no significant alteration in phosphorylation of FAK by the treatment of breast cancer with 15d-PGJ 2. We have reported actin cytoskeleton morphology in response to 15d-PGJ 2 occurs via the disruption of the F-actin cytoskeleton and metastasis in RCC.

The down-regulation of PI3K–Akt signaling has been reported to mediate the inhibition of metastasis in RCC, suggesting that 15d-PGJ 2 might inhibit the metastasis of Caki-2 cells via the suppression of PI3K–Akt signaling. In addition, topoisomerase inhibitors such as camptothecin, etoposide and doxorubicin enhanced the 15d-PGJ 2 -induced apoptosis. Although their enhancements were also independent of PPARγ, they did not enhance the inhibitory effect of 15d-PGJ 2 on the migration of Caki-2 cell (data not shown).

Prior to a new generation of drug treatments for proliferation and metastasis in RCC, PPARγ agonists on the F-actin cytoskeleton were also investigated. Rosiglitazone and PGD 2 had no effect on the F-actin cytoskeletal morphology (data not shown), suggesting that the alteration of the F-actin cytoskeleton morphology in response to 15d-PGJ 2 occurs via PPARγ or DP2 independent pathways. Further studies are required to clarify how 15d-PGJ 2 suppressed the FAK signaling.

In conclusion, 15d-PGJ 2 attenuated Caki-2 cell migration through the down-regulation of FAK signaling independent of PPARγ pathway. At the low concentration, 15d-PGJ 2 induced focal-adhesion disassembly and caused F-actin reorganization suggesting that 15d-PGJ 2 suppressed in cancer cell migration. Combined with our previous reports that 15d-PGJ 2 induces apoptosis in cancer cells at the high concentration, the present study implicates the therapeutic potential of 15d-PGJ 2 as a candidate to a new generation of drug treatments for proliferation and metastasis in RCC.

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Conflict of Interest The authors declare no conflict of interest.

Fig. 5. Effects of 15d-PGJ 2 on F-Actin Morphology
Caki-2 cells were treated with 15d-PGJ 2 (3 µM) for 30 min. After 30 min culture, cells were stained with Alexa Fluor 555 Phalloidin. Representative images of control cells (A) and 3 µM 15d-PGJ 2 -treated cells (B) are shown. Scale bar = 50 µm. (Color figure can be accessed in the online version.)
interest.

Supplementary Materials The online version of this article contains supplementary materials.

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