15d-PGJ₂ Induces Apoptosis by Reactive Oxygen Species–mediated Inactivation of Akt in Leukemia and Colorectal Cancer Cells and Shows In vivo Antitumor Activity

Sung-Won Shin,¹ ² Chi-Yeon Seo,² Hoon Han,² Jin-Yeong Han,² ³ Jin-Sook Jeong,² ⁴ Jong-Young Kwak,¹ ² and Joo-In Park¹ ²

Abstract

The peroxisome proliferator-activated receptor-γ (PPARγ) is a transcription factor belonging to the nuclear receptor superfamily (1, 2) and is expressed in some myeloid leukemic cell lines (3). PPARγ agonists include the natural ligands 15-deoxy-Δ¹², ¹⁴-prostaglandin J₂ (15d-PGJ₂), a natural ligand for peroxisome proliferator–activated receptor-γ (PPARγ), inhibits cell proliferation and induces apoptosis. The specific molecular mechanisms underlying this effect remain to be elucidated. We examined whether 15d-PGJ₂ has antitumor activity in vitro and in vivo, and investigated the underlying mechanism.

Experimental Design:
We examined 15d-PGJ₂–induced apoptosis in human leukemia cells in the context of mitochondrial injury, oxidative damage, and signaling pathway disturbances. In addition, we investigated the antitumor effect of 15d-PGJ₂ in a mouse CT-26 s.c. tumor model and HL-60 leukemia xenograft model.

Results:
15d-PGJ₂ induced apoptosis in leukemia and colorectal cancer cells in a dose-dependent manner and led to generation of reactive oxygen species (ROS) through mitochondria and NADPH oxidase activation, activation of JNK, and inactivation of Akt, a serine/threonine-specific protein kinase. Constitutive activation of Akt for an engineered myristoylated protein prevented 15d-PGJ₂–mediated apoptosis but not ROS generation. Collectively, these findings suggest a hierarchical model of apoptosis induced by 15d-PGJ₂ in human leukemia cells: oxidative injury represents a primary event resulting in Akt inactivation, which in turn leads to mitochondrial injury and apoptosis. Moreover, 15d-PGJ₂ markedly reduced growth of mouse CT-26 s.c. tumors and HL-60 xenograft tumors and down-regulated p-Akt and Akt expression in vivo.

Conclusions:
These results suggest that Akt inactivation through ROS production may contribute to 15d-PGJ₂–induced apoptosis in leukemia and colorectal cancer cell lines and that 15d-PGJ₂ may have therapeutic relevance in the treatment of human leukemia and colorectal cancer.

effects than expected on the regulation of cell growth. More detailed and broader studies will be required before PPARγ ligands might be employed as anticancer drugs in leukemia and colorectal cancer patients.

Akt is a serine/threonine kinase and a target of phosphatidylinositol 3 kinase (PI3K). It has potent antiapoptotic and proliferative functions (17), and constitutive activation of the PI3K/Akt pathway in leukemic cells (18, 19) may maintain the leukemic phenotype by regulating proliferation and apoptosis. Myeloid leukemic cells exhibit not only uncontrolled proliferation and resistance to apoptosis but also a block in differentiation (20, 21). In addition, in a study of colonic tissue with anti-phosphorylated Akt (anti-p-Akt) and anti-Akt2, normal colonic mucosa and hyperplastic polyps exhibited no significant Akt expression, in contrast to colorectal adenocarcinoma, which showed increased expression of Akt in 57% of tumors. P-Akt (Ser473) was detectable in tumors but not in normal colonic epithelium (22).

A recent report showed that rosiglitazone, a synthetic ligand for PPARγ, inhibits cell growth of human lung carcinoma cells by down-regulation of Akt/mTOR/p70S6K signal cascades and up-regulation of PTEN/AMPK (23). Inhibition of the PI3K/Akt pathway is believed to underlie this effect of the PPARγ up-regulation of PTEN/AMPK (23). Inhibition of the PI3K/Akt/mTOR/p70S6K signal cascades and inactivation of Akt, a serine/threonine-specific protein kinase. In addition, administration of 15d-PGJ2 significantly reduced tumor size and down-regulated expression of p-Akt and Akt without toxicity in a CT-26 s.c. tumor model and an HL-60 xenograft model. These findings suggest consistency between in vitro and in vivo data. Based on our observations, 15d-PGJ2 has potential as an anticancer drug in the treatment of leukemia and colorectal cancer patients who exhibit p-Akt expression.

## Translational Relevance

15-Deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2), an endogenous ligand for peroxisome proliferator-activated receptor-γ and a final metabolite of PGD2, inhibits cell proliferation and induces apoptosis in several cancer cell lines. Some reports show, however, that 15d-PGJ2 has procarcinogenic effects. We found that 15d-PGJ2 induces apoptosis of leukemic and colorectal cancer cells via a caspase-dependent pathway. The effect includes generation of reactive oxygen species through mitochondria and NADPH oxidase activation, activation of JNK, negative regulation of Akt transcript levels, and inactivation of Akt, a serine/threonine-specific protein kinase. In addition, administration of 15d-PGJ2 significantly reduced tumor size and down-regulated expression of p-Akt and Akt without toxicity in a CT-26 s.c. tumor model and an HL-60 xenograft model. These findings suggest consistency between in vitro and in vivo data. Based on our observations, 15d-PGJ2 has potential as an anticancer drug in the treatment of leukemia and colorectal cancer patients who exhibit p-Akt expression.
Fig. 1. 15d-PGJ2 inhibits cell growth and induces apoptosis in leukemic and colorectal cancer cells in a dose- and time-dependent manner. A, K562, HL-60, SNU-C4, and CT-26 cells were seeded 1 d before treatment with various concentrations of 15d-PGJ2 (0, 5, 10, or 20 μM) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of viability under controlled culture conditions. These data represent the mean ± SD of three independent experiments. *, P < 0.05, versus control cells; **, P < 0.01, versus control cells. B, K562, HL-60, SNU-C4, and CT-26 cells were seeded 1 d before treatment with 15d-PGJ2 (20, 10, 20, or 20 μM) for the indicated times, respectively. After treatment for the indicated times, cell viability was determined by MTT assay and expressed as a percentage of viability under controlled culture conditions. These data represent the mean ± SD of three independent experiments. *, P < 0.05, versus control cells; **, P < 0.01, versus control cells. C, K562, HL-60, SNU-C4, and CT-26 cells were seeded 1 d before treatment with various concentrations of 15d-PGJ2 (0, 5, 10, or 20 μM) for 24 h. The percentage of apoptotic cells was determined by Annexin V-FITC/propidium iodide (PI) staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. *, P < 0.05, versus control cells; **, P < 0.01, versus control cells. D, K562, HL-60, SNU-C4, and CT-26 cells were seeded 1 d before treatment with 15d-PGJ2 (20, 10, 20, or 20 μM) for indicated times, respectively. After treatment for the indicated times, the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. *, P < 0.05, versus control cells; **, P < 0.01, versus control cells.

Transfection and establishment of stable cell lines expressing the Myr-Akt vector. SNU-C4 cells (1 × 10^6) were transfected with 6 μg of myc-tagged myristoylated Akt expression vector (Myr-Akt) or empty vector (pUSEamp, Upstate Technology) using LipofectAMINE according to the manufacturer's procedure. Dominant-negative INK1 (DN-INK1) represents a catalytically inactive dominant-negative mutant of INK1 with an NH2-terminal FLAG tag (32). Transfections with plasmids were also done using LipofectAMINE according to the manufacturer's procedures. After transfection, cells were cultured in 10% fetal bovine serum–supplemented RPMI-1640 for 24 h, then subjected to 0.1% DMSO or 15d-PGJ2 treatment for 24 h. These cells were then used for Annexin V staining, ROS production, and Western blot analysis. CT-26 cells (1 × 10^5) were transfected with 2 μg of Myr-Akt vector or empty vector (pUSEamp, Upstate Technology) using LipofectAMINE according to the manufacturer's procedures. Stable cell lines were established after G418 selection (400 μg/mL) for 14 d.

Assessment of ROS production. ROS production was monitored by flow cytometry using DCF-DA. K562 cells were treated with 15d-PGJ2 for the indicated times and washed twice with PBS to remove the extracellular compounds. DCF-DA (100 μM) was added for an additional hour. Green fluorescence was excited using an argon laser and was detected using a 525-nm band-pass filter by flow cytometric analysis.

Establishment of mouse CT-26 s.c. tumors and HL-60 leukemia xenograft in a nude mouse and experimental design. All animal
Fig. 2. Treatment of K562 cells with 15d-PGJ2 leads to inhibition of Akt phosphorylation and expression. A, left panel, K562 cells were treated with 15d-PGJ2 (5, 10, or 20 μmol/L) for 24 h. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against Akt, p-Akt, Gsk-3β, and p-Gsk-3β. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. Right panel, SNU-C4 and CT-26 cells were treated with 15d-PGJ2 (20 μmol/L) for 12 h. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against p-Akt and Akt. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. B, K562 cells were treated with 15d-PGJ2 (20 μmol/L) for the indicated times. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against p-Akt, Akt, procaspase-8, procaspase-9, procaspase-3, cleaved caspase-3, and PARP. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. C, K562 cells (1 x 10^5 cells/well) were incubated in the presence of 15d-PGJ2 (20 μmol/L) for the indicated times. After treatment for the indicated times, the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. In a separate experiment, cells were stained with DiOC6 and reduction in \( \Delta \varphi_m \) was determined by monitoring uptake of DiOC6 using flow cytometry as described in Materials and Methods. “Low \( \Delta \varphi_m \)” values are expressed as the percentage of cells exhibiting a diminished mitochondrial membrane potential. The values obtained from DiOC6 assays represent the mean ± SD of three independent experiments. D, upper panel, cells were treated with several caspase inhibitors for 1 h before treatment with 15d-PGJ2 (20 μmol/L) for 24 h. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. *, \( P < 0.05 \), versus 15d-PGJ2–treated cells. Lower panel, cells were pretreated with several caspase inhibitors for 1 h before treatment with 15d-PGJ2 (20 μmol/L) for 24 h. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against p-Akt, Akt, and PARP. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments.
procedures and care were approved by the Institutional Animal Care and Usage Committee of Dong-A University. To determine the in vivo activity of 15d-PGJ$_2$, viable CT-26 cells ($2 \times 10^7/100 \mu$L PBS per mouse) and HL-60 cells ($2 \times 10^7/100 \mu$L PBS per mouse), as confirmed by trypan blue staining, were injected into the right flank of 6- to 7-week-old female Balb/c mice and Balb/c nude mice, respectively (Orient Bio Inc.). When average s.c. tumor volume reached 60 to 100 mm$^3$, the mice were assigned into two treatment groups: (a) control (vehicle only) and (b) 15d-PGJ$_2$ given at a dose of 5 mg/kg via tail vein every 3 d. Control groups were treated with vehicle. Tumor size was measured daily with a caliper (calculated volume = shortest diameter$^2 \times$ longest diameter / 2). The mice were followed for tumor size and body weight and were sacrificed on the 14th day. Tumors were resected, weighed, and frozen or fixed in formalin and paraffin-embedded for immunohistochemical studies.

Histology and immunohistochemical analysis. At the termination of experiments, tumor tissues were removed for histologic examination. Sections of tumor tissues were stained with H&E, and immunohistochemistry was done with the DAKO EnVision Kit (DakoCytomation). Briefly, tissue sections 4 μm in thickness were dewaxed and rehydrated at graded alcohols. Endogenous peroxidase was blocked by dipping sections in 3% aqueous hydrogen peroxide for 10 min. Antigen retrieval was achieved for 10 min in a microwave by treatment with 10 mmol/L citrate buffer (pH 6.0). Primary antibodies against p-Akt and Akt (1:50 dilution, respectively) were applied for 1 h at room temperature. Next, the sections were incubated with EnVision reagent (DakoCytomation), which is a peroxidase-conjugated polymer backbone, carrying secondary antibody molecules. The sections were lightly counterstained with hematoxylin. After washing with PBS, VectorShield (Vector Laboratories) mounting medium was applied, and sections were coverslipped and imaged by ScanScope (Aperio Technologies, Inc.).

Statistical analysis. Statistical analyses were done with the SPSS 14.0 statistical package for Windows (SPSS). Data are expressed as mean values ± SD. One-way ANOVA was applied to determine whether there were significant differences in cell viability between 15d-PGJ$_2$-treated and control cells. Differences in tumor volumes between treated and control groups were evaluated using the unpaired Student’s t test. Statistical significance was defined as $P < 0.05$.

Results

15d-PGJ$_2$ induces apoptosis of leukemic and colorectal cancer cells. To evaluate the effect of the natural ligand for PPARγ, 15d-PGJ$_2$, on the growth of K562 cells, cells were treated with 15d-PGJ$_2$ (0, 5, 10, or 20 μmol/L) for 24, 48, or 72 hours. Cell viability was determined by MTT assay. As shown in Fig. 1A and B, 15d-PGJ$_2$ significantly inhibited the growth of K562 cells in a dose- and time-dependent manner.

To investigate whether 15d-PGJ$_2$ induces apoptosis, K562 cells were treated with various concentrations of 15d-PGJ$_2$ for a range of times and costained with Annexin V conjugated with FITC, which marks apoptotic cells, and propidium iodide as a general cell marker. 15d-PGJ$_2$ treatment resulted in dose- and time-dependent increases in the proportions of apoptotic cells (Fig. 1C and D). To evaluate whether the induction of apoptosis by 15d-PGJ$_2$ is specific to K562 cells or a more general effect, the same experiment was done in HL-60, SNU-C4, and CT-26...
Fig. 4. Exposure of K562 cells to 15d-PGJ2 induces the generation of ROS through mitochondria and NADPH oxidase. A, K562 cells were incubated with 15d-PGJ2 (20 μmol/L) or CAT10410 (20 μmol/L) in the presence or absence of catalase (300 U/mL) for various intervals as indicated, after which they were labeled with an oxidative-sensitive dye (DCF-DA) and analyzed by flow cytometry. B, left panel, K562 cells were treated with 15d-PGJ2 for 24 h as described above in the absence or presence of catalase, EUK134, electron transport chain inhibitors (rotenone, antimycin A), or NADPH oxidase inhibitor (apocynin). At the end of the incubation period, the percentage of cells exhibiting apoptosis was determined as described in Materials and Methods. Values represent the mean ± SD of three separate experiments. *, P < 0.05, significantly less than values obtained for cells treated with 15d-PGJ2 in the absence of catalase, EUK134, rotenone, antimycin A, or apocynin. Right panel, K562 cells were treated with 15d-PGJ2 for 3 h as described above in the absence or presence of catalase, EUK134, electron transport chain inhibitors (rotenone, antimycin A), or NADPH oxidase inhibitor (apocynin), after which they were labeled with an oxidant-sensitive dye (DCF-DA) and analyzed by flow cytometry. **, P < 0.01, significantly less than values obtained for cells treated with 15d-PGJ2, the absence of catalase, EUK134, rotenone, antimycin A, or apocynin. C, in parallel, whole lysates from K562 cells incubated with 15d-PGJ2 for 24 h in the presence or absence of catalase were prepared and analyzed by Western blot as described. In each case, 30 μg of protein were separated by SDS-PAGE, after which blots were probed with the corresponding antibodies. Blots were subsequently stripped and reprobed with antibodies directed against β-actin to ensure equivalent loading and transfer. K562 cells were treated with 15d-PGJ2 for 12 h in the presence or absence of catalase. Total RNA was extracted and reverse transcription-PCR was done with primers specific to mRNA for each Akt isoform. Reverse transcription-PCR products for Akt-1 (383 bp), Akt-2 (276 bp), or GAPDH (203 bp) were separated by electrophoresis on a 1.5% agarose gel. The data shown are representative of three separate experiments. D, SNU-C4 cells were transfected with constructs encoding constitutively active forms of Akt or an empty vector. Cells were treated with 20 μmol/L 15d-PGJ2 for 3 h, after which ROS production was analyzed using flow cytometry as described in Materials and Methods.
cells. We also observed the growth inhibition and induction of apoptosis by 15d-PGJ₂ in these additional cell lines (Fig. 1A-D).

15d-PGJ₂ down-regulates Akt and p-Akt expression in leukemic and colorectal cancer cells. The PI3K/Akt signaling pathway has been implicated in the regulation of cell cycle progression and cell proliferation. In certain cell types (e.g., human embryonic kidney 293 cells and intestinal epithelial cells), rosiglitazone blocks this pathway (33, 34). To investigate the effect of 15d-PGJ₂ on Akt expression and phosphorylation, K562 cells were treated with 5, 10, or 20 μmol/L 15d-PGJ₂ for 24 hours.
Following treatment, whole cell extracts were analyzed by Western blot. As shown in Fig. 2A, after treatment with 20 μmol/L PGJ2 for 24 hours in K562 cells, the levels of Akt and p-Akt proteins were dramatically diminished. The down-regulation of p-Akt and Akt expression by 15d-PGJ2 treatment was also observed in SNU-C4 and CT-26 cells (Fig. 2A).

Enhanced Akt dephosphorylation in cells exposed to 15d-PGJ2 could be explained by reductions in total Akt protein levels. A more detailed time-course study revealed that down-regulation of p-Akt expression occurred as early as 3 or 6 hours following treatment (Fig. 2B), whereas an increase of apoptotic cells, loss of Δψm, activation of caspases, and cleavage of PARP were not detected until the 24-hour time point (Fig. 2B and C), suggesting that factors other than caspase activation might cause Akt inactivation.

Because caspase-3 has been reported to degrade Akt, we sought to characterize the hierarchy of cellular events induced by treatment with 15d-PGJ2. K562 cells were exposed to 20 μmol/L 15d-PGJ2 for 12 hours in the presence or absence of caspase-3 inhibitor Z-DEVD-FMK (50 μmol/L), caspase-8 inhibitor Z-IETD-FMK (20 μmol/L), caspase-9 inhibitor Z-LEHD-FMK (20 μmol/L), and pan-caspase inhibitor Z-VAD-FMK (25 μmol/L), and the levels of Akt and p-Akt were monitored. As shown in Fig. 2D, expression of Akt was partially recovered, but the reduction in p-Akt was not reversed by caspase-3 inhibitor, caspase-8 inhibitor, caspase-9 inhibitor, or pan-caspase inhibitor, suggesting that the down-regulation of p-Akt by treatment with 15d-PGJ2 occurred upstream of caspase activation, but that the reduction of Akt represented in part a consequence of caspase activation.

To investigate whether treatment with 15d-PGJ2 affects the expression of Akt mRNA, a reverse transcription-PCR experiment was done. Treatment with 15d-PGJ2 for 12 hours inhibited the expression of Akt1 and Akt2 mRNA (Supplementary Fig. S1A). The down-regulation of Akt1 and Akt2 mRNA by 15d-PGJ2 treatment was not reversed by pan-caspase inhibitor (Supplementary Fig. S1B), indicating that the down-regulation of Akt mRNA by 15d-PGJ2 occurred upstream of caspase activation.

To explore the functional significance of Akt inactivation in 15d-PGJ2-mediated cell death, SNU-C4 cells were transiently transfected with a constitutively active myristoylated Akt expression construct, Myr-Akt, and compared with those transfected with the parental vector (pUSEamp). Myr-Akt expression was confirmed by Western blot analysis using antibodies against p-Akt and myc (Fig. 3A). As shown in Fig. 3B, p-Akt levels were not reduced in Myr-Akt–transfected cells exposed to 15d-PGJ2. The extent of apoptosis was monitored in cells exposed to 15d-PGJ2. Expression of constitutively active Akt protected cells from apoptosis induced by 15d-PGJ2 at 24 hours (Fig. 3C). These findings indicate that down-regulation of Akt plays a functional role in the death of leukemia and colorectal cancer cells resulting from treatment with 15d-PGJ2.

Generation of ROS through mitochondria and NADPH oxidase activation in 15d-PGJ2–induced apoptosis. The generation of ROS represents a primary event in the cell death induced by 15d-PGJ2 (24). We measured ROS levels in K562 cells exposed to 15d-PGJ2. 15d-PGJ2 induced time-dependent production of ROS in K562 cells beginning at the 3-hour time point (Fig. 4A). NADPH oxidase and mitochondria are two major sources of ROS (35): in mitochondria, ROS are generated because of incomplete reduction of oxygen during normal oxidative phosphorylation (36). To determine whether ROS generation by 15d-PGJ2 involves the mitochondrial electron transport chain, electron transport chain inhibitors such as rotenone or antimycin A were tested for their effects on 15d-PGJ2–induced ROS production and apoptosis. Figure 4B shows that rotenone (complex I inhibitor) protected the cells from 15d-PGJ2–induced apoptosis and prevented ROS production by 15d-PGJ2. The complex III inhibitor antimycin A also protected cells and kept ROS levels low.

The next set of experiments was designed to test whether NADPH oxidase is also responsible for the ROS increase generated by 15d-PGJ2. Cytosolic NADPH oxidase was tested as a possible 15d-PGJ2 target using a specific inhibitor, apocyinin. In these experiments, ROS and apoptosis were measured by flow cytometry. As shown in Fig. 4B, apocyinin inhibited 15d-PGJ2–induced ROS production and apoptosis. Taken together, these findings suggest that mitochondria and NADPH oxidase are important sources of ROS by 15d-PGJ2 treatment. However, the detailed mechanisms for ROS production by 15d-PGJ2 are not clear, and further studies to clarify this mechanism are needed.

To investigate a functional relationship with cell death, K562 cells were exposed to 15d-PGJ2 in the absence or presence of catalase or EUK134 (SOD/catalase mimetic). As shown in Fig. 4A and B, coexpression of cells to catalase or EUK134 blocked 15d-PGJ2–induced ROS production and abolished apoptosis (Fig. 4B). Moreover, cells treated with 15d-PGJ2 plus catalase or apocyinin displayed a marked reduction in the activation of caspase-3, -8, and -9 in PARP degradation.

Fig. 5. Treatment of K562 cells with 15d-PGJ2 leads to activation of JNK. A, K562 cells were treated with 15d-PGJ2 (20 μmol/L) for the indicated times. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against JNK and p-JNK. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. B, cells were pretreated with catalase for 1 h before treatment with 15d-PGJ2 (20 μmol/L) for 24 h. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against JNK and p-JNK. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. C, left panel, K562 cells (1 × 105 cells/well) were pretreated with SP600125 before treatment with 15d-PGJ2 (20 μmol/L) for 24 h. After treatment for the indicated times, the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. *, P < 0.05, significantly less than values obtained for cells treated with 15d-PGJ2 in the absence of SP600125. Right panel, after treatment, protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against JNK, p-JNK, PARP, Akt, p-Akt, procaspase-3, and cleaved caspase-3. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments. D, left panel, SNU-C4 cells were transiently transfected with empty vector (pcDNA3.1) or DN-JNK. The culture medium was changed, and the cells were treated with or without 15d-PGJ2 (20 μmol/L) for 24 h. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining as described in Materials and Methods. These data represent the mean ± SD of three independent experiments. *, P < 0.01 (SNU-C4-DN-JNK cells treated with 15d-PGJ2, versus SNU-C4-pcDNA3.1) or DN-JNK. The culture medium was changed, and the cells were treated with or without 15d-PGJ2 (20 μmol/L) for 24 h. Protein lysates were prepared and subjected to Western blot analysis as described in Materials and Methods using antibodies against JNK, p-JNK, Akt, and p-Akt. Equal protein loading was ensured by showing uniform β-actin expression. The blot is representative of three separate experiments.
(Supplementary Fig. S2A and B). Pretreatment with catalase reversed the down-regulation of p-Akt protein, Akt protein, and Akt mRNA (Fig. 4C). In addition, the PPARgamma agonist CAY10410, a structural analog of 15d-PGJ2 lacking the electrophilic carbon of the 15d-PGJ2 cyclopenteno ring, did not induce apoptosis (Supplementary Fig. S3A) or trigger production of ROS (Fig. 4A). To confirm the relationship between ROS production and down-regulation of Akt, we measured both p-Akt and Akt expression under CAY10410 treatment. As predicted, expression of p-Akt and Akt was not down-regulated by CAY10421, in which there is no production of ROS (Supplementary Fig. S3). We also observed that H2O2 treatment resulted in induction of apoptosis through down-regulation of p-Akt and Akt (Supplementary Fig. S3B and C).

Finally, to investigate the hierarchy of events accompanying 15d-PGJ2-induced cell death, ROS generation was monitored in SNU-C4 cells expressing activated Akt. As shown in Fig. 4D, constitutive activation of Akt, which protected cells from 15d-PGJ2−induced apoptosis, had little effect on 15d-PGJ2−induced ROS production. These findings provide further support for a model in which 15d-PGJ2−mediated oxidative injury represents a primary cause, rather than a consequence, of Akt inactivation. Taken together, these results indicate that the generation of ROS in response to 15d-PGJ2 plays a critical role in 15d-PGJ2−induced apoptosis through the down-regulation of Akt and p-Akt.

15d-PGJ2 induces activation of JNK and inactivation of p-Akt as a downstream event of ROS production. To investigate whether 15d-PGJ2−induced ROS leads to the activation of JNK in K562 cells, we determined the phosphorylation state of JNK in K562 cells treated with 15d-PGJ2. As shown in Fig. 5A, 15d-PGJ2 treatment for 3 hours significantly increased the phosphorylation of JNK. Furthermore, pretreatment with catalase for 1 hour could prevent the phosphorylation of JNK caused by 15d-PGJ2 (Fig. SB). These results indicate that ROS production induced by 15d-PGJ2 contributes to the activation of JNK.

Increasing evidence shows that JNK plays an important role in apoptosis by directly initiating mitochondrial death pathways (37) or indirectly inhibiting Akt antiapoptotic signaling pathways (38). In the present study, K562 cells were pretreated with SP600125 (25 μmol/L; an inhibitor of JNK) for 1 hour and then treated with 15d-PGJ2. After treatment, the percentage of Annexin V−positive cells was determined by flow cytometry. As shown in Fig. 5C, SP600125 pretreatment partially blocked 15d-PGJ2−induced apoptosis and reversed down-regulation of p-Akt by 15d-PGJ2. In addition, DN-JNK−transfected SNU-C4 cells treated with 15d-PGJ2 exhibited less apoptosis compared with empty vector−transfected cells treated with 15d-PGJ2 (Fig. 5D). Taken together, these results suggest that ROS−mediated JNK activation may in part contribute to apoptosis through down-regulation of p-Akt.

Antitumor activity and down-regulation of p-Akt and Akt by 15d-PGJ2 in vivo. We observed the 15d-PGJ2−induced apoptosis and the down-regulation of p-Akt and Akt in CT-26 cells as well as in human leukemia cells. Thus, we evaluated the ability of 15d-PGJ2 to inhibit tumor growth of cancer cells in a mouse CT-26 s.c. tumor model, a model that has been extensively used to facilitate development of several new treatment modalities. CT-26 cells injected s.c. into the flank of Balb/c mice rapidly gave rise to exponentially growing tumors. As can be seen in Fig. 6A, 15d-PGJ2 significantly decreased tumor growth. After 14 days, the mean volume of tumors in mice treated with 15d-PGJ2 was more than 70% smaller than the tumors in the vehicle-treated mice (control group mean, 1,721.03 ± 583.69 mm3; 15d-PGJ2 group mean, 509.36 ± 189.91 mm3; P < 0.05). Mice treated with 15d-PGJ2 did not show signs of wasting, and body weights after 14 days were not significantly different from controls (data not shown).

In addition, 15d-PGJ2 markedly suppressed the growth of HL-60 leukemic xenografts. As shown in Fig. 6A, the mean volume of HL-60 tumors in the mice that received 15d-PGJ2 (758.83 ± 159.18 mm3) was significantly decreased compared with control mice (4,165.64 ± 680.74 mm3; P < 0.01). The tumors from control mice showed the typical histologic appearance of colorectal cancer cells and leukemia cells, respectively (Fig. 6B). The sections of 15d-PGJ2−treated CT-26 s.c. tumors showed that cancer cells were markedly decreased, with signs of necrosis with infiltration of inflammatory cells and fibrosis. HL-60 xenografts from mice treated with 15d-PGJ2 showed apoptotic regions and extensive necrosis with infiltration of phagocytic cells (Fig. 6B).

As expected, tumors from vehicle-treated control mice stained strongly for p-Akt and Akt (Fig. 6C). In contrast, immunohistochemical analysis of tumors from 15d-PGJ2−treated mice revealed down-regulation of p-Akt and Akt (Fig. 6C). These data are consistent with the in vitro findings.

We also assessed the toxicity of 15d-PGJ2 in Balb/c mice by checking the level of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. The dosage that we used in this experiment was safe based on the results for blood chemistry (data not shown). In addition, to confirm the role of Akt inactivation in the 15d-PGJ2−induced antitumor effect, the...
stable CT-26 cell lines expressing Myr-Akt or the empty vector were injected s.c. into the flanks of Balb/c mice. Stable cell lines expressing Myr-Akt gave rise to larger tumors compared with those expressing the empty vector (Supplementary Fig. S4). As can be seen in Fig. 6D, 15d-PGJ2 did not decrease tumor growth in mice expressing Myr-Akt; however, 15d-PGJ2 significantly decreased tumor growth in mice expressing the empty vector. These data suggest that reduction in Akt activity parallels the antineoplastic activity of 15d-PGJ2 and that these effects on Akt activity may contribute to lethality of human leukemia cells.

A recent study showed that 15d-PGJ2 induces apoptosis in K562 cells and mediates ROS production (24). Little is known, however, about the functional role of the Akt pathway in mediating 15d-PGJ2–induced lethality in human leukemia cells. The results of the present study show that Akt inactivation is a consequence of 15d-PGJ2–mediated ROS production and makes a key functional contribution to lethality of human leukemia cells.

Akt is activated in response to many growth factors (17). It inhibits apoptosis through multiple pathways (40) that induce direct phosphorylation and inactivation of proapoptotic proteins, such as BCL2-antagonist of cell death, or procaspase-9; increased expression of c-IAPs, c-FLIPr, Bcl-2, Mcl-1; and down-regulation of proapoptotic proteins (17, 41). Activation of Akt occurs in response to oxidative damage (25), which generally involves PTEN inactivation and results in an attenuation of lethality. The present findings suggest that the relationship between 15d-PGJ2–mediated oxidative injury and the effects on Akt activity differ from those of a previous report showing that ROS activate Akt (25). Most notably, in this study 15d-PGJ2 exposure resulted in diminished, rather than increased, Akt phosphorylation. This current finding is consistent with that of a previous study showing that 15d-PGJ2 induces down-regulation of p-Akt and apoptosis in leukemia (42).

It should also be noted that 15d-PGJ2 treatment reduced overall expression of Akt, an action that is partially reversed by coadministration of caspase-3 inhibitor. This result raises the possibility that Akt inactivation might represent a consequence of engagement of the caspase cascade, and the caspase-dependent down-regulation of Akt is a well-described phenom. However, caspase inhibitors did not restore normal levels of p-Akt in 15d-PGJ2–treated cells in this study. This outcome indicated that factors other than caspase-mediated events are involved. To investigate whether 15d-PGJ2 down-regulates steady-state levels of Akt protein at the level of Akt transcript expression, we used reverse transcription-PCR to assess Akt mRNA levels. 15d-PGJ2 treatment decreased expression of both Akt1 and Akt2 mRNAs in K562 cells, and pretreatment with a caspase inhibitor did not reverse this down-regulation. Thus, 15d-PGJ2 seems to down-regulate expression of Akt1 and Akt2 mRNA and may regulate Akt promoter activity, Akt RNA stability, or both.

Here we have presented a body of evidence that 15d-PGJ2–induced Akt inactivation plays a critical functional role in 15d-PGJ2–induced apoptosis. Constitutive activation of Akt largely reversed caspase activation, PARP cleavage, and apoptosis induced by 15d-PGJ2. On the other hand, constitutive activation of Akt did not block 15d-PGJ2–induced increases in ROS generation, eliminating the possibility that Akt prevents or attenuates 15d-PGJ2–mediated oxidative injury.

ROS play critical roles in the regulation of proliferation, apoptosis, and cellular transformation (25–27). It has recently been reported that generation of ROS is intimately involved in 15d-PGJ2–induced apoptosis (24); however, how 15d-PGJ2 induces ROS generation remains unknown. In this study, we used antioxidant catalase or EUK134 to investigate the involvement of ROS in 15d-PGJ2–mediated apoptosis and showed that mitochondria and NADPH oxidase are involved in ROS production by 15d-PGJ2. Several lines of evidence suggest that ROS play essential roles in 15d-PGJ2–mediated apoptosis in K562 cells. Catalase abrogates 15d-PGJ2–induced production of ROS in this cell line and significantly inhibits 15d-PGJ2–induced apoptosis. In addition, catalase blocks PARP cleavage resulting from 15d-PGJ2 treatment and prevents inactivation of Akt and the down-regulation of Akt mRNA and protein levels. Together, these data indicate that Akt activity represents a critical signaling node operating downstream of 15d-PGJ2–induced oxidative injury, which triggers mitochondrial injury and engagement of the apoptotic caspase cascade. Future studies will be aimed at defining the mechanisms by which ROS lead to a down-regulation of Akt RNA levels.

A previous study suggested that activation of JNK through ROS generation is important for 15d-PGJ2–induced apoptosis (43). To investigate the role of JNK activation in 15d-PGJ2–induced apoptosis, we used Western blot analysis to examine the expression of p-JNK after treatment with 15d-PGJ2. 15d-PGJ2 treatment resulted in activation of JNK, and SP600125 pretreatment and transfection of DN-JNK partially inhibited 15d-PGJ2–induced apoptosis. In addition, catalase blocks PARP cleavage resulting from 15d-PGJ2 treatment and prevents inactivation of Akt and the down-regulation of Akt mRNA and protein levels. Together, these data indicate that Akt activity represents a critical signaling node operating downstream of 15d-PGJ2–induced oxidative injury, which triggers mitochondrial injury and engagement of the apoptotic caspase cascade. Future studies will be aimed at defining the mechanisms by which ROS lead to a down-regulation of Akt RNA levels.
tumor model and HL-60 leukemic xenograft model. CT-26 cells expressing Myr-Akt gave rise to significantly larger tumors and showed less anti-tumor effect by 15d-PGJ2. These results are consistent with the in vitro data. In addition, the lack of toxicity in a mouse model points to 15d-PGJ2 as a potential candidate for therapeutic use. These data suggest that 15d-PGJ2 can be a possible anticancer agent in treatment of leukemia and colorectal cancer.

In summary, our study shows that 15d-PGJ2 induces apoptosis of leukemia and colorectal cancer cells via a caspase-dependent pathway. The effect includes generation of ROS through mitochondrial and NADPH oxidase activation, activation of JNK, negative regulation of Akt transcript levels, and inactivation of the Akt protein. These results suggest that 15d-PGJ2 may be of therapeutic importance in the treatment of leukemia as well as colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
15d-PGJ_{2} Induces Apoptosis by Reactive Oxygen Species–mediated Inactivation of Akt in Leukemia and Colorectal Cancer Cells and Shows In vivo Antitumor Activity

Sung-Won Shin, Chi-Yeon Seo, Hoon Han, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-08-3101

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/08/17/1078-0432.CCR-08-3101.DC1

Cited articles
This article cites 44 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/17/5414.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/15/17/5414.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.