Abstract. The cyclopentenone prostaglandin 15-deoxy-Δ^{12,14}-prostaglandin J_2 (15d-PGJ_2) has been shown to possess antineoplastic activity in human cancers of various origins. However, the mechanism of the antineoplastic activity of 15d-PGJ_2 remains to be completely elucidated. It has been reported that inhibiting the expression of human telomerase reverse transcriptase (hTERT), a major determinant of telomerase activity, induces rapid apoptosis in cancer cells. In this study, we investigated the effect of 15d-PGJ_2 on hTERT expression. Treatment with 30 μM 15d-PGJ_2 for 72 h induced apoptosis in the colon cancer cells LS180. 15d-PGJ_2 treatment decreased hTERT protein expression in a dose-dependent manner. Down-regulation of hTERT expression by hTERT-specific small inhibitory RNA induced apoptosis. These results indicate that the down-regulation of hTERT expression by 15d-PGJ_2 plays an important role in its proapoptotic properties. Since 15d-PGJ_2 reduced hTERT mRNA expression, we examined the effect of 15d-PGJ_2 on the DNA-binding activity of c-Myc, specificity protein 1 (Sp1) and estrogen receptor (ER) to the hTERT gene promoter using an electrophoretic mobility shift assay. 15d-PGJ_2 attenuated the DNA-binding of all three transcriptional factors. Further, we observed that 15d-PGJ_2 inhibited the DNA binding of these factors by different mechanisms; suppressed c-Myc mRNA expression, enhanced Sp1 protein degradation via the ubiquitin-proteasome pathway and inhibited ERβ phosphorylation at serine residues. We conclude that hTERT down-regulation by 15d-PGJ_2 plays an important role in its proapoptotic properties. Furthermore, 15d-PGJ_2 inhibits the transcriptional activity of c-Myc, Sp1 and ER by three different mechanisms and results in the transcriptional repression of the hTERT gene.

Introduction

Prostaglandins (PGs) are a family of biologically active molecules having a diverse range of actions that vary according to their PG type and target cells. Within this family, the cyclopentenone prostaglandins PGA_1, PGA_2 and PGJ_2 are formed by dehydration within the cyclopentenone rings of PGE_1, PGE_2 and PGD_2, respectively. Further, PGJ_2 is isomerized to 15-deoxy-Δ^{12,14}-prostaglandin J_2 (15d-PGJ_2). Cyclopentenone prostaglandins have been shown to possess anti-inflammatory and antiviral activities (1). In particular, 15d-PGJ_2 has been shown to possess antineoplastic activity in human cancers of various origins such as those of the stomach (2), colon (3), lung (4), breast (5) and bone marrow (6). However, the mechanism of its antineoplastic activity remains to be completely elucidated.

Telomerase, a ribonucleoprotein complex, helps to stabilize telomere length by adding TTAGGG repeats to telomeres (7.8). Telomerase activity has been detected in almost all human tumors, including those of the stomach (9) and colon (10) but not in adjacent normal cells (11,12). These results suggest that cancer cells stabilize their telomeres primarily through telomerase activation.

Human telomerase comprises human telomerase RNA, telomerase-associated protein 1 and human telomerase reverse transcriptase (hTERT) (7,13). It has been reported that the ectopic expression of hTERT in normal human cells increases...
their lifespan (14), while the expression of dominant-negative hTERT in human cancer cells results in telomerase inactivation and telomere shortening (15,16). Hence, hTERT is believed to be a major determinant of telomerase activity. In addition, up-regulation of hTERT gene expression has been observed in various cancer cells (17).

Previous studies demonstrated that the inhibition of hTERT expression in cancer cells by gene-targeting techniques induced rapid apoptosis without telomere shortening (18,19). These results indicate that besides its role in telomere length maintenance in cancer cells, hTERT might play an important role in the inhibition of apoptosis.

Here, we show that 15d-PGJ2 induces apoptosis in colon cancer cells and reduces hTERT expression. Inhibition of hTERT expression using hTERT gene-specific small inhibitory RNA (siRNA) rapidly induces apoptosis in colon cancer cells. Additionally, down-regulation of hTERT expression by 15d-PGJ2 was observed to occur via attenuation of c-Myc, specificity protein 1 (Sp1) and estrogen receptor (ER)-mediated gene transcription. Therefore, down-regulation of hTERT expression may play an important role in 15d-PGJ2-induced apoptosis.

Materials and methods

Materials. 15d-PGJ2 were obtained from Biomol International LP (Plymouth Meeting, PA). Anti-Sp1 antibody, anti-c-myc antibody, anti-ERα antibody, anti-ERβ antibody and anti-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ubiquitin antibody was from Upstate (Charlottesville, VA). Anti-hTERT antibody was from Calbiochem (San Diego, CA). Anti-phosphoserine antibody was from Chemicon International (Temecula, CA).

Cell culture. The human colon cancer cell lines LS180, SW480 and HT-29 were obtained from the American Type Culture Collection (University Boulevard Manassas, VA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and grown at 37°C in a humidified atmosphere of 5% CO2.

Measurement of cell viability. The viability of colon cancer cells treated with 15d-PGJ2 (10 to 30 μM) was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, by using the cell titer 96 aqueous one solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's protocol. The amount of soluble formazan produced as a result of MTS reduction by viable cells was assessed by measuring the absorbance at 490 nm using a microtiter plate reader (SpectraFluor, Tecxan, Maennedorf, Switzerland).

Detection of apoptosis. Apoptosis induced by 15d-PGJ2 was quantified by a combined staining of Annexin V and propidium iodide (PI) using Mecryo-apoptosis kit (Medical & Biological Laboratories, Nagoya, Japan). Briefly, 72 h after treatment with 15d-PGJ2 (30 μM), cells were harvested and resuspended in 85 μl of 1X binding buffer. After adding 10 μl of annexin V-FITC solution and 5 μl of PI solution, cells were incubated for 15 min at room temperature in the dark. At the end of incubation, 5,000 cells were analyzed by flow cytometry (EPICS XL-MCL cytometer; Beckman Coulter, Tokyo, Japan) after adding 400 μl of 1X binding buffer. All experiments were performed at least three times for each experimental condition.

Detection of telomerase activity. Telomerase activity was determined by a telomeric repeat amplification protocol (TRAP) assay using a TeloTAGGG Telomerase PCR ELISA PLUS (Roche Diagnostics, Penzberg, Germany), according to the manufacturer's protocol. Telomerase activity in the sample was calculated as the ratio of the absorbance value of the sample to that of the untreated cells. All assays were performed in triplicate.

Transfection of siRNA for hTERT into colon cancer cells. An siRNA was designed to target the coding region of the hTERT gene (nucleotides 598 to 618, relative to the start codon). As the transfection control, we used non-silencing siRNA which has no homology to any known mammalian gene. The siRNA duplexes used in this study were 5'-GAA CGG GCC UGG AAC CAU AGC-3' and 5'-UAU GGU UCC AGG CCC GUU CGC-3'. Transfection of these siRNAs was performed using the HiPerFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Quantification of hTERT, Sp1, c-Myc and ERβ mRNA. The expression of hTERT, c-Myc, Sp1 and ERβ mRNA was determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously (20,21). The gene-specific primers and fluorescent hybridization probes for these genes used in quantitative RT-PCR were as follows. For hTERT, the forward primer was 5'-GGG CGC GTA CGA CAG CAT-3' and reverse primer, 5'-ATG TAC GGC TGG AGG TCT GTG A-3'; and probe, 5'-(FAM) CGG AGG TCA TGC CCA GCA TCA TC (TAMRA)-3'. For c-Myc, forward primer was 5'-TCC CTC CAC TCG GAA GGA CTA-3' and reverse primer, 5'-CTC TGG...
CGC TCC AAG ACG TT-3'; and probe, 5'-FAM CGA GGA AAA TGT CAA GAG CGC AAC ACA (TAMRA)-3'. For Sp1, forward primer was 5'-GGG CCT CCC CCA ACC TGG CCG AGG G-3'; reverse primer, 5'-GCG TCT GGA ACT GTG GGA GTA A-3'; and probe, 5'-FAM TGT TGT GGC TGC CGC TCC CA (TAMRA)-3'. For ERß, forward primer was 5'-GGG AGA CGA CAA GCC CAA AT-3'; reverse primer, 5'-CTG GCT TCA CAC CAG CAG CTA C T-3'; and probe, 5'-FAM TCT CCT TTA GTG GTC CAT CGC TAG CTA TCA (TAMRA)-3'. The amounts of these mRNAs were normalized as ratios to the amounts of 18S ribosomal RNA (rRNA) which was quantified using a TaqMan Ribosomal RNA control reagents (Applied Biosystems). PCR products were also confirmed by agarose gel electrophoresis.

**Electrophoretic mobility shift assay (EMSA).** Nuclear proteins were extracted from the cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) along with a protease inhibitor cocktail (Sigma-Aldrich). Oligonucleotide probes containing the c-Myc-binding motif of the hTERT gene promoter (5'-GGG CCT CCC CCA ACC TGG CCG AGG G-3'), Sp1-binding motif of the hTERT gene promoter (5'-TCC TTT CCG CCG CCC CCG CCT CTC TCT GCC GGA G-3') and ERß-binding motif of the hTERT gene promoter (5'-TGG TGG TCA TGG TCA TGG A G A-3') were prepared; these were 5'-end labeled with biotin from Sigma Genosys Japan (Ishikari, Japan). Detection of c-Myc-, Sp1- and ERß DNA binding was determined by competition reactions in which a 200-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Products of the binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel (Invitrogen) using 1/2X TBE buffer (Invitrogen). The protein-oligonucleotide complexes obtained were electroblotted to a nylon membrane (Invitrogen). After incubation in blocking buffer at room temperature for 15 min, the membrane was incubated with a streptavidin-horseradish peroxidase conjugate at room temperature for 15 min. The membrane was incubated with a chemiluminescent substrate (Pierce) according to the manufacturer's protocol. The specificity of c-Myc, Sp1, or ERß DNA binding was determined by competition reactions in which a 200-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Products of the binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel (Invitrogen) using 1/2X TBE buffer (Invitrogen). The protein-oligonucleotide complexes obtained were electroblotted to a nylon membrane (Invitrogen). After incubation in blocking buffer at room temperature for 15 min, the membrane was incubated with a streptavidin-horseradish peroxidase conjugate at room temperature for 15 min. The membrane was incubated with a chemiluminescent substrate for 5 min.

**Immunoprecipitation.** Nuclear proteins (350-500 μg) extracted from the cells were immuno-precipitated by incubating with 20 μl of Protein G Plus-Agarose (Santa Cruz Biotechnology) and 4 μg of rabbit anti-Sp1 antibody, anti-c-Myc antibody, or 2 μg of anti-ERß antibody in phosphate-buffered saline (PBS) overnight at 4°C. The protein-antibody-protein G complexes were pelleted at 1,000 x g and then washed four times with PBS. Immunoprecipitates were resuspended in the Tris-glycine SDS sample buffer (Invitrogen) with 0.05 M dithiothreitol and boiled for 3 min. The immunoprecipitate was then electrophoresed on a 4-20% gradient Tris-glycine gel (Invitrogen) and immunoblot analysis was performed.

**Results**

**15d-PGJ2 induces apoptosis in colon cancer cells.** 15d-PGJ2 has been reported to have a growth inhibitory effect in different types of cancers and in malignant human hematopoietic cells (2-6). To investigate the growth inhibitory effect of 15d-PGJ2 in colon cancer cells, we cultured LS180, SW480 and HT-29 cells with various concentrations of 15d-PGJ2 (10-30 μM) for up to 72 h and determined the number of viable cells by an MTS assay. Since 15d-PGJ2 was dissolved in ethanol, we treated the cells with an identical concentration of ethanol to exclude the effect of ethanol on cell growth (vehicle-treated cells). As shown in Fig. 1A, 15d-PGJ2 inhibited the growth of all three cell lines in a dose-dependent manner, while no significant growth inhibition was observed in the vehicle-treated cells.

Using annexin V-PI staining, we determined the proportion of apoptotic cells after treating the LS180 cells with 30 μM of 15d-PGJ2 for 72 h. As shown in Fig. 1B, there was no significant difference observed between the proportion of annexin V-positive cells and untreated and vehicle-treated cells. There were 75.1% more annexin V-positive cells among the 15d-PGJ2-treated cells as compared to among the vehicle-treated cells, indicating that 15d-PGJ2 induced apoptosis in the LS180 cells.

**15d-PGJ2 down-regulates hTERT expression in colon cancer cells.** Elevated hTERT expression has been reported in cancer cells (17) and reduced hTERT protein expression by gene-targeting techniques resulted in the induction of apoptosis (18,19). To identify the possible molecular mechanism underlying 15d-PGJ2-induced apoptosis, we examined the expression level of the hTERT protein in the LS180 cells treated with 10-30 μM of 15d-PGJ2 for 48 h by immunoblot analysis. As shown in Fig. 2, 15d-PGJ2 attenuated hTERT protein expression in a dose-dependent manner; the protein expression in the cells treated with 30 μM of 15d-PGJ2 decreased to <50% in the vehicle-treated cells.

Since hTERT is a major determinant of telomerase activity, we examined whether 15d-PGJ2 decreased telomerase activity by down-regulating hTERT protein expression. Telomerase activity in the LS180 cells treated with 10-30 μM of 15d-PGJ2 for 48 h was measured using the TRAP assay. As shown in Table I, no significant reduction in telomerase activity was observed in the vehicle-treated cells. However, 15d-PGJ2 decreased telomerase activity in a dose-dependent manner.

**Inhibition of hTERT protein expression induces apoptosis in colon cancer cells.** To investigate the contribution of hTERT down-regulation to 15d-PGJ2-induced apoptosis, we inhibited hTERT protein expression by introducing siRNA targeting the hTERT gene (hTERT-siRNA) in LS180 cells; we then determined the change in the proportion of apoptotic cells. Fig. 3A shows hTERT protein expression as detected by immunoblot analysis in hTERT-siRNA-transfected cells. The reduced hTERT-siRNA attenuated hTERT protein expression at 6 days after transfection. No reduction in the level of hTERT protein was observed in cells transfected with non-silencing siRNA, which has no homology to any known mammalian gene.

Next, we determined the proportion of apoptotic cells in hTERT-siRNA-transfected cells at 6 days after transfection by annexin V-PI staining (Fig. 3B). When compared with the
untreated cells, the introduced hTERT-siRNA increased the proportion of annexin V-positive cells; we observed a 26.9% increase in the proportion of apoptotic cells. In contrast, non-silencing siRNA-transfected cells did not show a significant change in the proportion of annexin V-positive cells. These results indicate that hTERT down-regulation resulted in the induction of apoptosis.

Down-regulation of hTERT expression by 15d-PGJ₂ occurs at the transcription level. To clarify whether 15d-PGJ₂-induced down-regulation of hTERT expression at the transcriptional level, we examined hTERT mRNA expression in the LS180 cells treated with 30 μM of 15d-PGJ₂ for 48 h using RT-PCR (Fig. 4A). hTERT mRNA was also quantified by quantitative RT-PCR (Fig. 4B). hTERT mRNA expression in the 15d-PGJ₂-treated cells decreased in a dose-dependent manner; it decreased to undetectable levels on treatment with 30 μM of 15d-PGJ₂.

15d-PGJ₂ inhibits the binding of Sp1, c-Myc and ER to the hTERT gene promoter. Previous studies demonstrated that the transcription factors c-Myc and Sp1 were required for basal transcription from the hTERT gene promoter (22). In addition, ER enhances the transcription of the hTERT gene (23). Therefore, we investigated the DNA binding of c-Myc, Sp1 and ER to the hTERT gene promoter in the LS180 cells treated with 30 μM 15d-PGJ₂ for 48 h by using EMSA. In this assay, we used three types of biotinated oligonucleotides, namely, one containing the c-Myc-, one containing the Sp1- and one containing the ER-binding motif. Each oligonucleotide encompassed nucleotide positions from -173 to -152, -21 to +14, or -2682 to -2661 relative to the hTERT translation initiation site. As shown in Fig. 5, the production of c-Myc-,
Sp1- and ER-oligonucleotide complexes was inhibited in the 15d-PGJ2-treated cells. These results indicate that 15d-PGJ2 down-regulates hTERT protein expression by inducing transcriptional repression of the hTERT gene. 

Reduced c-Myc, Sp1 and ER protein expression appear to be one mechanism by which 15d-PGJ2 inhibits DNA binding of these three transcriptional factors to the hTERT gene promoter. We then determined the expression of c-Myc, Sp1 and ER in the LS180 cells treated with 30 μM of 15d-PGJ2 for 48 h by an immunoblot analysis. As shown in Fig. 6A, the expression levels of both c-Myc and Sp1 proteins in 15d-PGJ2-treated cells were reduced as compared to their expression levels in untreated or vehicle-treated cells.

Table I. Telomerase activity in 15d-PGJ2-treated cells.

<table>
<thead>
<tr>
<th>15d-PGJ2 (μM)</th>
<th>Relative telomerase activity (%)a</th>
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<tr>
<td>Vehicleb</td>
<td>97.8±0.0</td>
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<tr>
<td>10</td>
<td>80.0±5.0</td>
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<tr>
<td>20</td>
<td>58.5±1.6</td>
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<tr>
<td>30</td>
<td>27.6±5.3</td>
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Table: Relative telomerase activity in 15d-PGJ2-treated cells. Data represent the percentage of telomerase activity in untreated cells. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ2-treated cells.

Sp1- and ER-oligonucleotide complexes was inhibited in the 15d-PGJ2-treated cells. These results indicate that 15d-PGJ2 down-regulates hTERT protein expression by inducing transcriptional repression of the hTERT gene.

Sp1 and c-Myc protein expression is reduced by 15d-PGJ2. Decreased c-Myc, Sp1 and ER protein expression appear to be one mechanism by which 15d-PGJ2 inhibits DNA binding of these three transcriptional factors to the hTERT gene promoter. We then determined the expression of c-Myc, Sp1 and ER in the LS180 cells treated with 30 μM of 15d-PGJ2 for 48 h by an immunoblot analysis. As shown in Fig. 6A, the expression levels of both c-Myc and Sp1 proteins in 15d-PGJ2-treated cells were reduced as compared to their expression levels in untreated or vehicle-treated cells.
ER has two isoforms, i.e., ERα and ERβ. We attempted to identify the ER isoform expressed by the LS180 cells prior to studying the effect of 15d-PGJ2 on ER protein expression (Fig. 6B). Although the breast cancer cells MCF-7 expressed both ERα and ERβ proteins, the LS180 cells expressed only the ERβ protein. The SW480 cells and HT-29 cells also expressed only the ERβ protein (data not shown). We then determined whether 15d-PGJ2 down-regulated ERβ protein expression similar to the manner in which it down-regulated Sp1 and c-Myc protein expression. As shown in Fig. 6C, no reduction in the level of ERβ protein was observed in the 15d-PGJ2-treated cells.

Qin et al demonstrated that 15d-PGJ2 induced the down-regulation of ERα expression in MCF-7 cells (24). In order to confirm that 15d-PGJ2 fails to reduce ERβ protein expression, we investigated the expression of both ERα and ERβ proteins in the LS180 cells expressed only the ERβ protein. The SW480 cells and HT-29 cells also expressed only the ERβ protein (data not shown). We then determined whether 15d-PGJ2 down-regulated ERβ protein expression similar to the manner in which it down-regulated Sp1 and c-Myc protein expression. As shown in Fig. 6D, no reduction in the level of ERβ protein was observed in the 15d-PGJ2-treated cells.

Qin et al demonstrated that 15d-PGJ2 induced the down-regulation of ERα expression in MCF-7 cells (24). In order to confirm that 15d-PGJ2 fails to reduce ERβ protein expression, we investigated the expression of both ERα and ERβ proteins in the LS180 cells expressed only the ERβ protein. The SW480 cells and HT-29 cells also expressed only the ERβ protein (data not shown). We then determined whether 15d-PGJ2 down-regulated ERβ protein expression similar to the manner in which it down-regulated Sp1 and c-Myc protein expression. As shown in Fig. 6C, no reduction in the level of ERβ protein was observed in the 15d-PGJ2-treated cells.

These results indicate that the mechanism by which 15d-PGJ2 inhibits Sp1 and c-Myc protein binding to the hTERT gene promoter is by reducing the expression of these two proteins. In the case of ERβ, it is not the down-regulation of protein expression but protein modification that may block the DNA-binding of ERβ to the hTERT gene promoter.

15d-PGJ2 suppresses c-Myc mRNA expression. To explore the mechanism by which 15d-PGJ2 reduces the expression of the c-Myc and Sp1 proteins, we determined the level of c-Myc and Sp1 mRNA expression in the LS180 cells treated with 30 μM of 15d-PGJ2 for 48 h. The level of c-Myc mRNA expression was markedly decreased as compared to that in the untreated or vehicle-treated cells, while the level of Sp1 mRNA expression remained the same. In contrast, ERβ mRNA expression was up-regulated in the 15d-PGJ2-treated cells. According to these results, down-regulation of c-Myc protein expression by 15d-PGJ2 may be mediated by the suppression of c-Myc mRNA expression.

Ubiquitination of Sp1 is enhanced by 15d-PGJ2. Since 15d-PGJ2 down-regulated Sp1 protein expression without suppressing Sp1 mRNA expression, we hypothesized that 15d-PGJ2 enhanced Sp1 degradation via the ubiquitin-proteasome pathway. We then investigated the ubiquitination of Sp1 in 15d-PGJ2-treated cells. In the initial study, we treated LS180 cells with several proteasome inhibitors, including MG132 and lactacystin in addition to 15d-PGJ2, to detect the levels of ubiquitinated Sp1 protein. However, combination treatment using these proteasome inhibitors and 15d-PGJ2 resulted in severe cytotoxicity. Therefore, we extracted Sp1 protein from the untreated cells, vehicle-treated cells, or 15d-PGJ2-treated cells by immunoprecipitation using the anti-Sp1 antibody. After adjustment of the samples to ensure that they contained an equal amount of Sp1 protein, ubiquitinated Sp1 protein expression was analyzed by immunoblot analysis using the anti-ubiquitin antibody. As illustrated in Fig. 8, the intensity of the ubiquitinated Sp1 band increased in 15d-PGJ2-treated cells. In addition, 15d-PGJ2-treated cells showed an additional band, indicating polyubiquitination of Sp1. On the other hand, 15d-PGJ2 showed no effect on c-Myc ubiquitination. These results
indicate that 15d-PGJ2 down-regulates Sp1 protein by enhancing the ubiquitin-proteasome pathway-dependent degradation of the Sp1 protein.

Although the level of ERß protein expression was not reduced in 15d-PGJ2-treated cells, the intensity of the ubiquitinated ERß band was increased by 15d-PGJ2 and also Sp1. Since ERß mRNA expression is up-regulated by 15d-PGJ2, the level of newly synthesized ERß protein may be increased. Thus, 15d-PGJ2 appeared to have no influence on ERß protein expression.

Phosphorylation of ERß at serine residues is inhibited by 15d-PGJ2. We then investigated the mechanism by which 15d-PGJ2 inhibited the DNA-binding of ERß to the hTERT gene promoter without inhibiting its protein expression. Tremblay et al showed that the phosphorylation of ERß at serine 106 and serine 124 in the activation function 1 (AF-1) domain located in the NH2-terminal A/B region is critical for transcriptional activation (25). Therefore, we examined the effect of 15d-PGJ2 on the phosphorylation of ERß at serine residues. The ERß protein was immunoprecipitated from nuclear extracts of the LS180 cells treated with 30 μM of 15d-PGJ2 for 48 h by using the anti-ERß antibody. The ERß protein immunoprecipitates were analyzed by immunoblot analysis using either the anti-ERß or anti-phosphoserine antibody. As illustrated in Fig. 9, band intensity of phosphorylated ERß in the 15d-PGJ2-treated cells was clearly lower than that in the untreated or vehicle-treated cells.

Discussion
In this study, we showed that 15d-PGJ2 induces apoptosis and down-regulates hTERT expression. Since the inhibition of hTERT expression by siRNA targeting of the hTERT gene resulted in the induction of apoptosis, we conclude that hTERT down-regulation plays an important role in 15d-PGJ2-
induced apoptosis in colon cancer cells. Down-regulation of hTERT by 15d-PGJ2 was also observed in pancreatic cancer cells (MIAPaCa-2 and PANC-1) and breast cancer cells (MCF-7 and T-47D; data not shown). Therefore, 15d-PGJ2-mediated down-regulation of hTERT may be a generalized event in cancer cells.

The molecular mechanism underlying hTERT expression inhibition that induces apoptosis is not yet elucidated. The extreme end of a telomere is a G-rich single-stranded overhang of the 3' end, that is normally well protected by intercalation into the telomeric double strand and forms a loop structure, which is stabilized by certain telomeric proteins including hTERT and telomeric-repeat binding factors (TRF1 and TRF2) (7,8). Saretzki et al demonstrated that the introduction of the G-rich telomeric oligonucleotide (TTAGGG)2 into fibroblasts and glioblastoma cells led to p53-dependent growth inhibition (26). Karlseder et al showed that producing a free single-stranded telomeric end by introducing mutant TRF2, which cannot bind to telomere DNA, induced p53-dependent apoptosis in cervical and breast cancer cells (27). In addition, constitutive hTERT expression results in increased survival following the activation of exogenous temperature-sensitive p53 in BL41 Burkitt lymphoma cells (28). According to these observations, the inhibition of hTERT expression may produce a free single-stranded telomeric end that induces the p53-dependent apoptosis. However, it has been reported that the induction of apoptosis by inhibition of the hTERT gene was observed not only in LS180 cells with wild-type p53 but also in SW480 cells with mutant p53 (19). Therefore, down-regulation of hTERT expression may also induce the p53-independent apoptosis.

In addition, we observed that 15d-PGJ2 attenuated hTERT gene transcription by inhibiting the binding of c-Myc, Sp1, and ERß to the hTERT gene promoter. Interestingly, 15d-PGJ2 inhibited the DNA binding of these three transcriptional factors by different mechanisms.

15d-PGJ2 suppressed c-Myc mRNA expression. It has been reported that estradiol induced up-regulation of c-Myc mRNA expression in breast cancer cells (29,30). Since estradiol failed to increase the level of c-Myc mRNA expression in ER-negative cells and the ER antagonist tamoxifen reduced c-Myc mRNA levels in ER-positive cells (29), we conclude that ER up-regulates c-Myc mRNA expression. Moreover, Miller et al demonstrated that Sp1 binds to the promoter regions of the c-Myc gene (31). Therefore, 15d-PGJ2 may suppress c-Myc...
gene transcription by inhibiting the binding of Sp1 and ERβ to the c-Myc gene promoter. We observed that 15d-PGJ2, up-regulated ERβ mRNA expression but not c-Myc mRNA expression. 15d-PGJ2, possesses ligand activity toward the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) that regulates gene transcription by a distinct mechanism, including ligand-dependent trans-activation, ligand-dependent transrepression and ligand-independent transactivation (32). Vignati et al demonstrated that the PPAR-γ ligand ciglitazone induced apoptosis in ovarian cancer cells (33). They performed microarray-based gene profiling which revealed that 436 genes, including p21 and PTEN, were up-regulated and that 241 genes, including cyclin D1, were down-regulated. Therefore, it is not strange that 15d-PGJ2 has a different effect on the gene transcription of c-Myc and ERβ.

Second, 15d-PGJ2 enhanced the degradation of the Sp1 protein via the ubiquitin-proteasome pathway. Abdelrahim et al showed that cyclooxygenase 2 (COX-2) inhibitors decreased Sp1 protein expression (34). Similarly to our observation, they revealed that COX-2 inhibitors enhanced Sp1 protein ubiquitination. Interestingly, ERβ protein ubiquitination was also enhanced by 15d-PGJ2, while the level of ERβ protein expression was not affected by 15d-PGJ2. Since 15d-PGJ2, up-regulated ERβ mRNA expression, the newly synthesized ERβ protein may compensate for the degraded ERβ protein. Qin et al demonstrated that in MCF-7 cells, the down-regulation of ERα by 15d-PGJ2 is mediated by enhanced ubiquitination of the protein (24). We examined the mRNA expression of ERα in MCF-7 cells treated with 15d-PGJ2 and observed that ERα mRNA expression was suppressed by 15d-PGJ2, while ERβ mRNA expression was increased (data not shown). Therefore, 15d-PGJ2 reduced ERα protein expression via the suppression of ERα protein synthesis by inhibition of ERα mRNA in addition to the promotion of ERα protein degradation.

Third, 15d-PGJ2 inhibited ERβ phosphorylation at serine residues. ERs regulate transcription through their estrogen-independent and estrogen-dependent activation domains (AF-1 and AF-2, respectively) by recruiting coactivator proteins (35). The AF-2 domain of ERs is regulated by ligand-induced changes in receptor conformation, but the activities of AF-1 domains can be modulated by phosphorylation. Tremblay et al demonstrated that the phosphorylation of ERβ at serine 106 and serine 124 in the AF-1 domain stimulates recruitment of the co-activator protein SRC-1 (25). Therefore, 15d-PGJ2 may block SRC-1 recruitment to ERβ through the inhibition of phosphorylation at these serine residues thus attenuating ERβ-mediated gene transcription. The above mentioned authors also demonstrated that the phosphorylation of ERβ at these serine residues is mediated by the mitogen-activated protein kinase (MAPK) pathway. Accordingly, there is a possibility that 15d-PGJ2 may inhibit the activation of MAPK.

In summary, our studies demonstrate that down-regulation of hTERT expression plays an important role in 15d-PGJ2-induced apoptosis in cancer cells. Furthermore, 15d-PGJ2 inhibits the transcriptional activity of c-Myc, Sp1 and ER via three different mechanisms and results in the transcriptional repression of the hTERT gene.

References


