Down-regulation of hTERT expression plays an important role in 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced apoptosis in cancer cells

MIKAKO MORIAI*, NAOKI TSUJI*, DAISUKE KOBAYASHI, KAGEAKI KURIBAYASHI and NAOKI WATANABE

Department of Clinical Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan

Received October 22, 2008; Accepted January 9, 2009

DOI: 10.3892/ijo_00000263

Abstract. The cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-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 ERß 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.

Correspondence to: Dr Naoki Watanabe, Department of Clinical Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan
E-mail: watanabn@sapmed.ac.jp

*Contributed equally

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; hTERT, human telomerase reverse transcriptase; Sp1, specificity protein 1; ER, estrogen receptor; PG, prostaglandin; siRNA, small inhibitory RNA; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt; TRAP, telomeric repeat amplification protocol; RT-PCR, reverse transcription polymerase chain reaction; rRNA, ribosomal RNA; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase

Key words: 15-deoxy-$\Delta^{12,14}$-prostaglandin, human telomerase reverse transcriptase, estrogen receptor, prostaglandin, small inhibitory RNA

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-$\Delta^{12,14}$-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-PGJ_2 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-PGJ_2 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-PGJ_2-induced apoptosis.

Materials and methods

Materials. 15d-PGJ_2 were obtained from Biomol International LP (Plymouth Meeting, PA). Anti-Sp1 antibody, anti-c-myc antibody, anti-ERα antibody, anti-ERß 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% CO_2.

Measurement of cell viability. The viability of colon cancer cells treated with 15d-PGJ_2 (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, Tecan, Maennedorf, Switzerland).

Detection of apoptosis. Apoptosis induced by 15d-PGJ_2 was quantified by a combined staining of Annexin V and propidium iodide (PI) using Mebcyto-apoptosis kit (Medical & Biological Laboratories, Nagoya, Japan). Briefly, 72 h after treatment with 15d-PGJ_2 (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.

Immunoblot analysis. Cytoplasmic and nuclear proteins were extracted from cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) with a protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of proteins were suspended in Tris-glycine SDS sample buffer (Invitrogen) with 0.05 M dithiothreitol and were separated on a 4 to 20% gradient Tris-glycine gel (Invitrogen) under denaturing conditions using Tris-glycine SDS running buffer (Invitrogen). For detection of hTERT protein, 6% Tris-glycine gel (Invitrogen) was used. Proteins were electroblotted to a nitrocellulose membrane (Invitrogen). Proteins were detected using a Western Breeze chemiluminescent immunodetection kit (Invitrogen) according to the manufacturer's protocol. Chemiluminescent was detected by radiographic film or ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA). Band intensity was semiquantified using Photoshop Elements software after conversion to digitalizing image using an image scanner (GT9700 F, EPSON, Tokyo, Japan).

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 reagnet (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Quantification of hTERT, Sp1, c-Myc and ERß mRNA. The expression of hTERT, c-Myc, Sp1 and ERß 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 CAC CAT-3'; reverse primer, 5'-ATG TAC GGC TGG AGG TCT GTC A-3'; and probe, 5'-(FAM) CGG AGG TCA TCG CCA GCA TCA TC (TAMRA)-3'. For c-Myc, forward primer was 5'-TCC CTC CAC TCG GAA GGA CTA-3'; reverse primer, 5'-CTC TGG

CGC TCC AAG ACG TT-3'; and probe, 5'-(FAM) CGA GGA GAA TGT CAA GAG GCG AAC ACA (TAMRA)-3'. For Sp1, forward primer was 5'-GGG CTA CCC CTA CCT CAA AGG-3'; reverse primer, 5'-CGG TCT GGA ACT GTG GGA TTA-3'; and probe, 5'-(FAM) TGT TGT GGC TGC CGC TCC CA (TAMRA)-3'. For ERß, forward primer was 5'-GGC AGA CCA CAA GCC CAA AT-3'; reverse primer, 5'-CTT GCT TCA CAC CAG GGA CTC T-3'; and probe, 5'-(FAM) TCT CCT TTA GTG GTC CAT CGC CAG TTA 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'-GCG CTT CCC ACG TGG CGG AGG G-3'), Sp1-binding motif of the hTERT gene promoter (5'-TCC TTT CCG CGG CCC CGC CCT CTC CTC GCG GCG CGA-3') and ER-binding motif of the hTERT gene promoter (5'-TGT TGG TCA GGC TGA TCT CAA A-3') were prepared; these were 3' end-labeled with biotin from Sigma Genosys Japan (Ishikari, Japan). Detection of c-Myc-, Sp1-and ER-oligonucleotide complexes was performed using the Light Shift chemiluminescent EMSA kit (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-PGJ$_2$ induces apoptosis in colon cancer cells. 15d-PGJ$_2$ 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-PGJ$_2$ in colon cancer cells, we cultured LS180, SW480 and HT-29 cells with various concentrations of 15d-PGJ$_2$ (10-30 μM) for up to 72 h and determined the number of viable cells by an MTS assay. Since 15d-PGJ$_2$ 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-PGJ$_2$ 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.

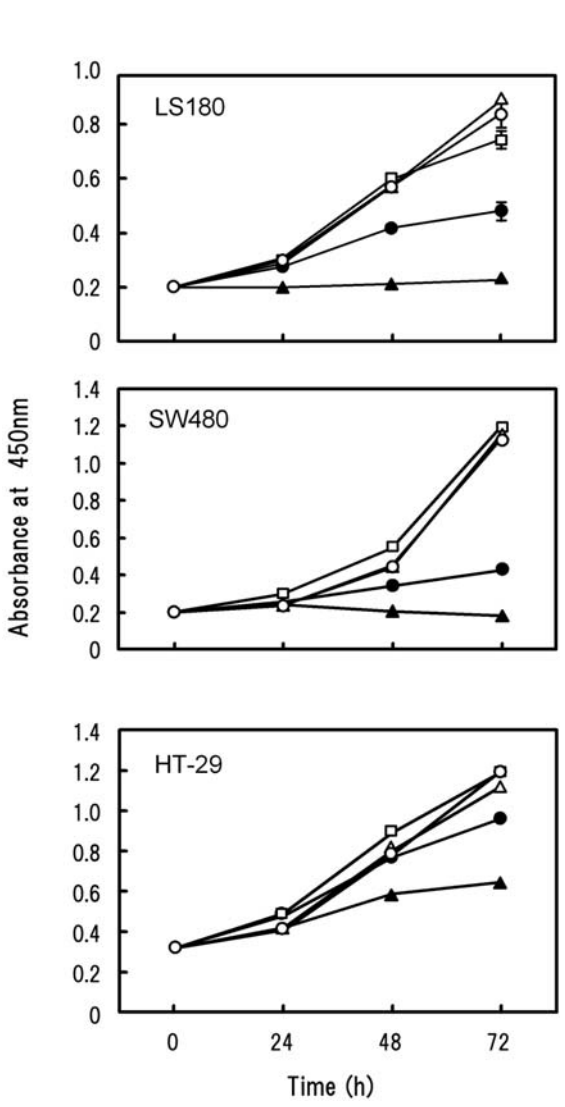
Using annexin V-PI staining, we determined the proportion of apoptotic cells after treating the LS180 cells with 30 μM of 15d-PGJ$_2$ for 72 h. As shown in Fig. 1B, there was no significant difference observed between the proportion of annexin V-positive cells and untreated and vehicle-treated cells. There were 75.1% more annexin V-positive cells among the 15d-PGJ$_2$-treated cells as compared to among the vehicle-treated cells, indicating that 15d-PGJ$_2$ induced apoptosis in the LS180 cells.

15d-PGJ$_2$ 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-PGJ$_2$-induced apoptosis, we examined the expression level of the hTERT protein in the LS180 cells treated with 10-30 μM of 15d-PGJ$_2$ for 48 h by immunoblot analysis. As shown in Fig. 2, 15d-PGJ$_2$ attenuated hTERT protein expression in a dose-dependent manner; the protein expression in the cells treated with 30 μM of 15d-PGJ$_2$ decreased to <50% in the vehicle-treated cells.

Since hTERT is a major determinant of telomerase activity, we examined whether 15d-PGJ$_2$ decreased telomerase activity by down-regulating hTERT protein expression. Telomerase activity in the LS180 cells treated with 10-30 μM of 15d-PGJ$_2$ 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-PGJ$_2$ 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-PGJ$_2$-induced apoptosis, we inhibited hTERT protein expression by introducing siRNA targeting for 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 introduced 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-PGJ_2 occurs at the transcription level. To clarify whether 15d-PGJ_2-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-PGJ_2 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-PGJ_2-treated cells decreased in a dose-dependent manner; it decreased to undetectable levels on treatment with 30 μM of 15d-PGJ_2.

15d-PGJ_2 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-PGJ_2 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-,

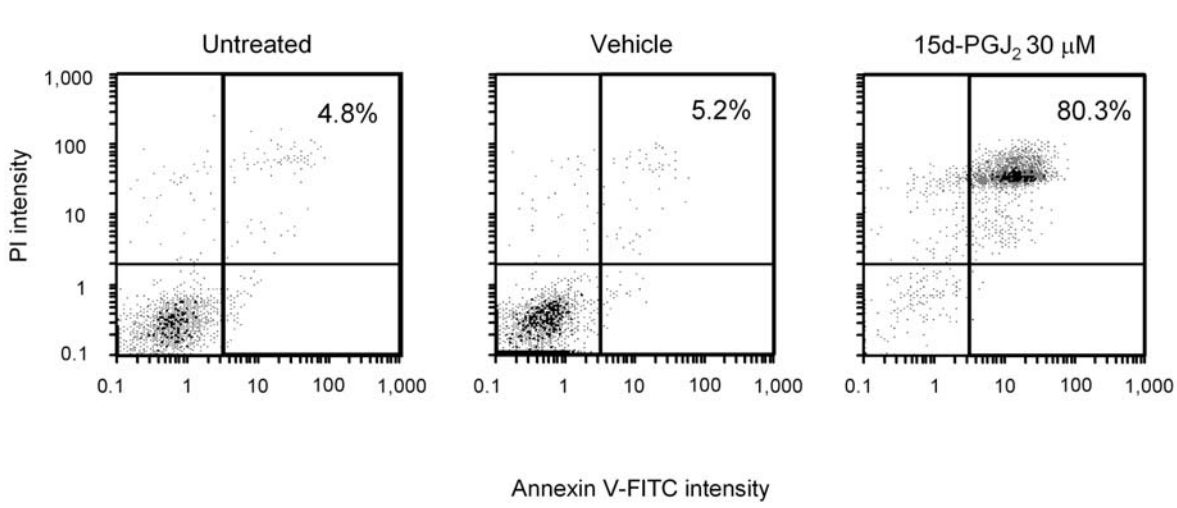


Figure 1. 15d-PGJ_2 induced apoptosis in colon cancer cells. (A) Growth curve of colon cancer cells treated with 15d-PGJ_2. The cells were incubated with different concentrations of 15d-PGJ_2 for up to 72 h. Cell viability was assessed by an MTS assay. Open circle, untreated; open triangle, ethanol; open square, 10 μM 15d-PGJ_2; closed circle, 20 μM 15d-PGJ_2; closed triangle, 30 μM 15d-PGJ_2. Data are the mean ± SD values. (B) Detection of apoptosis in 15d-PGJ_2-treated colon cancer cells. LS180 cells were cultured with 30 μM of 15d-PGJ_2 for 72 h. The apoptotic cells were quantified by combined staining with annexin V and propidium iodide. The percentages of annexin V-positive cells are indicated for each test condition. Vehicle represents the cells treated with the same concentration of ethanol as that used for 15d-PGJ_2-treated cells.

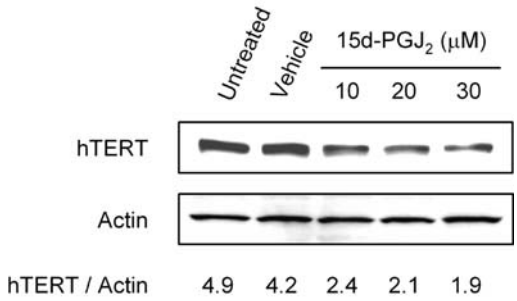


Figure 2. 15d-PGJ_2 down-regulated hTERT protein expression. LS180 cells were cultured with 10-30 μM of 15d-PGJ_2 for 48 h. The level of hTERT protein expression was examined by immunoblot analysis. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells. Band intensities of hTERT and actin were semiquantified using Photoshop Elements software after converting the bands to digital images using an image scanner.

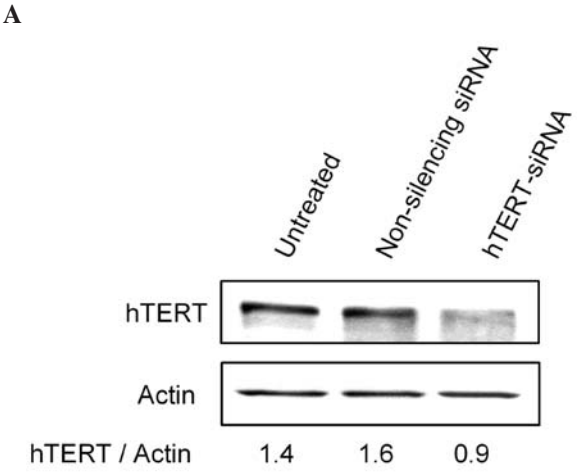
Table I. Telomerase activity in 15d-PGJ_2-treated cells.

15d-PGJ_2 (μM)	Relative telomerase activity (%)[a]
Vehicle[b]	97.8±0.0
10	80.0±5.0
20	58.5±1.6
30	27.6±5.3

[a]Telomerase activity was assessed using TRAP assay. Data represent the percentage of telomerase activity in untreated cells. [b]Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

Sp1- and ER-oligonucleotide complexes was inhibited in the 15d-PGJ_2-treated cells. These results indicate that 15d-PGJ_2 down-regulates hTERT protein expression by inducing transcriptional repression of the hTERT gene.

Sp1 and c-Myc protein expression is reduced by 15d-PGJ_2. Decreased c-Myc, Sp1 and ER protein expression appear to be one mechanism by which 15d-PGJ_2 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-PGJ_2 for 48 h by an immunoblot analysis. As shown in Fig. 6A, the expression levels of both c-Myc and Sp1 proteins in 15d-PGJ_2-treated cells were reduced as compared to their expression levels in untreated or vehicle-treated cells.



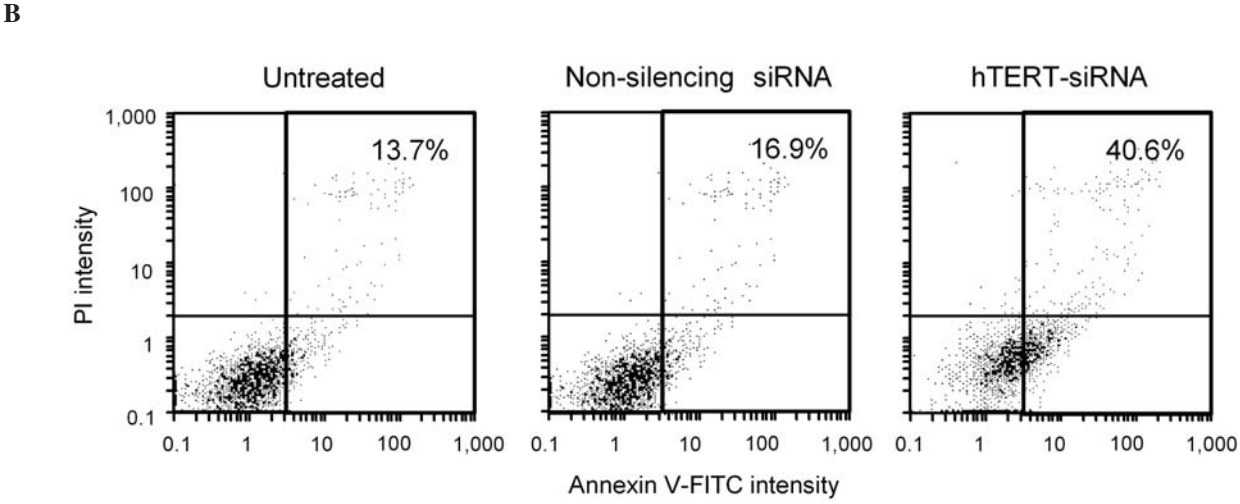


Figure 3. Inhibition of hTERT protein expression by hTERT-specific siRNA induced apoptosis in colon cancer cells. (A) hTERT protein expression in colon cancer cells transfected with hTERT-specific siRNA. LS180 cells were transfected with siRNA targeting the hTERT gene (hTERT-siRNA), and hTERT protein expression was assessed by immunoblot analysis at 6 days after transfection. Non-silencing siRNA, which has no homology to any known mammalian gene, was used as the transfection control. The band intensities of hTERT and actin were semiquantified using Photoshop Elements software after converting the bands to digital images using an image scanner. (B) Detection of apoptosis in colon cancer cells transfected with hTERT-specific siRNA. The apoptotic cells were quantified by combined staining with annexin V and propidium iodide at 6 days after transfection. The percentages of annexin V-positive cells are indicated for each test condition.

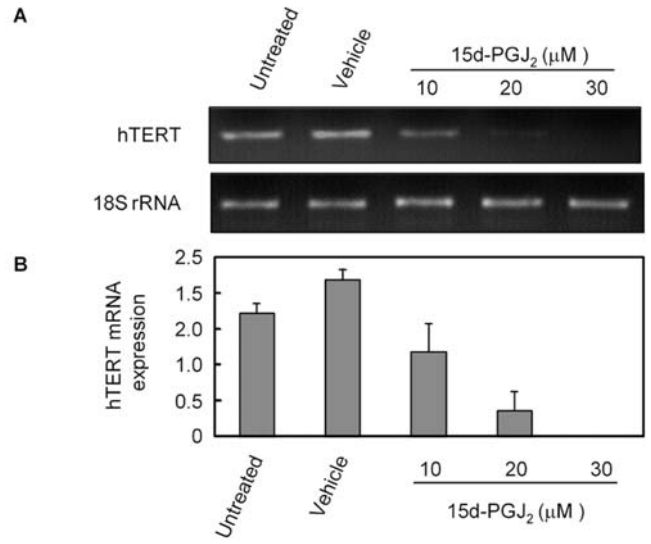


Figure 4. 15d-PGJ_2 decreased the level of hTERT mRNA expression. LS180 cells were cultured with 10-30 μM of 15d-PGJ_2 for 48 h. The level of hTERT mRNA expression was examined by RT-PCR. PCR products were analyzed by agarose gel electrophoresis (A). hTERT mRNA was also quantified by quantitative RT-PCR (B). The amounts of hTERT mRNA were normalized as ratios to the amount of 18S rRNA. Data are the mean ± SD. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

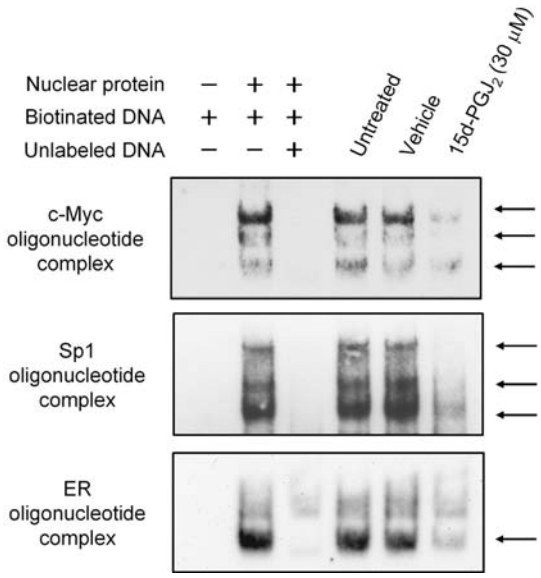


Figure 5. DNA-binding of c-Myc, Sp1 and ER to the hTERT gene promoter was attenuated by 15d-PGJ_2. LS180 cells treated with 30 μM of 15d-PGJ_2 for 48 h. DNA binding of c-Myc, Sp1, and ER to the hTERT gene promoter was examined by EMSA. Arrows indicate the protein-oligonucleotide complexes. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

ER has two isoforms, i.e., ERα and ERß. We attempted to identify the ER isoform expressed by the LS180 cells express prior to studying the effect of 15d-PGJ_2 on ER protein expression (Fig. 6B). Although the breast cancer cells MCF-7 expressed both ERα and ERß proteins, the LS180 cells expressed only the ERß protein. The SW480 cells and HT-29 cells also expressed only the ERß protein (data not shown). We then determined whether 15d-PGJ_2 down-regulated ERß 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 ERß protein was observed in the 15d-PGJ_2-treated cells.

Qin *et al* demonstrated that 15d-PGJ_2 induced the down-regulation of ERα expression in MCF-7 cells (24). In order to confirm that 15d-PGJ_2 fails to reduce ERß protein expression, we investigated the expression of both ERα and ERß proteins in the MCF-7 cells treated with 15d-PGJ_2 (Fig. 6D). In agreement with a previous study, ERα protein expression was clearly reduced by 15d-PGJ_2. On the other hand, as observed in LS180 cells, 15d-PGJ_2 did not influence the ERß protein expression.

These results indicate that the mechanism by which 15d-PGJ_2 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 ERß, it not the down-regulation of protein expression but protein modification that may block the DNA-binding of ERß to the hTERT gene promoter.

15d-PGJ_2 suppresses c-Myc mRNA expression. To explore the mechanism by which 15d-PGJ_2 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-PGJ_2 for 48 h by quantitative RT-PCR (Fig. 7). The level of c-Myc mRNA expression in the 15d-PGJ_2-treated cells 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, ERß mRNA expression was up-regulated in the 15d-PGJ_2-treated cells. According to these results, down-regulation of c-Myc protein expression by 15d-PGJ_2 may be mediated by the suppression of c-Myc mRNA expression.

Ubiquitination of Sp1 is enhanced by 15d-PGJ_2. Since 15d-PGJ_2 down-regulated Sp1 protein expression without suppressing Sp1 mRNA expression, we hypothesized that 15d-PGJ_2 enhanced Sp1 degradation via the ubiquitin-proteasome pathway. We then investigated the ubiquitination of Sp1 in 15d-PGJ_2-treated cells. In the initial study, we treated LS180 cells with several proteasome inhibitors, including MG132 and lactacystin in addition to 15d-PGJ_2, to detect the levels of ubiquitinated Sp1 protein. However, combination treatment using these proteasome inhibitors and 15d-PGJ_2 resulted in severe cytotoxicity. Therefore, we extracted Sp1 protein from the untreated cells, vehicle-treated cells, or 15d-PGJ_2-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-PGJ_2-treated cells. In addition, 15d-PGJ_2-treated cells showed an additional band, indicating polyubiquitination of Sp1. On the other hand, 15d-PGJ_2 showed no effect on c-Myc ubiquitination. These results

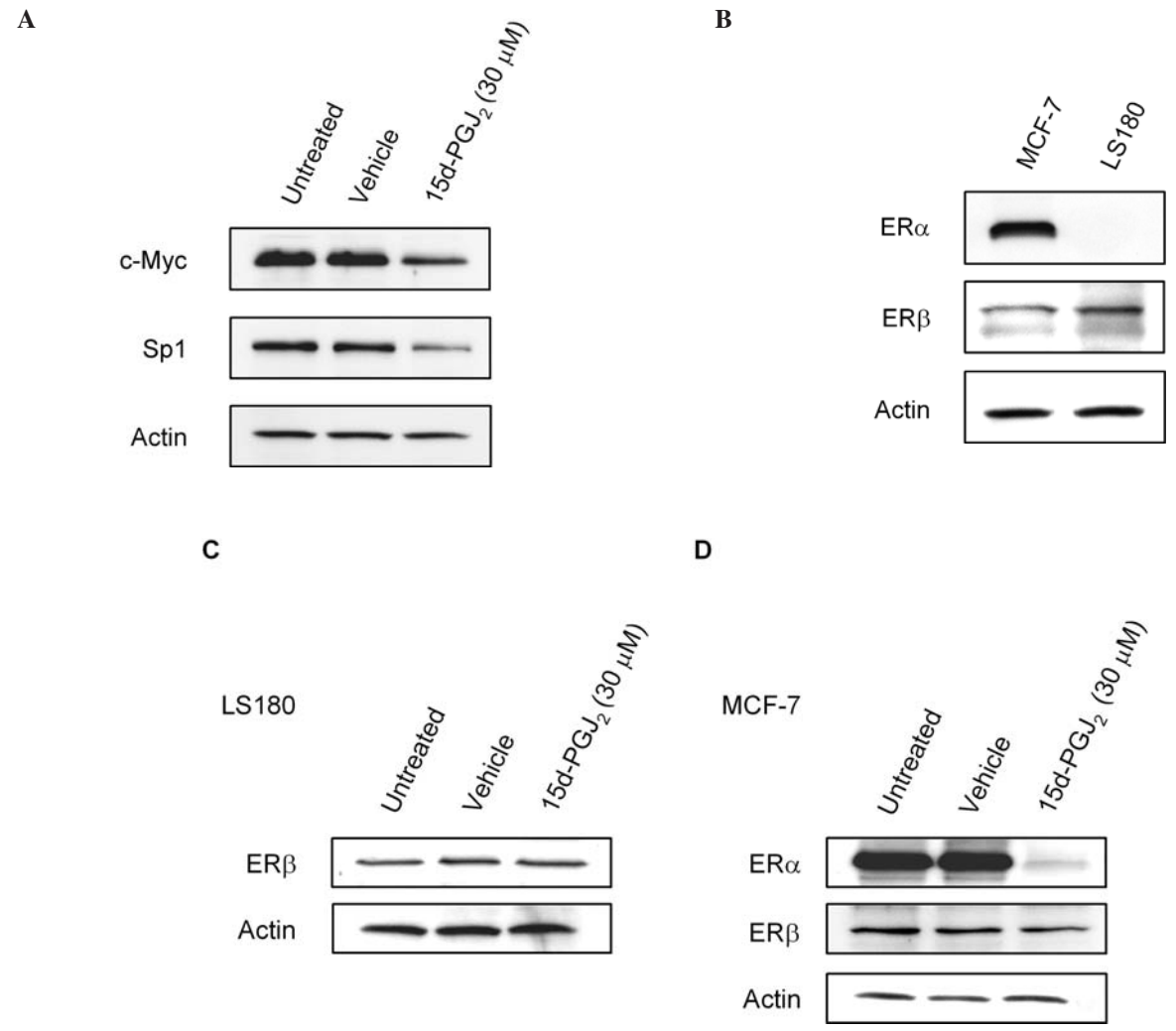


Figure 6. Sp1 and c-Myc protein expression is reduced by 15d-PGJ_2. (A) Effect of 15d-PGJ_2 on the level of c-Myc and Sp1 protein expression. LS180 cells treated with 30 μM of 15d-PGJ_2 for 48 h. c-Myc and Sp1 protein expressions were examined by immunoblotting. (B) Expression of ERα and ERß protein in breast cancer cells (MCF-7) and colon cancer cells (LS180). ERα and ERß protein expression were examined by immunoblotting. (C and D) 15d-PGJ_2 down-regulated the expression of ERα protein but not ERß protein. LS180 cells (C) or MCF-7 cells (D) were treated with 15d-PGJ_2 for 48 h. ERα and ERß protein expression were examined by immunoblotting. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

indicate that 15d-PGJ_2 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-PGJ_2-treated cells, the intensity of the ubiquitinated ERß band was increased by 15d-PGJ_2 and also Sp1. Since ERß mRNA expression is up-regulated by 15d-PGJ_2, the level of newly synthesized ERß protein may be increased. Thus, 15d-PJG_2 appeared to have no influence on ERß protein expression.

Phosphorylation of ERß at serine residues is inhibited by 15d-PGJ_2. We then investigated the mechanism by which 15d-PGJ_2 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 NH_2-terminal A/B region is critical for transcriptional activation (25). Therefore, we examined the effect of 15d-PGJ_2 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-PGJ_2 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-PGJ_2-treated cells was clearly lower than that in the untreated or vehicle-treated cells.

Discussion

In this study, we showed that 15d-PGJ_2 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-PGJ_2-

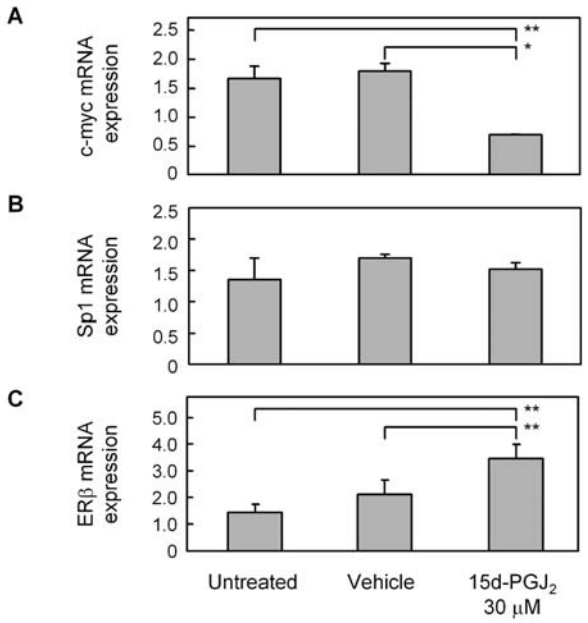


Figure 7. 15d-PGJ_2 decreased c-Myc mRNA expression. LS180 cells were cultured with 30 μM of 15d-PGJ_2 for 48 h. The expression levels of c-Myc mRNA (A), Sp1 mRNA (B), and ERß (C) were examined by quantitative RT-PCR. The amounts of hTERT mRNA were normalized as ratios to the amount of 18S rRNA. Data are the mean ± SD. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells. $^{*}P<0.01$ and $^{**}P<0.05$ by t-test.

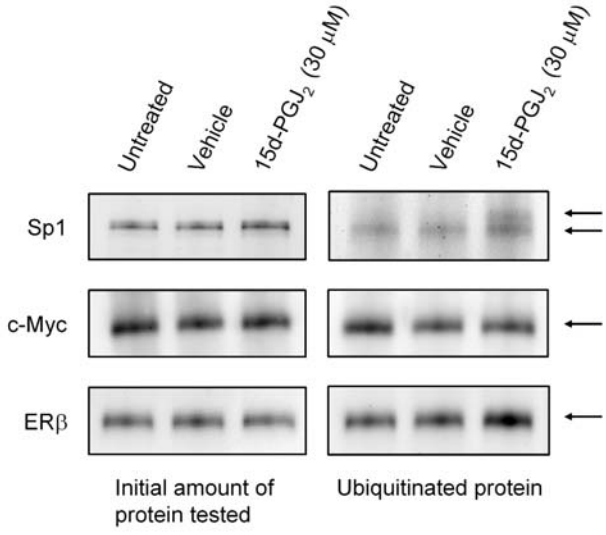


Figure 8. Ubiquitination of the Sp1 protein was enhanced by 15d-PGJ_2. LS180 cells were treated with 30 μM of 15d-PGJ_2 for 48 h. Sp1, c-Myc, or ERß protein from nuclear extracts was immunoprecipitated using the anti-Sp1, anti-c-Myc, or anti-ERß antibody, respectively. These three protein immunoprecipitates were analyzed by immunoblot analysis with anti-Sp1, anti-c-Myc, anti-ERß (left), or anti-ubiquitin antibody (right). Arrows indicate the ubiquitinated proteins. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

induced apoptosis in colon cancer cells. Down-regulation of hTERT by 15d-PGJ_2 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-PGJ_2-

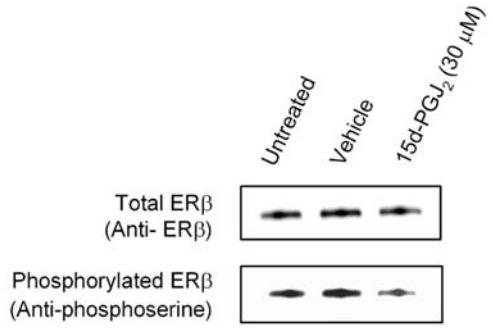


Figure 9. 15d-PGJ_2 inhibited the phosphorylation of ERß protein at serine residues. LS180 cells were treated with 30 μM of 15d-PGJ_2 for 48 h. ERß protein from nuclear extracts was immunoprecipitated using the anti-ERß antibody and immunoblotted with the anti-ERß or anti-phosphoserine antibody. Vehicle represents the cells treated with the same concentration of ethanol as that used to treat 15d-PGJ_2-treated cells.

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-PGJ_2 attenuated hTERT gene transcription by inhibiting the binding of c-Myc, Sp1, and ERß to the hTERT gene promoter. Interestingly, 15d-PGJ_2 inhibited the DNA binding of these three transcriptional factors by different mechanisms.

15d-PGJ_2 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-PGJ_2 may suppress c-Myc

gene transcription by inhibiting the binding of Sp1 and ERß to the c-Myc gene promoter. We observed that 15d-PGJ_2 up-regulated ERß mRNA expression but not c-Myc mRNA expression. 15d-PGJ_2 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-PGJ_2 has a different effect on the gene transcription of c-Myc and ERß.

Second, 15d-PGJ_2 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, ERß protein ubiquitination was also enhanced by 15d-PGJ_2, while the level of ERß protein expression was not affected by 15d-PGJ_2. Since 15d-PGJ_2 up-regulated ERß mRNA expression, the newly synthesized ERß protein may compensate for the degraded ERß protein. Qin *et al* demonstrated that in MCF-7 cells, the down-regulation of ERα by 15d-PGJ_2 is mediated by enhanced ubiquitination of the protein (24). We examined the mRNA expression of ERα in MCF-7 cells treated with 15d-PGJ_2 and observed that ERα mRNA expression was suppressed by 15d-PGJ_2, while ERß mRNA expression was increased (data not shown). Therefore, 15d-PGJ_2 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-PGJ_2 inhibited ERß 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 ERß at serine 106 and serine 124 in the AF-1 domain stimulates recruitment of the co-activator protein SRC-1 (25). Therefore, 15d-PGJ_2 may block SRC-1 recruitment to ERß through the inhibition of phosphorylation at these serine residues thus attenuating ERß-mediated gene transcription. The above mentioned authors also demonstrated that the phosphorylation of ERß at these serine residues is mediated by the mitogen-activated protein kinase (MAPK) pathway. Accordingly, there is a possibility that 15d-PGJ_2 may inhibit the activation of MAPK.

In summary, our studies demonstrate that down-regulation of hTERT expression plays an important role in 15d-PGJ_2-induced apoptosis in cancer cells. Furthermore, 15d-PGJ_2 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

1. Straus DS and Glass CK: Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. Med Res Rev 21: 185-210, 2001.
2. Sato H, Ishihara S, Kawashima K, *et al*: Expression of peroxisome proliferator-activated receptor (PPAR) γ in gastric cancer and inhibitory effects of PPAR γ agonists. Br J Cancer 83: 1394-1400, 2000.
3. Shimada T, Kojima K, Yoshiura K, Hiraishi H and Terano A: Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. Gut 50: 658-664, 2002.
4. Tsubouchi Y, Sano H, Kawahito Y, *et al*: Inhibition of human lung cancer cell growth by the peroxisome proliferator-activated receptor-gamma agonists through induction of apoptosis. Biochem Biophys Res Commun 270: 400-405, 2000.
5. Pignatelli M, Sanchez-Rodriguez J, Santos A and Perez-Castillo A: 15-Deoxy-$\Delta^{12,14}$- prostaglandin J_2 induces programmed cell death of breast cancer cells by a pleiotropic mechanism. Carcinogenesis 26: 81-92, 2005.
6. Laurora S, Pizzimenti S, Briatore F, *et al*: Peroxisome proliferator-activated receptor ligands affect growth-related gene expression in human leukemic cells. J Pharmacol Exp Ther 3059: 32-42, 2003.
7. Dhaene K, Van Marck E and Parwaresch R: Telomeres, telomerase and cancer: an up-date. Virchows Arch 437: 1-16, 2000.
8. Shay JW and Wright WE: Telomerase therapeutics for cancer: challenges and new directions. Nat Rev Drug Discov 5: 577-584, 2006.
9. Hiyama E, Yokoyama T, Tatsumoto N, *et al*: Telomerase activity in gastric cancer. Cancer Res 55: 3258-3262, 1995.
10. Chadeneau C, Hay K, Hirte HW, Gallinger S and Bacchetti S: Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res 55: 2533-2536, 1995.
11. Bodnar AG, Ouellette M, Frolkis M, *et al*: Extension of life-span by introduction of telomerase into normal human cells. Science 279: 349-352, 1998.
12. Vaziri H and Benchimol S: Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol 8: 279-282, 1998.
13. Meeker AK and De Marzo AM: Recent advances in telomere biology: implications for human cancer. Curr Opin Oncol 16: 32-38, 2004.
14. Morales CP, Holt SE, Ouellette M, *et al*: Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat Genet 21: 115-118, 1999.
15. Hahn WC, Stewart SA, Brooks MW, *et al*: Inhibition of telomerase limits the growth of human cancer cells. Nat Med 5: 1164-1170, 1999.
16. Delhommeau F, Thierry A, Feneux D, *et al*: Telomere dysfunction and telomerase reactivation in human leukemia cell lines after telomerase inhibition by the expression of a dominant-negative hTERT mutant. Oncogene 21: 8262-8271, 2002.
17. Ramakrishnan S, Eppenberger U, Mueller H, Shinkai Y and Narayanan R: Expression profile of the putative catalytic subunit of the telomerase gene. Cancer Res 58: 622-625, 1998.
18. Saretzki G, Ludwig A, von Zglinicki T and Runnebaum IB: Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. Cancer Gene Ther 8: 827-834, 2001.
19. Hao ZM, Luo JY, Cheng J, *et al*: Intensive inhibition of hTERT expression by a ribozyme induces rapid apoptosis of cancer cells through a telomere length-independent pathway. Cancer Biol Ther 4: 1098-1103, 2005.
20. Asanuma K, Tsuji N, Endoh T, Yagihashi A and Watanabe N: Survivin enhances Fas ligand expression via up-regulation of specificity protein 1-mediated gene transcription in colon cancer cells. J Immunol 172: 3922-3929, 2004.
21. Endoh T, Tsuji N, Asanuma K, Yagihashi A and Watanabe N: Survivin enhances telomerase activity via up-regulation of specificity protein 1- and c-Myc-mediated human telomerase reverse transcriptase gene transcription. Exp Cell Res 305: 300-311, 2005.
22. Kyo S, Takakura M, Taira T, *et al*: Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res 28: 669-677, 2000.
23. Kyo S, Takakura M, Kanaya T, *et al*: Estrogen activates telomerase. Cancer Res 59: 5917-5921, 1999.

24. Qin C, Burghardt R, Smith R, Wormke M, Stewart J and Safe S: Peroxisome proliferator-activated receptor gamma agonists induce proteasome-dependent degradation of cyclin D1 and estrogen receptor alpha in MCF-7 breast cancer cells. Cancer Res 63: 958-964, 2003.
25. Tremblay A, Tremblay GB, Labrie F and Giguere V: Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. Mol Cell 3: 513-519, 1999.
26. Saretzki G, Sitte N, Merkel U, Wurm RE and von Zglinicki T: Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. Oncogene 18: 5148-5158, 1999.
27. Karlseder J, Broccoli D, Dai Y, Hardy S and de Lange T: p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science 283: 1321-1325, 1999.
28. Rahman R, Latonen L and Wiman KG: hTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene 24: 1320-1327, 2005.
29. Santos GF, Scott GK, Lee WM, Liu E and Benz C: Estrogen-induced post- transcriptional modulation of c-myc proto-oncogene expression in human breast cancer cells. J Biol Chem 263: 9565-9568, 1988.
30. Leygue E, Gol-Winkler R, Gompel A, *et al*: Estradiol stimulates c-myc proto-oncogene expression in normal human breast epithelial cells in culture. J Steroid Biochem Mol Biol 52: 299-305, 1995.
31. Miller TL, Jin Y, Sun JM, Coutts AS, Murphy LC and Davie JR: Analysis of human breast cancer nuclear proteins binding to the promoter elements of the c-myc gene. J Cell Biochem 60: 560-571, 1996.
32. Glass CK and Ogawa S: Combinatorial roles of nuclear receptors in inflammation and immunity. Nat Rev Immunol 6: 44-55, 2006.
33. Vignati S, Albertini V, Rinaldi A, *et al*: Cellular and molecular consequences of peroxisome proliferator-activated receptor-gamma activation in ovarian cancer cells. Neoplasia 8: 851-861, 2006.
34. Abdelrahim M and Safe S: Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins. Mol Pharmacol 68: 317-329, 2005.
35. Glass CK and Rosenfeld MG: The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14: 121-141, 2000.