**PARP1** gene expression is downregulated by knockdown of **PARG** gene

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**Abstract.** Poly(ADP-ribosyl)ation is a modification of nuclear proteins that regulates DNA replication, repair and transcription. In order to investigate the biological effects of degradation of poly(ADP-ribose), knockdown of the poly(ADP-ribose) glycohydrolase (**PARG**) gene was performed by introducing a short interfering RNA (siRNA)-pool into HeLa S3 cells. Notably, poly(ADP-ribosyl)ated proteins did not accumulate in the cells. Western blotting, quantitative RT-PCR analysis and a transient transfection assay revealed that poly(ADP-ribose) polymerase 1 (**PARP1**) gene/protein expression and its promoter activity were reduced in the **PARG** knockdown cells. These results suggest that the amount of poly(ADP-ribose) in a cell is regulated under the control of **PARP1/PARG** gene expression balance. Furthermore, in this study, we showed that **PARG**-siRNA enhanced cell death induced by staurosporine (STS). Thus, we propose a **PARG**-siRNA utilizing gene-therapy for cancer treatment.

**Introduction**

Poly(ADP-ribosylation) is a NAD+-dependent post-transcriptional modification of chromosomal proteins mediated by poly(ADP-ribose) polymerases (**PARPs**) and poly(ADP-ribose) glycohydrolase (**PARG**) (1). Reversible poly(ADP-ribosylation) of chromosomal proteins has been suggested to play important roles in various biological processes, including DNA replication (2,3), repair (4-6), spindle assembly (7), transcription (8), telomere and chromosomal maintenance (9) and epigenetic gene regulation (10). Previous studies have suggested that poly(ADP-ribose) metabolism is associated with differentiation and proliferation (11,12), cell death (13) and apoptosis (14,15). Thus, poly(ADP-ribosylation) is significantly involved in various biological activities, suggesting that it requires precise controlling systems to adjust the amount and length of poly(ADP-ribose) in eukaryotic cells.

Previous studies indicated that several transcription factors, including Sp1 (16), YY1 (17), and NF-kB (18), are poly(ADP-ribosyl)ated and reduce the transcription activity (16). If there are binding sites for these transcription factors in the promoter region, transcription might be affected by the poly(ADP-ribosylation). To date, the promoter regions of the human **PARP1** (19) and **PARG** (20,21) genes have been isolated and characterized. In this study, we investigated the effect of short interfering RNAs (siRNAs) for the human **PARG** cDNA on poly(ADP-ribosylation) and **PARP1/PARG** gene expression in HeLa S3 cells. The results showed that expression and promoter activity of the **PARP1** gene were reduced by the knockdown of the **PARG** gene and that the amounts of poly(ADP-ribose) in the cells did not increase compared to the control cells. Moreover, **PARG** knockdown cells showed stronger cell death sensitivity to staurosporine (STS) than the control cells, suggesting that retarded turnover of poly(ADP-ribose)-NAD+ metabolism might induce intracellular apoptosis signals.

It is well known that **PARP1** activity is downregulated by its augmented auto-poly(ADP-ribosylation) (22,23), and artificially accumulated poly(ADP-ribose) induces apoptosis (13). Collectively, our results indicate that reduced poly(ADP-ribose) degradation subsequently suppresses transcription of the **PARP1** gene to escape excessive poly(ADP-ribose) accumulation, thereby achieving a balance in poly(ADP-ribose) levels for cell survival. Therefore, poly(ADP-ribose) may act as a dual regulator for **PARP1** activity not only at the post-translational level but also at the transcriptional level. Hence, we propose a molecular mechanism that prevents cells from accumulating excess amounts of poly(ADP-ribose) by regulating transcription of the **PARP1** gene.

**Materials and methods**

**Cell culture.** Human cervical carcinoma (HeLa S3) cells (24) were grown in Dulbecco's modified Eagle's medium (DMEM; Nacarai, Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS) (Sanko Pure Chemicals, Tokyo, Japan) and penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO2.
Transfection of siRNA. The ON-TARGETplus SMARTpool siRNAs used for knockdown of the human PARP gene were purchased from Thermo Fisher Scientific Inc. (Lafayette, CO, USA). They were introduced into HeLa S3 cells with DharmaFECT Transfection reagent following the manufacturer’s protocol (Thermo Fisher Scientific). In brief, 2 µM siRNA (50 µl) were added to serum-free DMEM (50 µl) in one tube, and DharmaFECT1 (1.5 µl) was added to 98.5 µl of serum-free medium in the other tube. They were gently mixed and incubated for 5 min at room temperature, and were then combined, mixed and further incubated for 20 min at room temperature. Subsequently, complete medium (800 µl) was added and cells were cultivated with the medium in a 35-mm culture dish.

Cell viability MTS assay. An MTS assay was performed according to the manufacturer’s instructions. In brief, mock- or siRNA-transfected cells were cultured in microwell plate wells. MTS solution (20 µl) (Promega, Madison, WI, USA) was added to each well (containing 100 µl of cell culture) and incubated for 3 h in a 37°C, 5% CO₂-humidified incubator. Then, the absorbance at 492 nm was measured by a microwell plate reader (Thermo Electron Corp., Vantaa, Finland) and normalized by the absorbance at 630 nm.

Reverse transcriptase and quantitative real-time polymerase chain reaction (RT-qPCR). RT-qPCR was carried out as previously described (24). First-strand cDNAs were synthesized with ReverTra Ace (Toyobo Corp., Tokyo, Japan), random primers (Takara, Kyoto, Japan) and total RNAs were extracted from HeLa S3 cells. A primer pair to amplify the human GAPDH cDNAs was previously reported (24), and those for amplifying the PARP1 and PARG cDNAs were: hPARP1S514, 5'-GCAGAGTATGCCAAGTCCAACAG-3' and hPARP1A813, 5'-ATCCACCTCATTGCGCTTTTTC-3'; and hPARG-S, 5'-ATGTGTAAGTGGCAAAATGAAGGG-3'; and hPARG-A952, 5'-CTTCTCTGAGGCCATCGAACATCTA-3' and AhPARP1-2895; and hPARG-2660 and AhPARG-2851, 5'-ATCTCGAGATTTGCTGCTCGGC-3'; respectively. Real-time PCR analysis was carried out using the Mx3000P Real-Time QPCR system (Stratagene, La Jolla, CA, USA) as previously described (24). For PCR amplification, cDNAs were amplified using SYBR-Green real-time PCR Master Mix (Toyobo) and 0.3 µM of each primer pair. Amplification of the PARP1 cDNA was carried out, starting with an initial step for 1 min at 94°C, followed by 42 cycles (94°C 30 sec, 55°C 30 sec and 72°C 1 min). The conditions for amplification of the PARG and GAPDH cDNAs were 1 min at 94°C, followed by 42 cycles (94°C 15 sec, 55°C 10 sec, and 72°C 15 sec).

Western blot analysis. Western blotting was carried out as previously described (24,25) with antibodies against PARP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PAR (Calbiochem, Darmstadt, Germany) followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem). Signal intensities were quantified with a LAS4000 system and Multi Gauge Software (Fuji Film, Tokyo, Japan).

Construction of luciferase (Luc) reporter plasmids. Luc reporter plasmids carrying 75-bp of the human PARG promoter regions were designated pKBST-Δ6 (21). The 5′-flanking regions of the human PARP1 gene were obtained by PCR with PrimeStar Taq polymerase (Takara) and the template genomic DNA from HeLa S3 cells as previously described (26). The sense and antisense primers used for PCR were: hPARP1-2660, 5'-TCGGTACCTGCTCCAGAAGCTAC-3' and AhPARP1-2895, 5'-ATCTCGAGGCCACCGCAACCG CGC-3', respectively. The amplified DNA fragments were digested with KpnI and Xhol and ligated into the MCS of the pGGL4-basic (pGL4.10[luc2]), vector (Promega) to make pGL4-PARP1. Deletion derivatives, pGL4-PARP1A1 and pGL4-PARP1Δ2, were generated by PCR with pGL4-PARP1 as the template and primer sets: hPARP1-2851, 5'-TCGGTACCGCCAGCATGCAATCTA-3' and AhPARP1-2895; and hPARG-2660 and AhPARG-2851, 5'-ATCTCGAAGTA GTTGCTGATGCTGGC-3', respectively. The nucleotide sequences of the PCR products were determined by a DNA Sequencing system (Applied Biosystems, Foster City, CA, USA) with Rv (5'-TAGCAAAAATAGGCTGTCCCATCCC-3') and GL (5'-CTTATTTTTTGGTCCTCTCC-3') primers.

Transient transfection and Luc assays. Plasmid DNAs were transfected into HeLa S3 cells by the DEAE-dextran method (24-26). After a further 24 h of incubation, cells were collected and lysed with 100 µl of 1X cell culture lysis reagent, mixed and centrifuged at 12,000 x g for 5 sec. The supernatant was stored at -80°C. The Dual Luciferase assay was performed with the Dual Luciferase assay system (Promega), as previously described (21).

Results

Decrease in the amounts of PARG gene transcripts after introducing its siRNAs. In order to suppress PARG gene expression, ON-TARGETplus SMARTpool siRNAs (PARG-siRNA) were transfected into HeLa S3 cells with DharmaFECT 1 reagent. The relative amounts of PARG transcripts were reduced by PARG-siRNA treatment (Fig. 1A). In 100 nM of PARG-siRNA-treated cells, the PARG gene expression level decreased to almost half of that in the mock-transfected cells. Since this treatment did not affect viability of cells compared with the mock-transfected cells (data not shown), further experiments were performed with 100 nM of PARG-siRNA.

Treatment of PARG-siRNA reduces the amounts of poly(ADP-ribose) and PARP1. As the PARG gene encodes the main poly(ADP-ribose) degrading enzyme, the level of poly(ADP-ribose)-modified proteins was assumed to increase following the introduction of PARG-siRNA into HeLa S3 cells. Western blotting revealed that the amounts of poly(ADP-ribose) decreased in the PARG-siRNA-transfected HeLa S3 cells (Fig. 1B). In addition, the decrease of poly(ADP-ribose) in the whole-cell extracts was accompanied by a decrease in PARP1 protein levels (Fig. 1C), suggesting that the PARG-siRNA diminished poly(ADP-ribose) and the PARP1 protein.

PARP1 gene expression and its promoter activity are downregulated by transfection with PARG-siRNA. To examine whether the decrease in PARP1 protein level is caused by changes in gene expression and promoter activity, RT-qPCR analyses and transient transfection experiments were performed. As shown in
Fig. 2A, the relative PARP1 gene expression of PARG-siRNA-transfected cells was 60% that of the control (mock) cells. In addition, the transient transfection and Luc reporter assay indicated that the PARP1 promoter activity decreased in the PARG-siRNA-transfected cells compared to the control cells (Fig. 2B). We also tested whether the 75-bp core promoter of the PARP1 gene (21) responds to PARG-siRNA treatment (Fig. 2B, bars 5 and 6), but only a 10% decrease was observed. To limit the PARG-siRNA responsive element(s), we constructed two deletion plasmids that contained -187 to +6 and -13 to +50 bp of the human PARP1 promoter (Fig. 2C). The results showed that the 193-bp fragment, which contains the GC-box/Spl, NF-xB/c-Rel, GATA-1 and GATA-2 binding sequences, responded to the PARG-siRNA. The promoter activity obtained from pGL4-PARP1∆2-transfected cells was lower than that of the pGL4-basic vector-transfected cells, indicating that the 63-bp sequence from -13 to +50 is not essential for transcription of the PARP1 gene. These results suggest that the transfection of PARG-siRNA leads to suppression of PARP1 gene expression through the 193-bp sequence of the PARP1 promoter region.
Viability of PARG siRNA-introduced HeLa S3 cells following N-methyl-N\textquotesingle nitro-N-nitrosoguanidine (MNNG) and STS treatments. It has been demonstrated that poly(ADP-ribose) metabolism is associated with cell death or apoptosis (13-15). To examine the effects of MNNG and STS on PARP1 in HeLa S3 cells, western blot analysis was performed (Fig. 3). The treatment with STS (0.1-0.3 µM) induced cleavage of PARP1, suggesting that caspases were activated and cells underwent apoptosis. Although the relative amount of PARP1 was diminished to 50%, cleaved forms of PARP1 were not detected by the treatment with 30 µM of MNNG (Fig. 3).

Subsequently, the effect of MNNG and STS on cell viability was analyzed following the transfection of PARG-siRNA. Although 40 µM of MNNG treatment caused severe damage to the cells, transfection of PARG-siRNA did not further increase cell death (Fig. 4A). On the other hand, PARG-siRNA strongly impaired cell viability when cells were treated with 0.3 and 0.4 µM of STS (Fig. 4B). These results suggest that PARG-siRNA treatment enhances cell death in conditions that cause PARP1 cleavage.

Discussion

Several experiments concerning expression of the PARG gene that have utilized PARG-siRNA or PARG-shRNA overexpressing systems have been reported (27). Constitutive suppression of PARG gene expression by introducing shRNA into HeLa S3 cells caused accumulation of poly(ADP-ribose) and led to enhancement of mitotic catastrophe by X-ray irradiation (28). It was reported that poly(ADP-ribose) accumulation occurred after a PARG shRNA expression vector was stably introduced into A549 cells, a lung adenocarcinoma cell line (29). Of note, these PARG-suppressed A549 cells exhibited reduced PARP activity. The decrease in poly(ADP-ribose) was
reported in mouse 3T3 embryonic fibroblasts, PARG-A2.3 cells, which express PARG60 but not full-length PARG110 (30). In the present study, a PARG-siRNA pool that contains sequences located upstream of exon 5 was transfected into HeLa S3 cells. Therefore, PARG-siRNA used here may selectively reduce full-length PARG expression but may not affect PARG60. In addition, the suppressing effect by the introduction of 25-100 nM of the PARG-siRNA was approximately 50% of the control (Fig. 1A). This low efficiency might be caused by the reduced poly(ADP-ribose) level in the cells (Fig. 1B).

It has been suggested that poly(ADP-ribose)ylation of Sp1 impairs activation of the PARP1 promoter (16). It has also been reported that PARP1 binds to its own promoter to suppress transcription (31). Since PARP1 physically interacts with Sp1 (16), both may cooperatively regulate transcription of the PARP1 gene. In our study, the introduction of PARG-siRNA into HeLa S3 cells caused suppression of the PARP1 promoter activity and led to subsequent reduction of its gene/protein expression. These data suggest a possible mechanism that prevents cells from accumulating excess poly(ADP-ribose) by suppressing PARP1 promoter activity. It is noteworthy that inverted repeats or putative cruciform-like structures are found both in the PARP1 (31) and PARG (21) promoters. Since PARP1 has been suggested to recognize and bind to the cruciform-like structures of DNA (31), the PARP promoter is possibly one of the targets for PARP1. Moreover, the PARP1 promoter harbors overlapped TTCC motifs as 5′-GGGTTCCCGTTCCCGG-3′ (Fig. 2B and C). The duplicated GGAA (TTCC) motifs, which are also found in the human PARG promoter region (21), have been suggested to be one of the determinants of TSS in TATA-less promoters with responses to various stimuli, including cyto-
kine and differentiation inducing signals (32-34). In addition, previous reports suggested that PARP1 and PARG interact with each other (14) and coordinately regulate global patterns of gene expression, and that the binding of PARP1 to the promoters in the genome is dependent on the presence of PARG (27). Collectively, these observations indicate that the location of PARP1 with PARG on these promoters may play an important role in the regulation of poly(ADP-ribose) metabolism at the transcriptional level. This might be relevant to the cooperative function of PARP1 and PARG to repair single-strand break of DNA (35).

In this study, we showed that the introduction of PARG-siRNA enhances sensitivity to STS but not to MNNG (Fig. 4). Since PARP1 cleavage was detected in STS-treated cells (Fig. 3), the introduction of PARG-siRNA may cause elevation of cell damage by reducing PARP1 expression and lead to complete loss of native PARP1, and induce cell death by apoptosis. On the other hand, cell death induced by MNNG was not further affected by the PARG-siRNA, suggesting that the signals induced by the reduction of PARP1 have been adequately saturated by the MNNG treatment. However, this possibility remains to be elucidated in future analyses.

In conclusion, the data presented in this study provide a new theory that PARP1 gene expression is regulated by the amount of PARG transcripts, suggesting a mechanism to avoid accumulation of excess amounts of poly(ADP-ribose) in a cell by regulating their transcription. Since poly(ADP-ribose) metabolism also controls NAD+ levels, local changes in the concentrations of NAD+ might affect transcription of the PARP1 and PARG genes. Markedly, in accordance with the development of PARG inhibitors (36), the use of the PARG-siRNA may contribute to a treatment of cancer with a reduced dose of anti-cancer drugs or ionizing radiation.

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