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Short communication

Unexpectedly Weak Impacts of Decreased p53 and Retinoblastoma Protein Levels on Mutagenesis by 8-Oxo-7,8-dihydroguanine (8-Hydroxyguanine)

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The loss of tumor suppressor proteins, p53 and retinoblastoma (Rb), causes genomic instability. 8-Oxo-7,8-dihydroguanine (G^O, 8-hydroxyguanine) is a major oxidatively damaged base that induces G:C→T:A transversions in cells. In this study, the effects of p53 and Rb reductions on the mutagenicity of G^O in DNA were investigated, using a *supF* shuttle plasmid propagated in human U2OS and HT1080 cells. The p53 and Rb proteins were individually knocked-down by siRNAs, and then the plasmid DNA containing G^O was introduced into the knocked-down cells. The knock-downs of p53 and Rb had quite weak effects on mutagenesis by G^O in the shuttle plasmid. These results suggested that p53 and Rb minimally affect the G^O-induced mutagenic processes in living cells.

Key words: p53, Rb, 8-oxo-7,8-dihydroguanine, 8-hydroxyguanine

Introduction

DNA is continuously damaged by endogenous and exogenous (environmental) factors, and the damaged bases cause mutations (1). Several tumor suppressor proteins play key roles in preventing the deleterious effects of damaged DNA (2). The p53 tumor suppressor, the guardian of the genome, maintains genomic stability by modulating DNA repair and inducing a cell cycle checkpoint and apoptosis (3–5). Another tumor suppressor, retinoblastoma protein (Rb), arrests cell cycle progression after DNA damage, securing sufficient time for repair (6–8).

Reactive oxygen species (ROS) are important mutagens, and the oxidized bases cause mutagenesis, carcinogenesis, neurodegeneration, and aging (9,10). 8-Oxo-7,8-dihydroguanine (G^O, also known as 8-hydroxyguanine) is one of the major oxidized bases produced by ROS (11,12). The formation of G^O:C in DNA leads to G:C→T:A transversions in mammalian cells (13,14). The G^O base in DNA duplexes is a substrate of mammalian base excision repair (BER) enzymes (DNA glycosylases) *in vitro* (15–20). Moreover, the knock-down of BER enzymes increases the mutation frequency of G^O in human cells (21). Recent studies indicated that p53 stimulates BER (22–28). Thus, p53 is expected to suppress the G:C→T:A transversions caused by the oxidation of G bases in DNA. However, it is unclear whether p53 actually suppresses mutations induced by G^O in mammalian cells. In addition, the Rb protein is essential for cell cycle arrest in response to DNA damaging agents (6–8), and recent evidence has indicated that Rb regulates the nucleotide excision repair of DNA damaged by UV (29–31). However, it is unknown whether Rb is involved in the repair of DNA damaged by ROS. Therefore, it is important to define the roles of the two tumor suppressor proteins, p53 and Rb, in the mutagenesis caused by G^O in cells.

In this study, we examined the effects of p53- and Rb-reductions on the mutations caused by G^O in human cells. We introduced a shuttle plasmid containing a G^O:C pair in the *supF* gene (Fig. 1) into the cells, in which the target protein, p53 or Rb, was knocked-down by siRNAs. The results obtained in this study indicated that these tumor suppressors weakly affect the mutagenesis induced by G^O.

Materials and Methods

Materials: Phosphorylated oligodeoxyribonucleotides containing G^O and their control oligodeoxyribonucleotides (21) were purchased from Nihon BioService (Asaka, Japan) and were purified by HPLC, as described previously (32). Other oligodeoxyribonucleotides were obtained from Hokkaido System

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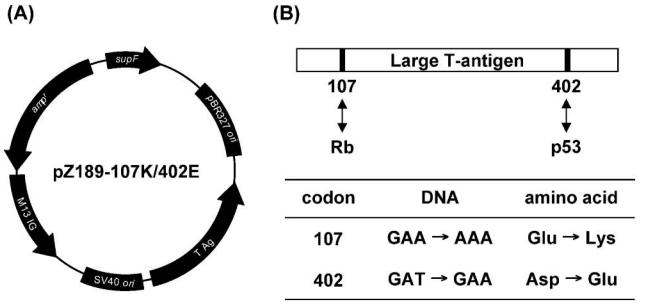

Fig. 1. (A) Structure of the pZ189–107K/402E plasmid. G^O was introduced at position 122 (G^O:C) in the *supF* gene. T Ag represents the SV40 large T antigen gene. (B) The mutant large T antigen, encoded by the gene in pZ189-107K/402E. The 107th and 402nd residues interact with Rb and p53, respectively, in the wt protein. Codons 107 and 402 are shown in the table.

Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan) in purified forms. The Validated Stealth RNAi DuoPak for p53 and the Stealth Select 3 RNAi were obtained from Life Technologies (Carlsbad, CA, USA). The following siRNAs were used: Rb#1 sense, 5′-UCAAGAUUCUGAGAUGUACUUCUGC; Rb#1 antisense, 5′-GCAGAAGUACAUCUCAGAAU CUUGA; Rb#2 sense, 5′-UUCAGUCUCUGCAUGAA GACCGAGU; Rb#2 antisense, 5′-ACUCGGUCUUC AUGCAGAGACUGAA. Stealth RNAi Negative Control Low or Medium duplexes (Life Technologies) were used as the negative controls, according to the recommended GC contents. The KS40/pOF105 *Escherichia coli* strain was provided by Prof. Tatsuo Nunoshiba, of International Christian University, and was used as an indicator strain for the *supF* mutants (33).

Construction of pZ189-107K/402E: Mutations were introduced into the SV40 large T antigen gene of pZ189-StuI (34) by site-directed mutagenesis, using the following mutagenic oligodeoxyribonucleotides: 5′-dTACACCACTGATTCCATTTTGGGC and 5′-dGG CATTTCTTTTGAGCAAAACAG. A DNA sequence analysis confirmed the presence of the expected mutations in codons 107 and 402 of the gene.

Construction of shuttle plasmid containing G^O: The following oligodeoxyribonucleotides were synthesized and chemically phosphorylated on the support: oligo G^O-122, 5′-dCGACTTCGAAGG^{O}TTCGAAT CC; oligo G-122, 5′-dCGACTTCGAAGGTTCGAAT CC. The single-stranded (ss) form of pZ189-107K/402E was obtained as described (35). The oligodeoxyribonucleotides were annealed with the ss form of pZ189-107K/402E, and the double-stranded (ds) DNAs containing G and G^O were obtained as described previously (36).

Mutagenesis experiments for shuttle plasmids containing G^O: U2OS and HT1080 cells (3.0×10^4 and 2.5×10^4 cells, respectively) were plated onto 24-well dishes and were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, at 37°C under a 5% CO_2 atmosphere for 24 h. The siRNA (10 pmol) was mixed with Lipofectamine 2000 (Life Technologies) and introduced into the cultured cells according to the supplier's recommendations. After 24 h, the plasmid containing G^O (100 ng, 29 fmol) was introduced by using Lipofectamine (Life Technologies). After 48 h of culture, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin (37). The recovered DNA was treated with *Dpn* I (New England Biolabs, Ipswich, MA, USA), to digest the unreplicated plasmids.

Determination of *supF* mutant frequency: The DNAs recovered from the cells were introduced into *E. coli* KS40/pOF105 by electroporation, using a Gene Pulser II transfection apparatus with a Pulse Controller II (Bio-Rad, Hercules, CA, USA). The *supF* mutant frequency was calculated according to the numbers of white and faint blue colonies on Luria–Bertani agar plates containing nalidixic acid (50 μg/ml), streptomycin (100 μg/ml), ampicillin (150 μg/ml), chloramphenicol (30 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg/ml), and isopropyl-β-D-thiogalactopyranoside (23.8 μg/ml), and the numbers of colonies on agar plates containing ampicillin and chloramphenicol.

Western blotting: The cells were extracted in radio immuno-precipitation assay buffer containing protease inhibitors. The whole cell extracts were fractionated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in 5% nonfat milk and probed with either a mouse anti-p53 monoclonal antibody (catalogue number sc-126; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a rabbit anti-Rb polyclonal antibody (catalogue number sc-50; Santa Cruz Biotechnology) overnight in Can Get Signal solution 1 (Toyobo, Osaka, Japan). After washing three times in phosphate-buffered saline containing 0.05% Tween 20, the membranes were incubated with either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) for 1 h. The p53 and Rb were then visualized using the Enhanced Chemiluminescence (ECL) System (GE Healthcare Bio-Sciences). The band intensities were determined using the ImageJ program (http://imagej.nih.gov/ij/). Amounts of p53 and Rb were normalized relative to β-actin contained in each sample and values relative to that in cells treated with the control siRNA were calculated.

Results

Knock-downs of p53 and Rb by siRNAs: In this study, siRNAs were used to knock-down p53 and Rb expression in human cells. Negative Control Low and

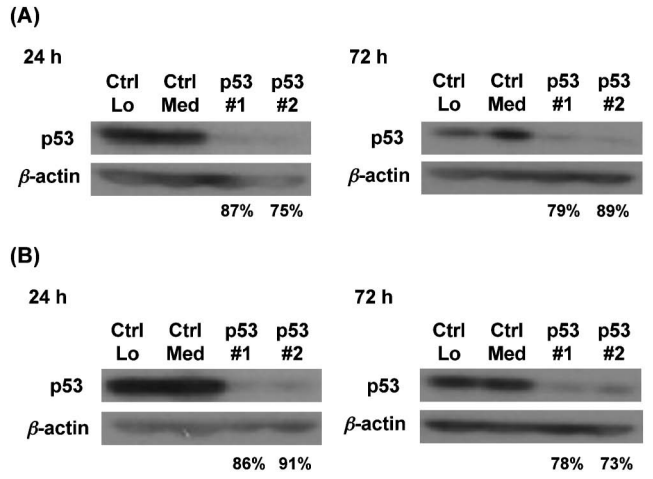

Fig. 2. Knock-down of p53 by siRNAs. The expression levels of p53 in (A) U2OS and (B) HT1080 cells were detected by western blot analyses. Ctrl Lo and Ctrl Med represent Stealth RNAi Negative Control Low and Medium duplexes, respectively, used as the negative controls. Knock-down efficiencies calculated as described in the Materials and Methods section are shown below the images.

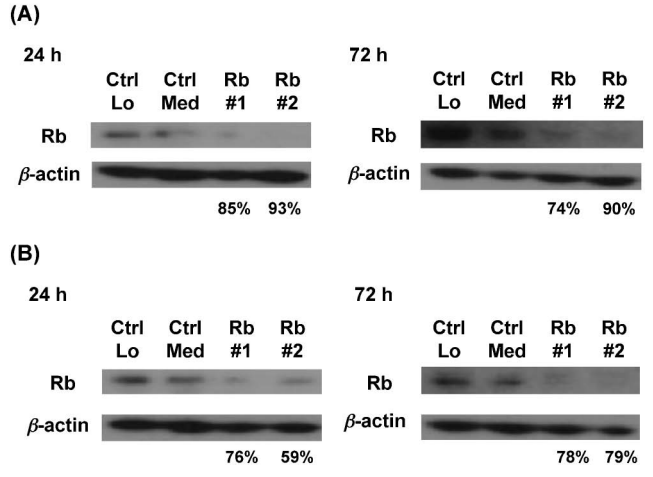

Fig. 3. Knock-down of Rb by siRNAs. The expression levels of Rb in (A) U2OS and (B) HT1080 cells were detected by western blot analyses. Ctrl Lo and Ctrl Med represent Stealth RNAi Negative Control Low and Medium duplexes, respectively, used as the negative controls. Knock-down efficiencies calculated as described in the Materials and Methods section are shown below the images.

Medium GC duplexes were used as control siRNAs. U2OS and HT1080 cells express wild-type (wt) p53 and Rb, and hence were used in this study. The knock-downs of the target proteins were confirmed by western blotting (Figs. 2 and 3). These target proteins were effectively knocked-down from 24 h (the time point of the shuttle plasmid introduction) to 72 h (the time point of the plasmid rescue) after the introduction of the siRNAs into both cell lines.

Effects of p53 and Rb knock-downs on G^O-induced mutations: We previously used a shuttle plasmid

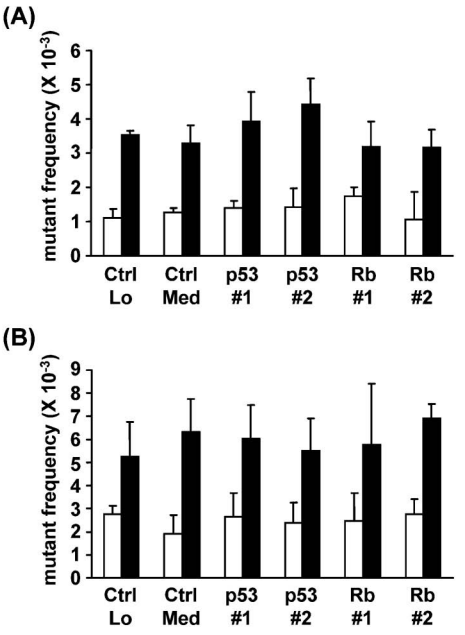

Fig. 4. Mutant frequencies induced by the G^O:C pair in (A) U2OS and (B) HT1080 cells with knocked-down p53 or Rb. Open columns, control plasmid containing G:C at position 122; closed columns, plasmid containing G^O:C at position 122. Data are expressed as means ± SD. Ctrl Lo and Ctrl Med represent Stealth RNAi Negative Control Low and Medium duplexes, respectively, used as the negative controls.

DNA containing the wt SV40 large T antigen gene (pZ189-StuI) to evaluate the roles of cellular proteins on G^O-induced mutations (21,38). The SV40 large T antigen interacts with p53 and Rb and inhibits their functions (39). These effects of the T antigen should be avoided to examine the influence of the p53 and Rb knock-downs. Thus, we constructed a novel shuttle plasmid, pZ189-107K/402E, expressing a mutant SV40 large T antigen (Fig. 1). The E107K and D402E large T antigen mutant is unable to bind p53 and Rb, but retains its replication activity (40). Indeed, the colony numbers of *E. coli* transformed by pZ189-107K/402E recovered from U2OS and HT1080 cells were similar to those obtained by pZ189-StuI recovered from the cells (data not shown). This result indicated that pZ189-107K/402E is normally replicated in these cultured cells.

We then introduced the pZ189-107K/402E plasmids, containing single G:C and G^O:C pairs at position 122 of the *supF* gene (Fig. 1), into cultured U2OS and HT1080 cells with knocked-down p53 and Rb. The replicated DNA was recovered from the treated cells, and then was transfected again into the indicator *E. coli* strain, KS40/pOF105 (33), for the calculation of the mutant frequencies of the *supF* gene. Judging by the numbers of colonies on the titer plates, which semi-quantitatively reflected the amounts of plasmid DNA replicated in the cells, no obvious effects of the p53 and Rb knock-downs

were observed (data not shown). Thus, the knock-downs of these proteins did not severely inhibit the replication of the plasmid DNA containing the G^O base. The observation that the numbers of cells at 48 h after siRNA treatment were similar for the knocked-down and control cells supports this conclusion (data not shown).

The knock-downs of p53 and Rb did not affect the mutant frequencies of the control plasmid containing the G:C pair in U2OS and HT1080 cells (Fig. 4). The mutant frequencies of the plasmid containing the G^O:C pair were higher than those of the control plasmid in both cells, indicating the induction of mutations by G^O during replication. Unexpectedly, however, the knock-downs of p53 and Rb affected the mutant frequencies by the G^O:C pair only weakly in both cell lines. Although the G^O-induced mutant frequency in the p53-knocked-down U2OS cells appeared slightly higher than that in the control cells, the difference was statistically insignificant (Student's *t*-test). These results indicated that the reductions of p53 and Rb had minimal effects on the mutagenesis by G^O in DNA.

Discussion

In this study, we investigated whether the knock-downs of the p53 and Rb tumor suppressors affected the mutagenesis induced by G^O in DNA. Since the loss of these proteins causes genomic instability (41,42), we expected that their reduction would increase the G^O-induced mutation frequency. However, their knock-downs did not affect the mutation frequency in both U2OS and HT1080 cells (Fig. 4). This result showed that p53 and Rb have quite weak effects on the mutations induced by G^O, produced by the direct oxidation of DNA.

The p53 protein is known to enhance BER activity (22–28). For example, p53 significantly enhances the sequential activities of OGG1 and APE in the removal of the G^O base from DNA *in vitro* (27). Moreover, the knock-downs of BER enzymes, including OGG1, increased the mutation frequency of G^O in human cells (21). However, our results suggested that p53 does not suppress mutations by G^O. The stimulation of BER by p53 might require oxidative stress, such as ROS, in addition to the increase in DNA damage. Actually, G^O in DNA accumulated in p53-deficient cells treated with hydrogen peroxide, as compared to wt cells (27). Thus, p53 might not have a significant impact on BER, unless the cells are exposed to exogenous stresses. Likewise, the reduction of Rb expression did not affect the mutant frequency induced by G^O in DNA. Thus, Rb has a minimal effect on mutagenesis by G^O, suggesting that it might not be involved in the BER process of G^O.

The p53 and Rb proteins are also known to suppress mutations by inducing a cell cycle checkpoint in response to DNA damage (3,6–8). However, our results indicated that the expected function of p53 and Rb, induction of cell cycle checkpoints, does not suppress G^O-induced mutations at least under our experimental conditions. First, the checkpoint by p53 or Rb may not be induced by damaged bases in episomal DNA, in contrast to those in genomic DNA. Alternatively, U2OS and HT1080 cells may lack the normal cell cycle checkpoint regulated by the p53-and Rb-dependent signal pathways. Although these cells express wt p53 and Rb, they are cancer cell lines. These cells might have aberrations in other genes involved in cell cycle regulation. As described in the Results section, the numbers of colonies on the titer plates that semi-quantitatively reflected the amounts of plasmid DNA replicated in the cells were similar in the control, and p53-and Rb-knocked-down cells, supporting these interpretations.

As shown in Fig. 4, G^O-induced mutant frequency was less than 1%, although G^O is known as a ''highly mutagenic base''. Two important factors determining the mutation frequency are the ratio of A to C incorporated opposite G^O by DNA polymerases and removal efficiency of G^O by DNA repair enzymes. We previously showed that the mutant frequency was affected when specialized DNA polymerases and DNA repair enzymes were knocked-down (21,38). Thus, the mutation frequency is dependent on activities of these proteins in cells. Comprehensive analysis of the important proteins would provide insight about mutagenicity of G^O in various cell lines and animal tissues.

In this study, we showed that the p53 and Rb tumor suppressors play minor roles in the suppression of mutations caused by G^O:C in human cells. Further studies are necessary to address the reasons for these unexpected findings.

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