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The role of DNA polymerase ζ in translesion synthesis across bulky DNA adducts and cross-links in human cells

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ABSTRACT

Translesion DNA synthesis (TLS) is a cellular defense mechanism against genotoxins. Defects or mutations in specialized DNA polymerases (Pols) involved in TLS are believed to result in hypersensitivity to various genotoxic stresses. Here, DNA polymerase ζ (Pol ζ)-deficient (KO: knockout) and Pol ζ catalytically dead (CD) human cells were established and their sensitivity towards cytotoxic activities of various genotoxins was examined. The CD cells were engineered by altering the DNA sequence encoding two amino acids essential for the catalytic activity of Pol ζ, i.e., D2781 and D2783, to alanines. Both Pol ζ KO and CD cells displayed a prolonged cell cycle and higher incidence of micronuclei formation than the wild-type (WT) cells in the absence of exogenous genotoxic treatments, and the order of abnormality was CD > KO > WT cells. Both KO and CD cells exhibited higher sensitivity towards the killing effects of benzo[a]pyrene diol epoxide, mitomycin C, potassium bromate, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and ultraviolet C irradiation than WT cells, and there were no differences between the sensitivities of KO and CD cells. Interestingly, neither KO nor CD cells were sensitive to the cytotoxic effects of hydrogen peroxide. Since KO and CD cells displayed similar sensitivities to the genotoxins, we employed only KO cells to further examine their sensitivity to other genotoxic agents. KO cells were more sensitive to the cytotoxicity of 4-nitroquinoline *N*-oxide, styrene oxide, cisplatin, methyl methanesulfonate, and ethyl methanesulfonate than WT cells. However, the KO cells displayed sensitivity camptothecin, etoposide, bleomycin, hydroxyurea, crotonealdehyde, and methylglyoxal in a manner similar to the WT cells. Our results suggest that Pol ζ plays an important role in the protection of human cells by carrying out TLS across bulky DNA adducts and cross-links, but has no or limited role in the protection against strand-breaks in DNA.

1. Introduction

The human genome is continuously exposed to various genotoxic stresses, the origins of which can be external or internal. To circumvent this DNA damage induced by the genotoxic stresses, cells possess a variety of defense mechanisms. Translesion DNA synthesis (TLS) is a defense mechanism where specialized DNA polymerases (Pols) carry out replication of DNA across DNA damage sites [1–3]. TLS is crucial to protect the cells from DNA damage because replicative Pols are unable to continue DNA replication beyond these lesions [4,5]. Inactivation of the specialized Pols confers hypersensitivity to genotoxic stresses [6,7]. However, the fidelity of the specialized Pols is markedly lower than that of the replicative Pols [8,9]. Therefore, TLS by the specialized Pols often accompanies sequence changes at or around the lesions, which, if left unrepaired, are carried forward in the next round of DNA replication [10].

Pol ζ is a specialized Pol that plays an important role in TLS [6]. Pol ζ belongs to B-family of Pols and is a heterodimer of REV3L, the catalytic subunit, and the auxiliary protein REV7, which associates with REV1 [11]. In addition, two accessory subunits of Pol δ, i.e., PolD2 and PolD3, are likely members of the active complex of Pol ζ [12]. Unlike yeast Rev3, which plays an important role in ultraviolet light (UV)-induced mutagenesis, human REV3L has a large exon encoding more than 1300 amino acids, the functions of which are not fully understood yet [12–15]. Knockout (KO) of the *Rev3l* gene resulted in embryonic lethality in mice, and increased

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chromosome aberrations in embryonic fibroblasts from the KO mice [16–18]. Deficiency of REV3L in human cells prolongs cell growth and increases chromosome aberrations even in the absence of genotoxic treatments [19]. REV3L-KO in chicken DT40 cell lines increases sensitivity to the cytotoxicity of genotoxic stresses such as UV irradiation, cisplatin (CisPt), and methyl methanesulfonate (MMS) [20]. Moreover, the protective roles of Pol ζ against genotoxic stresses have also been examined with several mutagens and UV irradiation [21–26]. However, its role has not been thoroughly investigated in human cells mainly because Pol ζ KO human cells are not easily available. In fact, the inactivation of REV3L in some of the human REV3L KO cell lines has been questioned [15,19]. Mouse embryonic fibroblasts lacking Pol ζ are available, but are deficient in p53 functions [27]. It is reported that the mouse cells do not exhibit similar TLS efficiency and accuracy as human cells [28].

To better understand the protective roles of human Pol ζ against genotoxic stresses, we engineered REV3L-KO and catalytically dead (CD) REV3L mutant human cell lines and examined their sensitivity to cytotoxicities of 16 genotoxic chemicals and UV-C irradiation. To this end, we used human Nalm-6 cells, which possess exceptionally high gene targeting efficiencies [29–33]. In addition, Nalm-6 cells possess normal p53 functions and near diploid karyotype. The CD mutant of Pol ζ was generated by knock-in of a targeting sequence that directs amino acid changes of both aspartates 2781 and 2783 to alanines (D2781A, D2783A). These amino acids are responsible for the binding to magnesium ions and are essential for the catalytic activities of Pol ζ. We generated a CD mutant because we postulated that Pol ζ, in particular the middle region, might have structural roles such as interaction with other proteins [15]. If so, the results with simple KO or knock down cells may not faithfully represent the protective roles of TLS mediated by Pol ζ. In fact, the REV3L CD cells generated in this study displayed more severe phenotypes than KO cells in the absence of genotoxic stresses. Recently, a non-catalytic role has been reported for human DNA pol κ in protection against hydrogen peroxide [34]. The present results also indicated that Pol ζ protects human cells from genotoxic stresses that induce bulky DNA lesions and cross-links, but not those that induce strand breaks in DNA. Furthermore, we discuss the specificity of the protective roles of Pol ζ in human cells against endogenous and exogenous genotoxic stresses.

2. Materials and methods

2.1. Cell culture and transfection

Nalm-6 cells were cultured in RPMI1640 supplemented with 10% calf serum, 50 μg/mL kanamycin, and 50 μM 2-mercaptoethanol at 37 °C under a 5% CO_2 atmosphere. Cells were transfected with DNA constructs by using Nucleofector I (LONZA) as previously described [35]. Transfected cells were cultured for 48 h at 37 °C and then the optimum numbers of the cells were seeded onto 96-well plates in medium containing 0.5 μg/mL puromycin (Wako Pure Chemical Industries).

2.2. Establishment of REV3L mutant cells

We have established heterozygous mutants of REV3L KO and REV3L CD cells as previously described [35]. To establish homozygous mutant cells, we introduced the targeting vectors with the puromycin-resistance gene and then the drug-resistance genes were excised by introduction of a Cre recombinase expression vector. The absence of exon 5 in the mRNA of REV3L in the homozygous KO cells was confirmed by RT-PCR using primers of REV3 Fw (5′-GGATAGTGACTGCAGACTACT-3′) and Rv (5′-GCCATGTAGATGAAGACATGTCT-3′). The presence of the designed mutations in the *REV3L* gene in the homozygous CD cell was demonstrated by RT-PCR followed by DNA sequencing, as previously described [35].

2.3. Doubling time and spontaneous micronuclei frequency

Cells were seeded at a concentration of 1×10^5 cells/mL. The cell number was counted every 24 h using a Coulter Counter Z2 (Beckman Coulter). The doubling times were calculated from the slopes of the growth curves. For the micronucleus assay, approximately 1×10^6 cells suspended in 0.075 M KCl solution were incubated for 6 min at room temperature, fixed twice with ice-cold Carnoy's fluid (75% methanol, 25% acetic acid), then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/mL acridine orange solution and immediately observed with a Olympus BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact cells were examined, and the cells containing micronuclei were scored.

2.4. Cytotoxicity assay

We seeded cells into 96-well plates at 20,000 cells/100 μL/well in the presence of serially diluted concentrations of genotoxic chemicals and cultured for 48 h at 37 °C under a 5% CO_2 atmosphere. UV-C irradiation was performed with a germicidal lamp (Toshiba GL10). The UV-C dose was elevated by increasing exposure time. UV-C irradiated cells were cultured for 48 h at 37 °C. After the treatments, 10 μL of Cell Counting Kit8 solution (DOJINDO) was added into each well and then incubated for 4 h at 37 °C. The absorbance at 450 nm was measured and the cell viability was calculated. The names of chemicals, CAS number, and supplier used in this study are listed in Supplementary Table 1. The half maximal inhibitory concentration (IC_{50}) of cell growth was determined by non-linear regression analysis.

2.5. Statistical analysis

Statistical significance was examined by the Student's *t*-test with a Bonferroni correction. Levels of $P < 0.05$ were considered to be significant.

3. Results

3.1. Establishment of REV3L mutants

The KO cell line was generated by replacing of both alleles of exon 5 of *REV3L* with the drug-resistance genes (Supplementary Fig. 1A, [35]). The CD mutation was introduced into both alleles of exon 30, which resulted in the substitution of amino acids in the catalytic site of Pol, i.e., D2781A/D2783A (Supplementary Fig. 1B, [35]). Transcription of *REV3L* gene in KO and CD cells was analyzed by RT-PCR and DNA sequencing. We confirmed the absence of exon 5 of REV3L in the mRNA in the KO clone (Fig. 1A). The cDNA sequence of the *REV3L* gene of the CD clone indicated that the knock-in allele was transcribed (Fig. 1B). We obtained 9 heterozygous clones out of 36 hygromycin-resistant clones for KO cells and 9 heterozygous mutants that exhibited sensitivity to *Nar*I out of 68 hygromycin-resistant clones for CD cells by the first targeting (Supplementary Table 2). In addition, we obtained 6 homozygous clones out of 295 puromycin-resistant clones for KO cells and 1 homozygous mutant that exhibited sensitivity to *Nar*I out of 518 puromycin-resistant clones for CD cells by the second targeting (Supplementary Table 2). Then, the drug-resistance genes of KO and CD cells were removed by transient expression of Cre recombinase (Supplementary Fig. 3). The growth of REV3L KO and CD cells was slower than the wild-type