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Multiple repair pathways mediate cellular tolerance to resveratrol-induced DNA damage

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ABSTRACT

Resveratrol (RSV) has been reported to exert health benefits for the prevention and treatment of many diseases, including cancer. The anticancer mechanisms of RSV seem to be complex and may be associated with genotoxic potential. To better understand the genotoxic mechanisms, we used wild-type (*WT*) and a panel of isogenic DNA-repair deficient DT40 cell lines to identify the DNA damage effects and molecular mechanisms of cellular tolerance to RSV. Our results showed that RSV induced significant formation of γ-H2AX foci and chromosome aberrations (CAs) in *WT* cells, suggesting direct DNA damage effects. Comparing the survival of *WT* with isogenic DNA-repair deficient DT40 cell lines demonstrated that single strand break repair (SSBR) deficient cell lines of *Parp1*$^{-/-}$, base excision repair (BER) deficient cell lines of *Polβ*$^{-/-}$, homologous recombination (HR) mutants of *Brca1*$^{-/-}$ and *Brca2*$^{-/-}$ and translesion DNA synthesis (TLS) mutants of *Rev3*$^{-/-}$ and *Rad18*$^{-/-}$ were more sensitive to RSV. The sensitivities of cells were associated with enhanced DNA damage comparing the accumulation of γ-H2AX foci and number of CAs of isogenic DNA-repair deficient DT40 cell lines with *WT* cells. These results clearly demonstrated that RSV-induced DNA damage in DT40 cells, and multiple repair pathways including BER, SSBR, HR and TLS, play critical roles in response to RSV- induced genotoxicity.

1. Introduction

Resveratrol (3, 4′, 5-trihydroxy stilbene; RSV), a naturally occurring polyphenol found in a variety of food and medicinal plants (Sobolev and Cole, 1999; Sanders et al., 2000), has been shown to induce apoptotic cell death in tumour cells but not in normal cells (Clement et al., 1998; Baarine et al., 2011). Numerous studies have shown consistent anticancer effects of RSV in a variety of cancer cell lines, including colon (Fuggetta et al., 2006; Demoulin et al., 2015), lung (Luo et al., 2013), breast (Kim et al., 2004), pancreatic (Cui et al., 2010) and glioblastoma (Leone et al., 2010) cancer cells. RSV is potent against cancer in both chemoprevention and chemotherapy (Jang et al., 1997). The mechanisms of anticancer activity of RSV seem to be complex. Studies have shown that RSV could inhibit the hydroperoxidase activity of type 1 cyclooxygenase (Jang et al., 1997), and act as a phytoestrogen (Gehm et al., 1997). In addition, most studies have indicated that RSV exerted an antiproliferative activity via the induction of cell cycle arrest (Ragione et al., 1998; Zhou et al., 2009) and apoptosis (Kim et al., 2004). The cell cycle arrest induced by RSV was also reported to be associated with its genotoxic potential (Ragione et al., 1998; Zhou et al., 2009). Numerous studies have shown that RSV could induce DNA damage, including DNA strand breaks (Hadi et al., 2010; Ullah et al., 2013; Arif et al., 2015), micronuclei (Schmitt et al., 2002), chromosomal aberrations and sister chromatid exchanges (Matsuoka et al., 2001; Matsuoka et al., 2002). However, seemingly contradictory results of RSV inducing DNA damage were also reported with respect to dose, time of application and cell types (De Salvia et al., 2002; Signorelli and Ghidoni, 2005; Stopper et al., 2005; Gatz and Wiesmuller, 2008). The underlying mechanisms of RSV in genotoxicity remain elusive.

The DT40 cell line has a remarkable advantage for determining genotoxicity. In DT40 cells, approximately 70% of the whole cell cycle time is in the S phase. DNA damage often leads to the formation of DNA double strand breaks (DSBs) when they encounter DNA replication forks. The formation of DSBs is more frequent in DT40 cells than in

Abbreviations: HR, homologous recombination; TLS, translesion DNA synthesis; SSBR, single-strand break repair; BER, base excision repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PPR, postreplication repair; DSBs, double- strand breaks; *WT*, wild-type; CAs, chromosome aberrations

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Table 1
DNA repair genes mutated in the analysed DT40 clones.

Gene	Function	Reference
Rev3	TLS[a], HR[b] (catalytic subunit of Polξ)	Sonoda et al., 2003
Parp1	Poly(ADP) ribosylation, related to single-strand and base excision repair	Masson et al., 1998
Rad18	TLS	Okada et al., 2002
Brca1	HR	Qing et al., 2011
Brca2	HR	Hatanaka et al., 2005
Fen1	Base excision repair, processing 5′flap in long-patch and lagging strand DNA replication	Asagoshi et al., 2010
Ubc13	The initial step of HR and postreplication repair	Zhao et al., 2007
Ku70	Initial step for NHEJ[c] dependent DSB[d] repair, associated with DNA-PK catalytic subunit	Takata et al., 1998
Polβ	Base excision repair	Prasad et al., 2012
XPA	An initial step of nucleotide excision repair	Liu et al., 2012

[a] TLS, translesion DNA synthesis.
[b] HR, homologous recombination.
[c] NHEJ, non-homologous end joining repair.
[d] DSB, double-strand break.

mammalian cell lines due to the very high proportion of the cell cycle spent in S phase (Qing et al., 2011). Therefore, DNA damage can be more sensitively identified in DT40 cells than in mammalian cells by counting individual DSBs. DSBs can be reliably identified by detection of γ-H2AX foci in DT40 cells (Dickey et al., 2009). In addition, the DT40 cell line possesses stable diploid genomes, which make it more convenient to identify the CAs in mitotic cells (Dodgson and Romanov, 2004). Furthermore, DT40 cell lines have well-studied DNA repair properties. The lack of different repair pathways leads to enhanced DNA damage and cellular proliferation inhibition in response to genotoxic chemicals. Isogenic DNA-repair deficient DT40 cell lines are a promising tool for sensitive and mechanism-specific screening of genotoxicity of chemicals and environmental samples (Ji et al., 2009; Evans et al., 2010; Ji et al., 2011a, 2011b). To help elucidate the molecular mechanism of genotoxicity of RSV, in this study, we determined the genotoxic potential of RSV by both the immunocytochemical analysis of γ-H2AX and CAs analysis. We also investigated the essential roles of DNA repair pathways including BER, SSBR, nucleotide excision repair (NER), non-homologous end joining (NHEJ) and two postreplication repair (PPR) pathways, HR and TLS, in response to RSV-induced DNA damage.

2. Materials and methods

2.1. Chemicals

The authentic standard of resveratrol (purity ≥ 99%), camptothecin (CPT) (purity ≥ 99%) were obtained from MedChem Express (Princeton, NJ, USA). Stock solution of RSV (25 mM) and CPT (100 μM) were prepared in DMSO and stored at − 20 °C in aliquots until use.

2.2. Cell lines and cell culture

WT and mutant DT40 cell lines were used in the study (mutant genes are listed in Table 1). The phenotypes of *WT* and isogenic DNA-repair deficient DT40 cell lines have been reported previously. Both *WT* and mutant cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, NY, USA), 1% chicken serum (Gibco, NY, USA), 1% penicillin streptomycin (Gibco, NY, USA) and 50 μM β-mercaptoethanol (Gibco, NY, USA) at 39 °C in a humidified atmosphere of 5% CO_2 (Sanyo, Osaka, Japan).

2.3. Cell viability assay

Cell viability was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay according to previously established methods (Carmichael et al., 1987). Briefly, the cells were plated in 96-well-plates (Costar Corning, Rochester, NY, USA) at a density of 5×10^4 cells/mL. The cells were incubated in the presence of various concentrations of RSV or CPT for 72 h, and DMSO (< 0.1%) was applied as control. At the end of the treatment time, 20 μL of 5 mg/mL MTT (Amresco, USA) was added, and the synthesized formazan dye crystals were dissolved in 50 μL of SDS over night. The absorbance was measured at 570 nm using a microplate reader, and the wells without cells were used as blanks. The 50% inhibiting concentration (IC50) was calculated by SPSS software version 13.0. All the experiments were conducted at least three times.

2.4. Chromosome aberration analysis

Karyotype analysis was performed as described previously (Sonoda et al., 1998). Briefly, cells were treated with RSV in the complete medium. Colcemid (0.1%) (Gibco-BRL, Grand Island, NY, USA) was added 3 h before harvest to arrest cells in metaphase. We suspended the cells in 1 mL of 75 mM KCl for 15 min at room temperature, and then fixed the cells with 5 mL of Carnoy's solution (mixture of acetic acid and methanol, 1:3) for 30 min. The cell suspension was then dropped onto ethanol-washed glass slides and dried by a flame. The dried slides were stained with 5% Giemsa solution and rinsed carefully with water before being air-dried. All the experiments were performed in triplicate. The chicken karyotype contains 80 chromosomes, including 11 major autosomal macrochromosomes, ZW sex chromosomes, and 67 microchromosomes (Yamamoto et al., 2011). In the present study, 50 metaphase cells each experiment were analysed by light microscope (with 1000 × magnification) with scoring limited to the 11 major macrochromosomes and the Z chromosome (Yamamoto et al., 2011). According to the International System for Human Cytogenetic Nomenclature (ISCN), a break is identified as a discontinuity of a chromosome that shows a clear misalignment of the distal fragment, and a gap is defined as a clear non-staining region on a chromosome that is equal to or less than the width of a chromatid (Ji et al., 2009).

2.5. Immunofluorescent analysis

Immunofluorescent analysis was conducted as described previously (Jiang et al., 2016). Briefly, the DT40 cell lines were harvested on glass slides after incubating with RSV for different time. The cells were fixed with 3% formaldehyde for 10 min at room temperature following by washing with PBS. The formaldehyde-fixed cells were permeabilized with 0.1% NP-40 for 15 min and washed with PBS. After blocking with 3% BSA for 30 min, the cells were incubated with anti-phospho-Histone H2AX (Ser139) mouse monoclonal antibody (1:500; Millipore, Billerica, MA, USA), then washed with PBS again and incubated with the secondary Cy3-labeled anti-mouse antibody (1:1000; Beyotime, Wuhan, China).

2.6. Statistical analysis

Statistical significance of differences was determined by Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.