Free Radical Biology & Medicine 48 (2010) 1197–1201

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Suppression of mutagenesis by 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (7,8-dihydro-8-oxo-2′-deoxyguanosine 5′-triphosphate) by human MTH1, MTH2, and NUDT5

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ARTICLE INFO

Article history:
Received 19 November 2009
Revised 25 January 2010
Accepted 1 February 2010
Available online 6 February 2010

Keywords:
8-Hydroxy-dGTP
Nucleotide pool sanitization enzymes
Oxidative mutagenesis
Nucleotide pool
Free radicals

ABSTRACT

To assess the functions of the three human MutT-type enzymes, MTH1, MTH2, and NUDT5, mutation induction by an oxidized form of dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP; 7,8-dihydro-8-oxo-2′-deoxyguanosine 5′-triphosphate), was examined using human 293T cells treated with their specific siRNAs. Shuttle plasmid DNA containing the *supF* gene was first transfected into the cells, and then 8-OH-dGTP was introduced by means of osmotic pressure. *Escherichia coli* cells were transformed with the DNAs replicated in the treated cells. The knockdown of the MTH1, MTH2, and NUDT5 proteins increased the A:T → C:G substitution mutations induced by 8-OH-dGTP. In addition, the increase in the induced mutation frequency was more evident in the triple-knockdown cells. These results indicate that all three of the human MTH1, MTH2, and NUDT5 proteins act as a defense against the mutagenesis induced by oxidized dGTP.

Normal cellular metabolism produces endogenous reactive oxygen species (ROS). ROS are generated as by-products of the mitochondrial electron transport chain, and certain cellular enzymes also generate ROS. Moreover, ROS are produced by environmental mutagens/carcinogens, including ionizing radiation and ultraviolet light. The formation of ROS leads to the oxidation of cellular components and disturbs their normal functions. The formation of oxidized DNA lesions is one of the causative factors of mutagenesis, carcinogenesis, neurodegeneration, and aging [1–5].

DNA precursors (2′-deoxyribonucleotides) are also subject to oxidative damage. The formation of oxidized DNA precursors is a potential source of mutagenesis [6]. 8-Hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP; 7,8-dihydro-8-oxo-2′-deoxyguanosine 5′-triphosphate) is the major oxidation product of dGTP in in vitro oxidation reactions [7]. 8-OH-dGTP was reportedly present at a concentration of 1–10% relative to the unmodified dGTP in the mitochondrial nucleotide pool [8]. This oxidized form of dGTP is highly mutagenic in living cells when added exogenously [9–11] and is expected to act as an endogenous mutagen.

Nucleotide pool sanitization is an important means by which organisms prevent the mutagenesis caused by damaged DNA precursors [6,12]. The *Escherichia coli* MutT (NudA) protein, encoded by the mutator gene *mutT*, was the first enzyme found to degrade an oxidized DNA precursor (8-OH-dGTP) in vitro [13]. Moreover, at least two *E. coli* MutT-type proteins, Orf135 (NudG) and Orf17 (NudB), in addition to MutT, catalyze the hydrolysis of oxidized DNA precursors in vitro [14,15]. *E. coli* strains lacking MutT and Orf135 exhibit a mutator phenotype [16–19], indicating the importance of nucleotide pool sanitization to prevent mutagenesis by oxidized DNA precursors. Mammalian cells also possess MutT-type enzymes. The MTH1 (NUDT1) protein catalyzes the hydrolysis of various oxidized DNA precursors, including 8-OH-dGTP, and greater numbers of tumors were formed in the lungs, livers, and stomachs of MTH1-deficient mice than in wild-type mice [20,21]. MTH2 (NUDT15) degrades 8-OH-dGTP in vitro, and the expression of the cDNA encoding MTH2 significantly reduced the elevated spontaneous mutation frequency in *E. coli mutT* cells [22]. The NUDT5 protein is unique, because the hydrolysis of 8-hydroxy-2′-deoxyguanosine 5′-diphosphate (8-OH-dGDP; 7,8-dihydro-8-oxo-2′-deoxyguanosine 5′-diphosphate) by this protein is much more efficient than that of 8-OH-dGTP [23,24]. However, the expression of NUDT5 in *mutT*-deficient *E. coli* mutant cells decreases the spontaneous mutation frequency to the normal level, suggesting the importance of its 8-OH-dGDPase activity in nucleotide pool sanitization [23]. Thus, it is quite important to examine whether the three proteins prevent the mutations induced by 8-OH-dGTP in mammalian (human) cells.

Recently, we showed that 8-OH-dGTP specifically induced A:T → C:G transversions in live simian and human cells [10,11]. In this study, to assess the contributions of these MutT-type proteins to the prevention of the mutagenesis caused by 8-OH-dGTP in human cells, we suppressed their expression by siRNAs and introduced 8-OH-dGTP into the knocked-down cells. The knockdowns of all of the

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doi:10.1016/j.freeradbiomed.2010.02.002

MutT-type proteins enhanced the A:T → C:G substitution mutations, suggesting their roles as functional nucleotide pool sanitization enzymes.

Materials and methods

Materials

8-OH-dGTP was purchased from TriLink BioTechnologies (San Diego, CA, USA). dGTP (FPLC grade) was obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Purified oligonucleotides were from Sigma Genosys Japan (Ishikari, Japan). siRNAs ("stealth RNAi"; Invitrogen, Carlsbad, CA, USA) were synthesized according to the BLOCK-iT RNAi Designer software, on the supplier's Web site. The following siRNAs were used: MTH1 sense, 5′-AUCUGAUCCAGCUG-GAACCAGCAUG; MTH1 antisense, 5′-CAUGCUGGUUCCAGCUGGAU-CAGAU; MTH2 sense, 5′-UUCCCAAGGAACCCACUCCCAACUU; MTH2 antisense, 5′-AAGUUGGGAGUGGGUUCCUUGGGAA; NUDT5 sense, 5′-AGAAGAUUCCGUUGGUUCUUGGCUC; NUDT5 antisense, 5′-GAGC-CAAGAACCAACGGAAUCUUCU. Negative Control Medium GC Duplex was used as a control siRNA (Invitrogen). The pZ189-StuI plasmid was constructed in our laboratory [11]. The *E. coli* strain KS40/pOF105 [25] was provided by Dr. Tatsuo Nunoshiba, of International Christian University, and was used as an indicator strain of the *supF* mutants.

Mutagenesis experiments

293T cells (3×10^4 cells) were plated into 24-well dishes and were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, at 37°C under a 5% CO_2 atmosphere for 24 h. siRNAs (7.2 pmol each) were mixed with Lipofectamine (Invitrogen) and introduced into the cultured 293T cells according to the supplier's recommendations. In the triple-knockdown experiment, an siRNA cocktail (MTH1, 3.6 pmol; MTH2, 7.2 pmol; and NUDT5, 3.6 pmol) was used. After 24 h, the pZ189-StuI plasmid (58 fmol, 200 ng) was mixed with Polyfect (Qiagen, Hilden, Germany) and transfected into the cultured 293T cells according to the supplier's recommendations. After 24 h, 8-OH-dGTP (2.4 pmol) was introduced by osmotic pressure, using a buffer solution (30 mM KCl, 10 mM Hepes–NaOH, pH 7.4), according to the literature [26]. After 24 h of culture, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin [27]. The recovered DNA was treated with DpnI, to digest the unreplicated plasmids.

The plasmids recovered from the 293T cells were transfected into *E. coli* KS40/pOF105 cells 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 pale 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, as described in Ref. [25].

Quantitative RT-PCR analysis of mRNA

Total RNA was extracted from 293T cells using an RNeasy Mini Kit (Qiagen) combined with RNase-free DNase I (Takara, Otsu, Japan), for the degradation of the genomic DNA in the total RNA samples. First-strand cDNA synthesis was performed with 500 ng of total RNA using an oligo(dT) primer and an RNA PCR kit (AMV) (Takara), according to the manufacturer's instructions. Each of the mRNA transcripts was measured by a quantitative PCR method with an ABI 7500 real-time PCR system and SYBR green chemistry (Applied Biosystems, Foster City, CA, USA) using the following primer sets: MTH1 upper, 5′-dAGGAGGAGAGCGGTCTGACA; MTH1 lower, 5′-dCACGAACTCAAACACGATCTG; MTH2 upper, 5′-dGAAAGGAGAA-GTGGATGTGAC; MTH2 lower, 5′-dGGAACCCACTCCCAACTTTC; NUDT5 upper, 5′-dGTTCTCCAGCGGTCTGTATG; NUDT5 lower, 5′-dCTTCGGCCTTGCGTTTTCG. Data are expressed as the ratio to the GAPDH mRNA, which was determined using the following primers: GAPDH upper, 5′-dAACTTTGGTATCGTGGAAGG; GAPDH lower, 5′-dGTCTTCTGGGTGGCAGTGAT.

Table 1
Amount of mRNA in cells with knocked-down nucleotide pool sanitization enzymes

siRNA	48 h	72 h
MTH1	0.21 ± 0.02 (79%)	0.27 ± 0.03 (73%)
MTH2	0.24 ± 0.03 (76%)	0.32 ± 0.04 (68%)
NUDT5	0.10 ± 0.02 (90%)	0.17 ± 0.03 (83%)

The amount of mRNA was measured by quantitative RT-PCR at 48 and 72 h after siRNA introduction. The amount of mRNA was normalized relative to the human GAPDH mRNA contained in each sample. Values relative to those in 293T cells treated with the control siRNA are shown. Data are expressed as means ± SEM ($n=4$). The values in parentheses show knockdown efficiencies.

Statistical analysis

Statistical significance was examined using the Student *t* test. Levels of $P<0.05$ were considered significant.

Results

Knockdown of MutT-type enzymes enhances the induced mutations

Recently, we introduced 8-OH-dGTP into human cells and found that it specifically induced A:T → C:G transversion mutations [11]. We first examined the effects of the single knockdown of MTH1, MTH2, and NUDT5 by siRNAs. These knockdowns were measured by quantitative RT (reverse transcription)-PCR (Table 1). The treatment with siRNA reduced the amounts of mRNAs encoding these MutT-type proteins.

When unmodified dGTP was introduced by means of osmotic pressure, the observed *supF* mutation frequency was $\sim1\times10^{-4}$ in the cells treated with the control siRNA (Fig. 1). The mutation frequencies upon treatment with siRNAs against MTH1, MTH2, and NUDT5 were similar to that of the control cells, indicating that the reduced amounts of these proteins did not affect the background mutation frequency in this experimental system. The introduction of 8-OH-dGTP enhanced the total mutation frequency to $\sim4\times10^{-4}$ in the cells treated with the

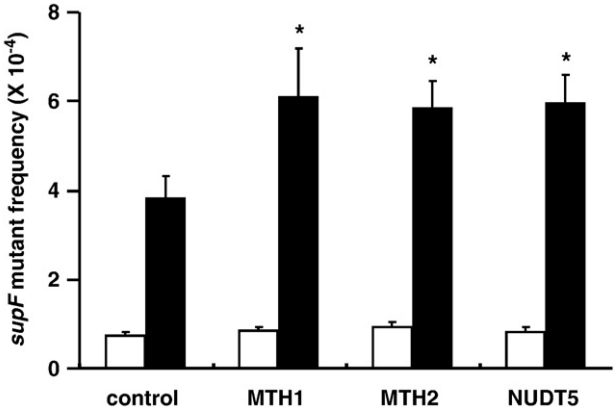

Fig. 1. Effects of knockdown of MTH1, MTH2, and NUDT5 on 8-OH-dGTP-induced mutation frequency. Open columns, dGTP introduction; closed columns, 8-OH-dGTP introduction. Data are expressed as means ± SEM ($n=6$ except for control siRNA, dGTP ($n=5$)). *$P<0.05$ vs control.