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Overexpression of human NTH1 DNA glycosylase and its catalysis-defective mutant in HeLa cells: Effect on sensitivity to oxidative and alkylation DNA damage

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Human NTH1 (hNTH1) is a DNA glycosylase that acts on oxidatively damaged pyrimidine bases such as thymine glycol, at an initial step of base excision repair. In the present study, we established HeLa cell lines overexpressing wild-type hNTH1 and a catalysis-defective mutant hNTH1, to study the roles of hNTH1 in thymine glycol repair *in vivo*. Expression of hNTH1 in these cells is inducible under a Tet-On system, and is turned on by addition of doxycycline. Mutation of Lys-212 to Gln (K212Q) destroys catalytic activity of hNTH1, but the mutant protein retains damage recognition and will bind to thymine glycol on DNA. Therefore, K212Q proteins overexpressed in cells probably interfere with normal hNTH1 function by dominant negative effect. Sensitivity of the stable cell lines against methylation and oxidative stresses was measured in the presence or absence of doxycycline. Overexpression of wild-type hNTH1 did not alter the viability of the cells, indicating that the amount of hNTH1 in normal cells is sufficient to repair all thymine glycol. Dominant negative interference of hNTH1 function had no effect, implying the presence of a backup enzyme for hNTH1 and an alternative repair system for thymine glycol.

1. Introduction

Reactive oxygen species (ROS) generated during oxidative phosphorylation in mitochondria damage various biomaterials including nucleic acids [for review see reference 1]. Macrophages and neutrophiles also produce ROS during normal inflammatory reaction. ROS attacks sugar moieties of DNA and cleaves the sugar-phosphate backbone, leaving single-strand breaks. ROS also causes ring saturation and fragmentation of DNA bases, producing thymine glycol and urea from thymine residue and 8-hydroxyguanine from guanine residue. DNA and RNA synthesis mediated by DNA polymerase and RNA polymerase, respectively, is interrupted by the inability to insert a nucleotide opposite thymine glycol; thus, the lesion causes lethal damage. Thymine glycol in DNA can be repaired by base excision repair, which begins with removal of the damaged base by DNA glycosylase [1]. *Escherichia coli* endonuclease III (*nth*) is a DNA glycosylase with AP lyase activity, which removes thymine glycol from sugar moiety and cleaves DNA backbone by β-elimination, leaving α,β-unsaturated aldehyde at 3' end and phosphate group at 5' end. Human cells have a structural and functional homologue of endonuclease III, hNTH1 [2]. Human NTH1 exhibits strong thymine glycol removal activity in human cells, but it is not known to what extent hNTH1 contributes to repair of such lesions *in vivo*. In the present study, we established HeLa cell lines overexpressing wild-type hNTH1 and its catalysis-defective mutant under regulation by the Tet-On system, and sensitivity of the stable cell lines to methylation and oxidative stress was

measured. Viability of the cells was not altered by overexpression of either type of hNTH1, implying the presence of a backup enzyme of hNTH1 and alternative repair system of thymine glycol.

2. Materials and Methods

2.1. Materials

The HeLa Tet-On cell line, which stably expresses the tetracycline-controlled transactivator protein, was purchased from BD Biosciences Clontech (Palo Alto, CA, USA) and maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Tet system approved, BD Biosciences Clontech) and 100 μg/ml Geneticin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2. Plasmids pTRE2pur and pTRE2-Luc were purchased from BD Biosciènces Clontech. Menadione sodium bisulfite, paraquat (methyl viologen) and methyl methanesulfonate were purchased from Sigma-Aldrich. Human NTH1 cDNA and its K212Q (Lys-212 to Gln) mutant are described elsewhere [2].

2.2. Construction of cell lines overexpressing hNTH1

Wild-type and K212Q mutant hNTH1 cDNA fragments were amplified by PCR and inserted into pTRE2pur expression vector between Bam HI and Eco RV sites. The plasmids with wild-type and mutant hNTH1 were named pTRE2pur-WT and pTRE2pur-K212Q, respectively. HeLa Tet-On cells (3.3×10^5) were inoculated into a 5-cm culture dish, and grown for 24 hrs. The hNTH1 expression plasmid (6.7 μg) was introduced into the cells using Calcium Phosphate Transfection Kit (Invitrogen Corp., Carlsbad, CA, USA). After 24 hrs, the medium was replaced with fresh complete medium. After an additional 24 hrs, the medium was replaced with medium containing 1 μg/ml puromycin (Sigma-Aldrich). Culturing was continued until puromycin-resistant colonies appeared (about 2 weeks), with replacement of the medium and puromycin every 4 days. Large, healthy colonies were peeled off by overlaying a filter disk soaked in 0.25% trypsin solution and transferring to individual wells of a 24-well culture plate. After cells were grown to confluence, each clone was transferred and maintained in a 5-cm dish containing medium with 1 μg/ml puromycin. The cell lines expressing wild-type and mutant hNTH1 were named Tet on-WT and Tet on-K212Q, respectively. To induce hNTH1 expression, doxycycline (BD Biosciences Clontech) was added to the medium at a concentration of 3 μg/ml.

2.3. Viability test

Cells from exponentially growing culture were seeded at 500 cells per 5-cm dish. After 6 hrs, the cells underwent 1 of 2 treatments: methyl methanesulfonate, by adding it to the medium; or hydrogen peroxide, by adding it to the plate dissolved in phosphate-buffered saline. After 1 hr exposure to these drugs, the culture medium or phosphate-buffered saline was removed, and the dishes were washed twice with the culture medium. After adding fresh culture medium, cell colonies were cultured for 8 to 10 days, fixed in 10% formaldehyde and stained with 0.25% crystal violet. Colonies containing more than 30 cells were counted.

Cells were cultured in a 96-well plate at 6000 cells per well for 24 hrs. Menadione or paraquat was added to the culture medium at the indicated concentration, and cells were cultured for 48 hrs. Surviving cells were counted using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), by measuring absorbance (at 450 nm) of formazan produced from water-soluble tetrazolium salts by cellular dehydrogenase activity.

2.4. Immunological detection of hNTH1

Cells were lysed in buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% Nonidet P-40, by repeatedly (3 times) freezing in liquid nitrogen and quickly thawing. Proteins were

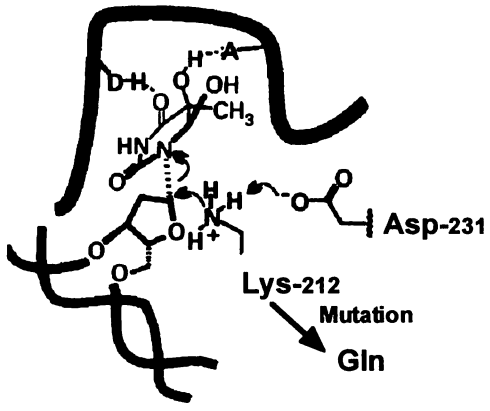

Fig. 1. Catalytic mechanism of hNTH1. Asp-231 of hNTH1 deprotonates the Lys-212 residue, which then attacks C-1 of deoxyribose of the lesion, causing release of the base and formation of a covalent Schiff intermediate with DNA. The Schiff base intermediate undergoes several transformations resulting in strand cleavage via β-elimination to leave 5'-phosphate and 3'-α,β-unsaturated aldehyde. Mutation of Lys-212 to Gln (K212Q) destroys catalytic activity of hNTH1, but does not damage recognition.

fractionated by SDS-PAGE (12.5% polyacrylamide), and transferred onto Hybond-P blotting membrane (Amersham Bioscience, Piscataway, NJ, USA). The hNTH1 band was visualized using anti-hNTH1 antibody (1:2000), horseradish peroxidase-labeled anti-rabbit Ig antibody (1:3000) and ECL Western Blotting Detection Reagents Kit (Amersham Bioscience), essentially as described previously [2]. Imaging of the chemiluminescence was performed with a LAS-1000 Plus luminescent image analyzer system (FUJIFILM, Tokyo, Japan).

3. Results and Discussion

3.1. Construction of HeLa cell lines overexpressing hNTH1

We used the Tet-On system for high-level, regulable expression of hNTH1 in human cells. In this system, gene expression is turned on when tetracycline or doxycycline is added [3]. In order to estimate induction level of gene expression in ready-made HeLa Tet-On cells, the firefly luciferase gene was introduced into the cells in a pTRE-Luc plasmid by lipofection, and was transiently expressed. Addition of doxycycline increased luciferase activity in cell extract more than 130-fold (data not shown), indicating that the HeLa Tet-On cell line can express a foreign gene under doxycycline regulation.

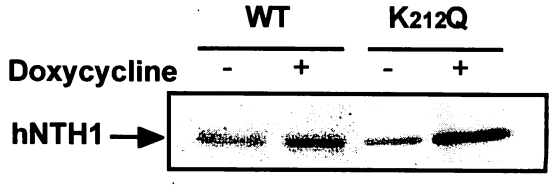

Fig. 2. Overexpression of hNTH1 in HeLa Tet-On cells. Stable cell lines transfected with pTRE2pur-WT and pTRE2pur-K212Q were grown in the presence or absence of 3 μg/ml doxycycline for 24 hrs. Wild type and K212Q mutant of hNTH1 were detected by Western blotting, as described in Materials and Methods. The amount of hNTH1 was measured by densitometry using NIH image 1.62 program.

To study roles of hNTH1 in DNA repair, we constructed HeLa cell lines that express wild-type and K212Q mutant hNTH1, using the Tet-On system. Lys-212 of hNTH1 plays a role in its DNA glycosylase and AP lyase activity (Fig. 1). Mutation of Lys-212 to Gln completely inactivates the enzyme, but damage-specific DNA binding activity remains fully intact [2]. Thus, K212Q protein expressed in cells binds to thymine glycol in DNA, and probably interferes with normal hNTH1 function by dominant negative effect. We constructed expression plasmids of wild-type and K212Q mutant hNTH1 using pTRE2pur as a vector, and transfected the plasmid DNA into HeLa Tet-On cells. Puromycine-resistant colonies were isolated, and expression of foreign hNTH1 in each cell line was immunologically assayed in the presence of doxycycline. In the present analyses, we used the cell line with the highest induction level (Fig. 2). The hNTH1 band of cells not induced with

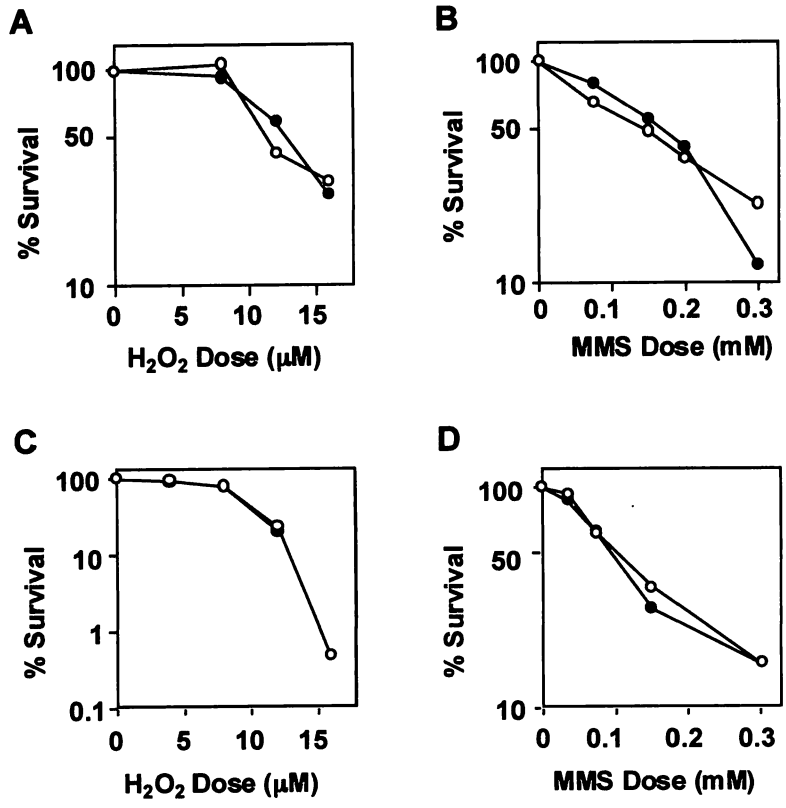

Fig. 3. Sensitivity of hNTH1-overexpressing cell lines to hydrogen peroxide (H_2O_2) and methyl methanesulfonate (MMS). Cells of lines Tet on-WT (A and B) and Tet on-K212Q (C and D) were treated with H_2O_2 (A and C) and MMS (B and D), and cultured in the presence (●) or absence (○) of 3 μg/ml doxycycline. Survival fractions were measured by colony formation.

doxycycline showed constitutive expression of endogenous hNTH1. Induction increased the total amount of hNTH1 about twofold in Tet on-WT cells and about 4-fold in Tet on-K212Q cells. Integration of hNTH1 cDNA in the genomic DNA of HeLa cells was confirmed by PCR and Southern hybridization (data not shown).

3.2. Sensitivity of hNTH1-overexpressing cells to oxidative and alkylation damage

HeLa cells overexpressing wild-type hNTH1 (Tet on-WT) or K212Q mutant hNTH1 (Tet on-K212Q) were challenged with oxidative and alkylation damage in the presence or absence of doxycycline (Figs. 3 and 4). Hydrogen peroxide, menadione and paraquat produce reactive oxygen species. Methyl methanesulfonate generates alkylated bases. With both Tet on-WT and Tet on-K212Q cells, there was no apparent difference in sensitivity between induced cells and non-induced cells. Overexpression of wild-type hNTH1 did not increase survival fraction against oxidative stresses, indicating that the amount of endogenous hNTH1 in HeLa cells is sufficient to remove all thymine glycol. Removal of thymine glycol by hNTH1 is apparently not the rate-limiting step of base excision repair for this lesion *in vivo*. Dominant negative effect of K212Q did not affect oxidative damage. This implies the presence of a backup enzyme of hNTH1 and an alternative thymine glycol repair system. Recently, several groups have generated mNth1 knock-out mice, have shown that they exhibit no overt abnormalities, and have found no difference in sensitivity against oxidative stresses between mNth1 -/- and +/- cells [4,5]. They concluded that a novel enzyme compensates for loss of mNth1 in mutant mice. In

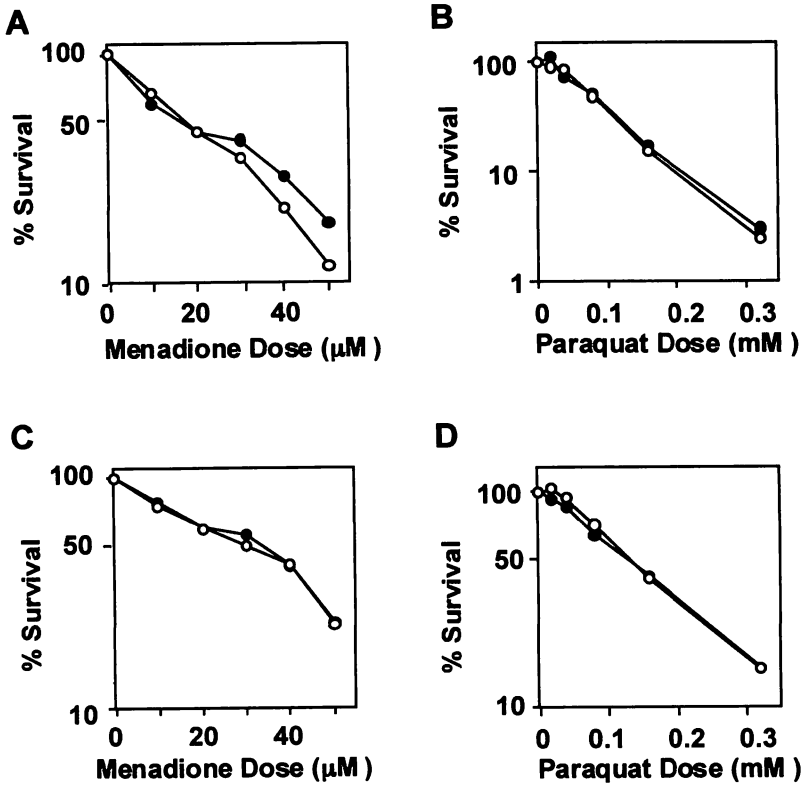

Fig. 4. Sensitivity of hNTH1-overexpressing cell lines to menadione and paraquat. Cells of lines Tet on-WT (A and B) and Tet on-K212Q (C and D) were treated with menadione (A and C) and paraquat (B and D), and cultured in the presence (●) or absence (○) of 3 μg/ml doxycycline. Survival fractions were measured using the Cell Counting Kit-8.

normal human cells, alkylated bases are removed by methyl purine DNA glycosylase and repaired via base excision repair pathway. The resulting apurinic sites (AP sites) are hydrolyzed at the 5' end by AP endonuclease, such as APEX nuclease. Human NTH1 cleaves the AP site at its 3' end by its AP lyase activity. Mutant proteins expressed in Tet on-K212Q cells compete with AP endonuclease for binding to AP sites. However, dominant negative effect of K212Q did not affect alkylation damage. This is probably due to the abundance of APEX nuclease in human cells [6].

3.3. DNA repair pathway of thymine glycol

The present results and those of investigators using mNth1 knock-out mice imply the existence of backup glycosylase for NTH1. *E. coli* expresses 2 types of thymine glycol DNA glycosylase: endonuclease III and endonuclease VIII (*nei*). Recently, by searching human genomic sequences, Hazra *et al.* [7] found 2 human DNA glycosylases genes (NEIL1 and NEIL2) with homology to endonuclease VIII, and showed that hNEIL1 is expressed ubiquitously and has substrate specificity that overlaps with that of hNTH1. Takao *et al.* [8] showed that mNeil1 activity compensates for loss of mNth1 in mutant mice. Thus, repair of thymine glycol via base excision repair can be initiated by at least 2 kinds of DNA glycosylase. Thymine glycol can be excised from DNA *in vitro* by reconstituted nucleotide excision repair [9]. XPG protein, a component of the nucleotide excision repair machinery, had been shown to activate removal of thymine glycol by hNTH1 *in vitro* [10], and appears to play a role in transcription-coupled repair of

thymine glycol [11,12]. During DNA replication, DNA polymerases δ and ε stall at sites opposite thymine glycol; i.e., this lesion is lethal. However, these replication polymerases can be replaced by a translesional DNA polymerase, like polymerse η, allowing DNA synthesis to continue, with some increase in error rate [13]. Up to 500 thymine glycol residues and similar oxidized pyrimidines are estimated to be generated daily in the human genome as endogenous DNA lesions [14]. Mammalian cells are thought to be equipped with redundant enzymes and pathways for repairing these lesions to avoid serious lethality and mutagenesis.

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