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Mutagenic Specificity of *N*-Nitrosotaurocholic Acid in *supF* Shuttle Vector Plasmids

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The mutagenic specificity of *N*-nitrosotaurocholic acid (NO-TCA) in human cells was investigated using *supF* shuttle vector plasmids. The plasmids pMY189 were treated with NO-TCA *in vitro* and introduced into normal fibroblasts (WI38-VA13) and nucleotide excision repair (NER)-deficient cells (XP2OS(SV)) for replication. The background mutation frequency of the *supF* gene was 4.1×10^{-4} and 2.0×10^{-4} after replication in normal and NER-deficient cells, respectively. The mutation frequency increased 5 and 15 times in normal and NER-deficient cells, respectively, after the treatment of the plasmid with 50 mg/mL of NO-TCA. The higher mutation frequency in NER-deficient cells indicates that the DNA damage induced by NO-TCA is repaired by NER. Base sequence analysis of 101 and 94 plasmids with mutations in the *supF* gene propagated in normal and NER-deficient cells, respectively, revealed that the majority of the mutations were base substitutions (about 89 and 90%) and the rest were deletions and insertions (about 11 and 10%) in both cell lines. About half of the mutant plasmids contained a single base substitution. Of the single base substitutions, the most frequent mutations were G:C to A:T transitions (about 37 and 36%), followed by G:C to C:G transversions (about 31 and 28%) in both cell lines. The mutations were not distributed randomly but were located at several hot spots in the *supF* gene, and almost all hot spots were at G:C sites. These observations accord with previous findings that NO-TCA forms DNA adducts with dC and induces G:C to A:T base substitution in *Salmonella typhimurium* TA100.

Key words: *N*-nitrosotaurocholic acid, mutagenic specificity, *supF* shuttle vector plasmid

Introduction

Bile acids are components of bile, which is a digestive secretion produced in the mammalian liver and stored in the gallbladder. Bile acids are conjugated with glycine or taurine after their synthesis in hepatocytes. The conjugates are then excreted into the digestive tract. Bile acids have been treated as carcinogens for more than

half a century, and recent reports indicate that bile acids cause DNA damage (1). Epidemiological studies indicate an association between bile acids and colorectal cancer (2,3), and high levels of secondary bile acids are present in the feces of populations at high risk of colorectal cancer (4,5).

Nitrosation of bile acid conjugates can be mediated by the acid-catalyzed reaction of amides with nitrite (6), and activated macrophages in infected and inflamed organs are also believed to be involved in such reactions. *N*-Nitroso bile acid conjugates have been suggested as plausible etiological factors in the development of gastric cancer in humans (7,8). *N*-Nitrosotaurocholic acid (NO-TCA), an *N*-nitroso bile acid conjugate, has already been demonstrated to exert mutagenic activity in both bacterial and mammalian assay systems (6,9). NO-TCA induces hepatocellular carcinoma and gastric tumors in male Fischer 344 rats (10). Recently, the chemical structures of DNA adducts derived from NO-TCA were identified. NO-TCA reacts directly with DNA and forms the DNA adduct, 3-ethanesulfonic acid-2'-deoxycytidine (3-ethanesulfonic acid-dC), *N*⁴-cholyl-2'-deoxycytidine (*N*⁴-cholyl-dC), and *N*⁶-cholyl-2'-deoxyadenosine (*N*⁶-cholyl-dA) (11). In a ³²P-postlabeling assay, adduct spots corresponding to 3-ethanesulfonic acid-dC were detected in both duodenal reflux and sham operation rats (12). NO-TCA induced mutations in *Salmonella typhimurium* TA100, and a mutation spectrum analysis revealed that NO-TCA predominantly induced G:C to A:T transitions (11). However, the mutation spectrum of NO-TCA in mammalian cells has not been investigated.

Shuttle vector plasmids are used to examine the spec-

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trum of mutations caused by environmental mutagens and carcinogens (13). The mutations induced in the plasmids by mutagens reflect the mutations that occur in endogenous genes of mammalian cells in which the plasmids are propagated (14). In the present study, we used the pMY189 plasmid (15), a derivative of the pZ189 shuttle vector plasmids, to analyze mutational specificity. The plasmids were treated with NO-TCA, and the frequencies and types of NO-TCA-induced mutations in the *supF* gene of plasmids in normal and repair deficient human cells were analyzed.

Materials and Methods

Chemicals: NO-TCA was purchased from the Nard Institute (Osaka, Japan). Ampicillin, chloramphenicol, nalidixic acid, isopropyl β -D-thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indoyl β -D-galactoside (X-gal) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Restriction endonuclease *DpnI* was purchased from New England Biolabs (Beverly, MA, USA).

Cells: SV40-transformed normal human fibroblast cells, WI38-VA13 (16), and group A nucleotide excision repair (NER)-deficient cells XP2OS(SV) were used (17). All cells were cultured in RPMI1640 medium (Sigma-Aldrich Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA).

Shuttle vector plasmid and bacterial strains: The shuttle vector plasmid pMY189, which is a derivative of the pZ189 plasmid (13), was used for the mutation analysis (15). The indicator *E. coli* strain KS40/pKY241 (18) is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 [*lacZ* (am) *CA7070 lacY1 HsdR HsdM D (araABC-leu)7679 galU galK rpsL thi*] (19), and was used to detect the mutated *supF* of pMY189. The plasmid pKY241 was constructed by Akasaka *et al.* (18) and contains a chloramphenicol resistant marker and *gyrA* (amber) genes. *E. coli* KS40/pKY241 cells that carry the active *supF* gene cannot form colonies on a plate containing nalidixic acid, whereas the cells that carry the mutated *supF* gene can form colonies on selection plates containing nalidixic acid, chloramphenicol, and ampicillin. *E. coli* cells containing the wild type *supF* gene produce blue colonies, whereas cells containing the mutated *supF* gene produce white or light blue colonies on the selection plates.

Treatment of the plasmid with NO-TCA, transfection into human cells, and plasmid recovery: Purified stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit (QIAGEN GmbH, Hilden, Germany). pMY189 (3 μ g) was incubated with various amounts of NO-TCA in 33 μ L of 100 mM phosphate buffer (pH 7.5) (11). The reaction was allowed to proceed for 6 h at 37°C followed by phenol/chloroform extraction and ethanol precipitation, and the plasmids were redissolved in 500 μ L of Dulbecco's phosphate

buffered saline (PBS) solution (pH 7.5). The WI38-VA13 or XP2OS(SV) (1×10^7 cells) cells and 3 μ g of NO-TCA-treated pMY189 in PBS solution (500 μ L) were placed in an electroporation cuvette (electrodes 0.4 cm apart), and the cells were transfected with the plasmids by an exponential electric pulse (350 V, 950 μ F) using a Gene pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). Transfection efficiency of the plasmids was almost same in both cell lines because the rate of plasmid recovery was not different in both cell lines using non-treated plasmid. The cells were plated in ϕ 10 cm dishes and incubated at 37°C for 72 h in a CO₂ incubator. Then, the plasmids were extracted from the cultured cells using the QIAprep-spin miniprep kit (QIAGEN GmbH). The purified plasmids were digested with the restriction endonuclease *DpnI* to eliminate plasmids that retained the bacterial methylation pattern.

Selection of mutated *supF* and determination of DNA base sequences: The *DpnI*-treated plasmid DNA was introduced into the indicator bacteria KS40/pKY241 by the Micro Pulsar electroporation apparatus (Bio-Rad Laboratories). The bacterial cells were plated on Luria-Bertani (LB) agar containing 50 μ g/mL nalidixic acid, 150 μ g/mL ampicillin and 30 μ g/mL chloramphenicol, IPTG, and X-gal to select the plasmids containing the mutated *supF* genes. A portion of the cells was plated on LB agar containing ampicillin and chloramphenicol to measure the total number of transformants. After the plates had been incubated for 24 h at 37°C, the colonies were counted, and mutation frequencies were calculated. After the *E. coli* that carried the mutated plasmids had been cultured overnight in 2 mL of LB medium, the plasmids were extracted and purified with the QIAprep-spin miniprep kit (QIAGEN GmbH), and the size of the mutated plasmid was checked by agarose gel electrophoresis. In order to reduce the contribution of spontaneous mutations, we selected *supF* mutants from plasmids treated with NO-TCA at the highest concentration. The base sequences of the *supF* gene of the plasmids that were not aberrant in size were determined with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems Inc., Foster City, CA, USA) and the M13-21 primer using an ABI3130 automatic DNA sequencer (Applied Biosystems Inc.). Mutants that had identical base changes derived from the same transfection plate were not scored to exclude clusters of the same clones. The χ^2 -goodness of fit test was used to determine if the NO-TCA-induced base substitution mutations were distributed randomly or non-randomly over the coding region of the *supF* tRNA gene.

Results

Mutation of plasmids: The mutation frequency increased in both cell lines with increasing concentrations

Fig. 1. The mutation frequency of the NO-TCA-treated pMY189 plasmids propagated in repair proficient WI38-VA13 (open circle) and repair deficient XP2OS(SV) cells (closed circle). The proportion of bacterial colonies containing the mutated *supF* gene in the ampicillin-resistant bacterial colonies is shown as the mutation frequency. The Y-axis represents the fold increase in the frequency when the background frequency is 1 (see text). The mean values of three independent experiments are plotted with the SD.

of NO-TCA (Fig. 1). The background plasmid mutation frequency was $2.0\text{--}4.1 \times 10^{-4}$ for both cell lines, and the mutation frequency increased about 5 and 15 times in the normal and XP cells, respectively, after treatment of the plasmid with 50 mg/mL of NO-TCA. The rate of plasmid recovery from the cells was not decreased by the NO-TCA treatment (data not shown).

Base sequence analysis: The majority (about 90 %) of the NO-TCA-induced mutations were base substitutions in both cell lines (Table 1). In XP cells, about 62 % and 25% of the mutant plasmids had single and multiple (at least two base substitutions except for tandem substitutions) base substitutions, respectively, and about 4% of them had tandem base substitutions (adjacent two base substitutions). In normal cells, about 51 and 36% of the plasmids had single and multiple base substitutions, respectively, and 3% of the plasmids had tandem base substitutions.

The types of single base substitutions found are shown in Table 2. The majority of the single base substitutions occurred at guanine or cytosine bases in both cell lines (about 94% and 86% in normal and XP cells, respectively). The most frequent mutations were G:C to A:T transitions, and the next most frequent mutations were G:C to C:G transversions in both cell lines.

Table 1. Types of sequence alterations in the *supF* gene in NO-TCA-treated plasmids pMY189 replicated in normal [WI38-VA13] or NER deficient [XP2OS(SV)] human fibroblasts

Type	No. of plasmids with mutations (%)	
	WI38-VA13	XP2OS(SV)
Base substitutions	90 (89.1)	85 (90.4)
Single	51 (50.5)	58 (61.7)
Multiple	36 (35.6)	23 (24.5)
Tandem	3 (3.0)	4 (4.2)
Frameshift	11 (10.9)	9 (9.6)
Deletion	10 (9.9)	8 (8.5)
Insertion	1 (1.0)	1 (1.1)
Total no. of plasmids sequenced	101 (100)	94 (100)

Table 2. Types of single base substitutions in the *supF* gene in NO-TCA-treated plasmids replicated in normal [WI38-VA13] or NER deficient [XP2OS(SV)] human fibroblasts

Base substitution	No. of base substitutions (%)	
	WI38-VA13	XP2OS(SV)
G:C to A:T	19 (37.2)	21 (36.2)
G:C to C:G	16 (31.4)	16 (27.6)
G:C to T:A	13 (25.5)	13 (22.4)
A:T to G:C	1 (2.0)	4 (7.0)
A:T to T:A	0 (0)	2 (3.4)
A:T to C:G	2 (3.9)	2 (3.4)
Total	51 (100)	58 (100)

Mutation spectrum: The locations of the NO-TCA-induced base substitutions in the coding region of the *supF* tRNA gene are shown in Fig. 2. The distribution was similar in normal and XP cells. Mutations were not distributed randomly ($p < 0.0001$ in both cells lines) but were mostly located at specific sites. There were several hot spots (at least three base changes occurred) at sites 133, 156, 159, 160, and 168 in normal cells and at sites 133, 156, 159, 160, 161, and 168 in XP cells, in which the number of the mutations observed was ≥ 4 -fold greater than the number expected for a random distribution. The most prominent hot spot was at site 160 in both cells. All hot spots, except for site 161 in XP cells, were located at G:C sites.

Discussion

In the previous study, we reported that NO-TCA reacts with calf thymus DNA and forms 3-ethanesulfonic acid-dC and two bulky adducts: *N*⁴-choly-dC and *N*⁶-choly-dA (11). In the present study, the shuttle vector plasmids were treated with NO-TCA and introduced into normal and NER deficient cells. NER is known to be a major pathway by which cells remove bulky DNA

Fig. 2. The distribution of the NO-TCA-induced single base substitutions in the *supF* tRNA gene. Sites corresponding to tRNA coding sequences (residues 99–183) are shown. Substituted bases after plasmid replication in WI38-VA13 and XP2OS(SV) cells are shown above and below the tRNA coding sequence, respectively. The arrows indicate the locations of mutation hotspots.

adducts that had been induced by UV light and chemicals. As shown in Fig. 1, the mutation frequency was higher in the NER deficient XP cells than in the normal WI38 cells. Similar results have been reported with UV (13), *cis*-diamminedichloroplatinum (II) (20), and aflatoxin B1 (21), DNA lesions of which are known to be repaired by the NER system. These results suggest that the enhanced mutation frequencies induced by NO-TCA are due to unrepaired DNA adducts in cells and that NO-TCA adducts are removed from DNA by the NER system.

In an *S. typhimurium* assay, NO-TCA induced mutations in a dose-dependent manner in the TA100 strain, which can detect base substitution mutations, but did not induce mutations in the TA98 strain, which can detect frameshift mutations (11). In the present experiment, the majority (~90%) of the mutations induced in human cells were base substitutions, and frameshift mutations were rare (Table 1). The NO-TCA-produced DNA lesions caused base substitution mutations in both bacterial and mammalian cells. As shown in Table 1, about 25–36% of the NO-TCA-induced mutations were multiple base substitutions. There have been several reports on multiple substitutions in shuttle vector plasmids propagated in mammalian cells (22,23). Seidman *et al.* (22) explained that multiple substitutions are induced at undamaged bases by error-prone DNA polymerases. Courtemanche *et al.* (23) suggested that multiple mutations may arise during translesion DNA synthesis and involve an error-prone polymerase, which would introduce base opposite misincorporation errors. In the present study, to reveal the specific mutations induced by NO-TCA, we focused on single base substitutions since multiple base substitutions can con-

tain base changes due to untargeted mutagenesis. As shown in Table 2, the most frequent single base substitution mutations were G:C to A:T transitions. This observation is consistent with the type of NO-TCA-induced mutations observed at the *hisG46* site of TA100 (11). Since this bacterial assay strain detects only reverse mutations, the information about base changes is limited. In contrast, the *supF* system can detect various types of mutation. In fact, the present study showed that about 31% and 26% of the single base substitutions were G:C to C:G and G:C to T:A transversions, respectively, in normal cells, while about 28% and 22% of them were G:C to C:G and G:C to T:A transversions, respectively, in XP cells (Table 2). We detected 3-ethanesulfonic acid-dC as a major adduct, and *N*⁴-cholyl-dC and *N*⁶-cholyl-dA as minor adducts in the reaction of NO-TCA with calf thymus DNA (11). We reported that the minor adduct signals were almost 500 times weaker than the major adduct signal in ³²P-postlabeling analysis (11). In the present work, most single base substitutions (about 94% and 86% in normal and XP cells, respectively) occurred at G:C sites and a small number of base substitutions (about 6% and 14% in normal and XP cells, respectively) were observed at A:T sites in the *supF* gene (Table 2). This base preference of mutations may reflect the relative amounts of dC and dA adducts in the plasmid.

As shown in Fig. 2, the single base substitutions occurred preferentially at certain sites. In the bacterial assay, NO-TCA induced G:C to A:T transitions at the CCC (*hisG46* site) of TA100. The *supF* gene contains several CCC (or GGG) sequences; however, none of the mutation hotspots were located at those sites in the present analysis. The CCC (or GGG) sequence might not be a hotspot of adduct formation. In fact, even if

NO-TCA induces base changes at other sequences, TA100 cannot detect them because its only mutation target site is *hisG46*. Other possibilities for the inconsistency of the mutation hotspots between these studies include differences in the damage-processing systems of *Salmonella* and human cells and differences in the method of NO-TCA treatment in these studies.

Specific mutations have been shown to be critical for the activation of oncogenes and the inactivation of tumor suppressor genes in human cancers. This work is the first report to describe the mutagenic specificity of NO-TCA in human cells. However, this study does not address the effect of chromatin structure, which may substantially affect adduct formation and mutagenesis. A comparison of NO-TCA-induced mutations in plasmid and chromosomal DNA is required for evaluation of its genetic effects in humans.

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