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Roles of translesion synthesis DNA polymerases in the potent mutagenicity of tobacco-specific nitrosamine-derived O^2-alkylthymidines in human cells

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ABSTRACT

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent human carcinogen. Metabolic activation of NNK generates a number of DNA adducts including O^2-methylthymidine (O^2-Me-dT) and O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O^2-POB-dT). To investigate the biological effects of these O^2-alkylthymidines in humans, we have replicated plasmids containing a site-specifically incorporated O^2-Me-dT or O^2-POB-dT in human embryonic kidney 293T (HEK293T) cells. The bulkier O^2-POB-dT exhibited high genotoxicity and only 26% translesion synthesis (TLS) occurred, while O^2-Me-dT was less genotoxic and allowed 55% TLS. However, O^2-Me-dT was 20% more mutagenic (mutation frequency (MF) 64%) compared to O^2-POB-dT (MF 53%) in HEK293T cells. The major type of mutations in each case was targeted T → A transversions (56% and 47%, respectively, for O^2-Me-dT and O^2-POB-dT). Both lesions induced a much lower frequency of T → G, the dominant mutation in bacteria. siRNA knockdown of the TLS polymerases (pols) indicated that pol η, pol ζ, and Rev1 are involved in the lesion bypass of O^2-Me-dT and O^2-POB-dT as the TLS efficiency decreased with knockdown of each pol. In contrast, MF of O^2-Me-dT was decreased in pol ζ and Rev1 knockdown cells by 24% and 25%, respectively, while for O^2-POB-dT, it was decreased by 44% in pol ζ knockdown cells, indicating that these TLS pols are critical for mutagenesis. Additional decrease in both TLS efficiency and MF was observed in cells deficient in pol ζ plus other Y-family pols. This study provided important mechanistic details on how these lesions are bypassed in human cells in both error-free and error-prone manner.

1. Introduction

Tobacco use is the single largest preventable cause of disease and premature death in the US, yet more than 45 million Americans still smoke cigarettes. Tobacco products contain over 5000 compounds and over 70 carcinogens [1]. Amongst the plethora of carcinogens present in tobacco and tobacco smoke, likely candidates for initiating cancers are the tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) [2–5]. Intermediates formed during metabolic activation of these compounds by P450 have been shown to covalently bind to DNA forming DNA adducts [6]. NNK thus far is the only carcinogen found in tobacco products that causes lung tumors in animal models, irrespective of the route of administration [7,8].

NNK can be metabolically activated to generate methylating agents or to generate pyridyloxobutyalting agents [6,7]. These agents react with DNA forming methyl (Me) and 4-(3-pyridyl)-4-oxobutyl (POB) adducts, respectively. The most

Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; O^2-Me-dT, O^2-methylthymidine; O^2-POB-dT, O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine; HEK293T, human embryonic kidney 293T; TLS, translesion synthesis; MF, mutation frequency; pol, DNA polymerase; Kf$^-$, exo-free Klenow fragment of DNA polymerase I; Dpo4, *Sulfolobus solfactaricus* DNA polymerase IV.

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common methylated DNA adducts identified in NNK-treated rodents formed in the methylation pathway are 7-Me-dG and O^6-Me-dG [9–11]; however, other methylation products such as O^2-Me-dC and O^2-Me-dT are also most likely formed [9]. While mutagenicity and carcinogenicity of the methylation pathway of NNK-derived O^6-Me-dG and its link to carcinogenesis is well documented [8,10–14], the relative importance of the POB-pathway is still under investigation.

The pyridyloxobutylating pathway generates four POB adducts including O^2-POB-dT, O^2-POB-dC, O^6-POB-dG and 7-POB-dG, which were detected in NNK treated rats and mice [15–17]. A/J mice treated with a pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), showed that three POB DNA adducts, O^2-POB-dT, O^6-POB-dG and 7-POB-dG, form and persist in lung DNA at significant levels, suggesting their likely involvement in lung carcinogenesis [18]. However, O^2-POB-dT was identified as the most abundant POB adduct in lung tissues of A/J female mice [19] as well as the most persistent adduct in lung and liver of male F344 rats treated with NNK [20]. NNKOAc-treated CHO cells produced point mutations mainly at the AT base pairs, implicating O^2-POB-dT as a candidate lesion responsible for these mutation [21].

In vivo mutagenesis study of O^2-Me-dT and O^2-POB-dT (structures shown in Plate 1) showed that these DNA lesions are highly mutagenic in *Escherichia coli* cells [22]. Both survival and the mutagenicity were increased with SOS induction, suggesting involvement of bacterial TLS pols for their bypass and mutagenesis. Replication of O^2-alkylthymidine lesions with varying side chains in *E. coli* showed that bypass efficiency decreased as the chain length of the alkyl group increased [23] and pol V is indispensable for T → A mutations [22]. In vitro kinetics using exo-free Klenow fragment of *E. coli* DNA polymerase I (Kf^-), *Sulfolobus solfactaricus* DNA polymerase IV (Dpo4), human polymerase κ (pol κ) and yeast polymerase eta (pol η) established that O^2-Me-dT is a strong block to DNA synthesis and that the correct nucleotide dATP is preferentially incorporated opposite O^2-Me-dT [24,25]. Likewise, bypass of O^2-POB-dT is inefficient by Kf^- and Dpo4, but, when bypass occurs, the wrong nucleotide dTTP is preferentially incorporated opposite O^2-POB-dT [25].

To test the hypothesis that O^2-POB-dT, the most abundant DNA adduct of the POB-pathway of NNK, is mutagenic, we have transfected a single-stranded (ss) pMS2 plasmid containing a single O^2-Me-dT or O^2-POB-dT in human embryonic kidney 293T cells (HEK293T). In addition, we investigated the involvement of TLS pols, including pol η, κ, ι, ζ and Rev1, for lesion bypass and mutagenesis by siRNA-induced knockdown of these TLS pols. We determined that both O^2-Me-dT and O^2-POB-dT are highly mutagenic in human cells, inducing predominantly T → A mutations. We also found that the lesion bypass is dependent on pol η, ζ, and Rev1.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was purchased from Du Pont New England Nuclear (Boston, MA). All enzymes, including *Eco*RV restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, uracil DNA glycosylase, and exonuclease III, were obtained from New England Bioloabs (Beverly, MA). HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA). *E. coli* DH10B cells was purchased form Life Technologies, Inc. (Grand Island, NY)

siRNAs: Synthetic siRNA duplexes against *POLH* (SI02663619), *POLK* (SI04930884), *POLI* (SI03033310), *REV1* (SI00115311), and All Stars negative control siRNA (1027280) were purchased from Qiagen (Valencia, CA). The siRNA for *REV3* was purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the siRNAs are listed in Table S1 of the Supporting information.

2.2. Synthesis and characterization of oligonucleotides

The modified oligonucleotides 5′-GTGCGT*GTTTGT-3′, (where T^* represents O^2-Me-dT or O^2-POB-dT) containing the DNA sequence of *p*53 codons 272–275 in which the lesion was located in codon 273, were synthesized and characterized as reported [22]. Oligonucleotides were analyzed by MALDI-TOF MS analysis, which gave a molecular ion with a mass within 0.005% of theoretical. The M + 1 for the O^2-Me-dT oligodeoxynucleotide is 3713 and we found an *m/z* of 3712. The M + 1 for the O^2-POB-dT oligodeoxynucleotide is 3846 and we found an *m/z* of 3847. Unmodified oligonucleotides were analyzed by MALDI-TOF MS analysis, which gave a molecular ion with a mass within 0.005% of theoretical.

2.3. Construction and characterization of pMS2 vectors containing a single O^2-Me-dT or O^2-POB-dT

The single-stranded (ss) pMS2 shuttle vector, which contains its only *Eco*RV site in a hairpin region was prepared as described [26]. Synthesis and characterization of the lesion-containing and control constructs followed a protocol reported earlier [27]. The final constructs were dissolved in 1 mM Tris–HCl—0.1 mM EDTA, pH 8, and a portion was run on 1% agarose gel to assess the amount of circular DNA.

2.4. Replication and analysis in HEK 293T cells

The HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum. The cells were grown to ~90% confluency and 50 ng of each construct was transfected using 6 μL of Lipofectamine cationic lipid reagent (Invitrogen, Carlsbad, CA). After transfection with control or lesion containing pMS2, the cells were grown at 37 °C and 5% CO_2 for 24 h and the plasmid DNA was collected and purified by the method of Hirt [28]. Each progeny DNA was then transformed *E. coli* DH10B, and the transformants were analyzed by oligonucleotide hybridization followed by DNA sequence analysis as described [27].

2.5. TLS assay in human cells

The lesion-containing or control pMS2 construct was mixed in a 2:1 ratio with a single- stranded pMS2 DNA containing the same DNA sequence as the construct, except in the 12mer insert it contained a C in place of G two nucleotides 5′ to the lesion site (i.e., 5′-GTCCGTGTTTGT- 3′). The mutant DNA was used as an internal control. The mixed DNA was used to transfect HEK293T cells and processed as described above. Oligonucleotide probes for the complementary sequences for both the lesion containing plasmid and the internal control plasmid were used to analyze the progeny. The internal control gave the same number of progeny as the control construct. Typically, three independent experiments were performed to determine the extent of TLS with each pol knockdown.

2.6. Mutational analyses of TLS products from human cells with pol knockdowns

The procedure described in Section 2.4 was adapted for the knockdown experiments as follows. Synthetic siRNA duplexes specific for TLS pols were transfected into HEK293T cells using