Enzymatic Removal of $O^6$-Ethylguanine from Mitochondrial DNA in Rat Tissues Exposed to $N$-Ethyl-$N$-nitrosourea in Vivo*

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Masahiko S. Sato†, Nam-ho Huh‡, Manfred F. Rajewsky§, and Toshio Kurokif

From the 1Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokane-dai, Minato-ku, Tokyo-108, Japan and the 2Institut für Zellbiologie (Tumorforschung), Universität Essen, Hufelandstrasse 55, D-4300 Essen 1, Federal Republic of Germany

The cellular genetic system possesses repair mechanisms to restore the integrity of DNA containing structural alterations caused by various DNA-reactive agents. Some of the DNA repair mechanisms operating in mammalian cell nuclei have been fairly well characterized for the nuclear DNA of eukaryotic cells as well as prokaryotes. However, little is known about DNA repair in mitochondria. Using highly sensitive immunolocalizational methods to detect specific DNA alkylation products, we found active removal of $O^6$-ethyl-2'-deoxyguanosine ($O^6$-EtdGuo) from rat liver mitochondrial DNA after pulse-exposure to $N$-ethyl-$N$-nitrosourea in vivo. In the kidney, $O^6$-EtdGuo was removed from mitochondrial DNA with moderate efficiency, but nearly no removal was observed from the DNA of brain mitochondria. Among the rat tissues examined, the kinetics of $O^6$-EtdGuo elimination from mitochondrial DNA was very similar to the kinetics of removal from nuclear DNA. $O^4$-Ethyl-2'-deoxythymidine, another premutagenic DNA ethylation product, was stable in both mitochondrial and nuclear DNA of rat liver.

In mammalian cells, there are two independent, but interactive genetic systems, i.e. nDNA and mtDNA. Although mtDNA only represents less than 1% of total cellular DNA, it also encodes essential gene products and, therefore, any damages to mtDNA may impair important cellular functions. Structural as well as functional alteration of mitochondria have been observed in tumor cells, suggesting mtDNA as a possible target of chemical or physical carcinogens (1). Indeed, a number of chemical carcinogens have been shown to bind to mtDNA to a significantly higher extent than nDNA (2-7).

The cellular genetic system possesses repair mechanisms to restore the integrity of DNA containing structural alterations caused by various DNA-reactive agents. Some of the DNA repair mechanisms operating in mammalian cell nuclei have been fairly well characterized (8). However, little is known about DNA repair in mitochondria. Clayton et al. (9) found no removal of UV-induced pyrimidine dimers from the mtDNA of various types of mammalian cells. DNA adducts formed by aflatoxin B1 or breaks in mtDNA caused by 4-nitroquinoline-1-oxide also remain stable with time, i.e. unreppaired (2, 10). These results indicate little or no excision repair activity in mitochondria. Anderson and Friedberg (11) detected uracil-DNA glycosylase activity in mitochondrial fraction of human KB cells.

We have investigated whether the enzymatic removal of $O^6$-EtdGuo from the DNA of cells exposed to alkylating $N$-nitroso compounds, a well-characterized repair mechanism in nDNA, also occurs in mitochondria. This study has become feasible only after the development of highly sensitive immunological detection methods for DNA alkylation products (12).

**EXPERIMENTAL PROCEDURES**

Materials—EtNU was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan) and deoxyribonuclease I (grade I) from Boehringer Mannheim (Federal Republic of Germany (F.R.G.)). $^{125}$I-Labeled anti-(rat Ig) F(ab')2 fragment from sheep (14 &iu/g) was obtained from Amersham International, Ltd. (Buckinghamshire, United Kingdom). All other reagents were of analytical grade.

**Preparation of nDNA and mtDNA—**Tissues were homogenized in ice-cold 0.25 M sucrose using a Teflon homogenizer and centrifuged at 800 x $g$ for 10 min. nDNA was prepared from the pellets by a conventional phenol/chloroform extraction (14). mtDNA was isolated from the supernatant according to Yonekawa et al. (15). Briefly, mitochondria were collected by centrifugation, treated with deoxyribonuclease I (200 ng/ml, 30 min) and then with sodium dodecyl sulfate (1%, 15 min). DNA extracted from the mitochondria with phenol/chloroform was treated with ribonucleases and then banded by EtBr-CsCl centrifugation. The bands corresponding to open and closed circular mtDNA were collected. After removing EtBr and CaCl, the mtDNA was further purified by electrophoresis in 1% agarose gel and recovered with silica slurry. DNA was finally dissolved in 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.0.

**Quantitation of DNA—**The amount of DNA was determined by fluorescence method as described by Down et al. (16), using rat liver DNA as standard.

**Quantitation of $O^6$-EtdGuo and $O^4$-EtdThd—**$O^6$-EtdGuo was quantitated by ISB as described previously (12). Briefly, 3 µg of heat-denatured DNA per slot of Minifold II (Schleicher & Schuell Inc., Dassel, F.R.G.) was applied onto nitrocellulose filter. The filters were treated with 15 µCi/ml anti-($O^6$-EtdGuo) monoclonal antibody (BR-6, 17) and then with 5 µCi/ml $^{125}$I-labeled anti-(rat Ig) F(ab')2 fragment from sheep. $O^6$-EtdGuo content in the sample DNA was determined by densitometric scanning of x-ray films after autoradiography. The detection limit was 2.0 fmol/3 µg of DNA/slot. The content of $O^6$-EtdGuo in DNA was expressed as the molar ratios to dGuo.

For determination of the amount of $O^4$-EtdThd, DNA was enzymatically hydrolyzed (18). $O^4$-EtdThd was fractionated by HPLC (18) and quantitated by competitive radioimmunoassay (19) using anti-
RESULTS AND DISCUSSION

Since mtDNA comprises only a minor fraction of total cellular DNA, a high yield and purity procedure was required for the preparation of mtDNA. After EtBr-CsCl gradient centrifugation, two bands were pooled which corresponded to closed circular and open circular mtDNA, respectively. O^6^-EtdGuo was removed with similar efficiency from both forms of mtDNA, as determined separately (data not shown). To estimate the level of possible contamination of mtDNA with nDNA, Southern analysis were performed (20). Nitrocellulose filters carrying HindIII-restricted mtDNA and nDNA fragments were hybridized with nick-translated total nDNA containing 0.5 ng of HindIII-related repetitive sequence of nDNA, the purity of mtDNA, as determined separately (data not shown). To estimate the level of possible contamination of mtDNA with nDNA, Southern analysis were performed (20). Nitrocellulose filters carrying HindIII-restricted mtDNA and nDNA fragments were hybridized with nick-translated total nDNA digested with HindIII. By comparing the relative intensity of HindIII-related repetitive sequence of nDNA, the purity of the mtDNA fractions from the liver, brain, and kidney were estimated to be 90, 75, and 95%, respectively.

O^6^-EtdGuo in rat liver mtDNA and nDNA was quantitated by an ISB. As shown in Fig. 1, the initial (at 1 h after injection of EtNU) O^6^-EtdGuo to dGuo molar ratio in mtDNA was 1.80 \( \times \) 10^-1, i.e. nearly 2-fold higher than the corresponding value for nDNA. O^6^-EtdGuo was rapidly removed from mtDNA with time, only 20% of the initial amount remaining in mtDNA at 25 h. Consistent with previous observations (21), O^6^-EtdGuo was eliminated efficiently from nDNA.

To further confirm the active removal of O^6^-EtdGuo from liver mtDNA, we tried to detect O^6^-EtdGuo in BamHI-digested mtDNA fragments on nitrocellulose filters after Southern blotting. Rat mtDNA has two restriction sites for BamHI, giving rise to 10.9- and 5.1-kilobase fragments (Fig. 2A). O^6^-EtdGuo in the DNA segments was visualized with the use of anti-O^6^-EtdGuo monoclonal antibody ER-6 (17) and a 125I-labeled anti-(rat Ig) F(ab')2 fragment from sheep. As shown in Fig. 2, O^6^-EtdGuo was detected in the bands formed by BamHI-digested mtDNA at 1 h after the EtNU-pulse (B, lane 1), but after 25 h, the signal was below the detection limit (B, lane 2). nDNA showed a smear and formed no discrete bands on the nitrocellulose filters after digestion with BamHI. From these results, we concluded that the observed elimination of O^6^-EtdGuo from liver mtDNA (Fig. 1) was due to an actual repair process in vivo, rather than to an artifact by the contamination of mtDNA with nDNA.

The observed reduction in the amount of O^6^-EtdGuo could, at least in part, also have been due to DNA replication. It is difficult, however, to determine the extent of DNA replication precisely under in vivo conditions. To circumvent this difficulty, we have quantitated another DNA ethylation product, O^4^-EtdThd, which is known to be hardly eliminated from the nDNA of rat liver, kidney, and brain (22). As shown in Fig. 1, the removal of O^6^-EtdThd from liver mtDNA and nDNA during a 25-h period following the EtNU-pulse was negligible. This indicates not only that neither mtDNA nor nDNA replicated to any significant extent during the 25-h period of observation but also that no efficient mechanism for removal of O^6^-EtdThd from DNA exists in the mitochondria of rat liver cells.

The efficiency to eliminate O^6^-EtdGuo from nDNA is known to vary among different types of rat cells being highest in parenchymal liver cells and lowest in brain cells (21, 22). We have studied the capacity of rat brain and kidney mitochondria to remove O^6^-EtdGuo from DNA under the similar conditions with those for liver (Fig. 3). In the brain, the initial O^6^-EtdGuo/dGuo molar ratios were 1.25 \( \times \) 10^-5 and 0.95 \( \times \) 10^-5 in mtDNA and nDNA, respectively, and almost no removal of O^6^-EtdGuo was observed, with 78% remaining in mtDNA and 86% in nDNA at 25 h after the EtNU-pulse (Fig. 3A). In the kidney O^6^-EtdGuo was removed with moderate efficiency from mtDNA as well as from nDNA (Fig. 3B). In kidney mtDNA, the O^6^-EtdGuo/dGuo molar ratio was 1.18 \( \times \) 10^-7 at 1 h and 0.39 \( \times \) 10^-5 at 25 h, while the corresponding values for kidney nDNA were 1.40 \( \times \) 10^-6 and 0.53 \( \times \) 10^-5, respectively. The stable O^6^-EtdThd content in nDNA in both

![Fig. 1. Elimination of O^6^-EtdGuo and O^4^-EtdThd from nDNA and mtDNA of rat liver after pulse exposure to EtNU (75 μg/g of body weight). The amount of O^6^-EtdGuo in mtDNA (○—○) and in nDNA (●—●) was determined by ISB. O^6^-EtdThd in mtDNA (□—□) and in nDNA (■—■) was measured by radioimmunoassay (see "Experimental Procedures"). Vertical bars, standard deviations of the O^6^-EtdGuo/dGuo molar ratios in DNA from four independent experiments. dThd, 2'-deoxythymidine.](image)

![Fig. 2. Detection of O^6^-EtdGuo in BamHI-digested mtDNA fragments. mtDNA isolated 1 h (lane 1) and 25 h (lane 2) after intravenous injection of EtNU (75 μg/g of body weight) was digested with BamHI. Three μg of each DNA was electrophoresed in 1% agarose gel, stained with 0.5 μg/ml of EtBr, and run overnight. The separated DNA fragments were then transferred onto a nitrocellulose filter by the conventional Southern transfer method (20). O^6^-EtdGuo was detected by sequential treatment with anti-(O^6^-EtdGuo) monoclonal antibody ER-6; (17) and 125I-labeled anti-(rat Ig) F(ab')2 fragment from sheep under the similar conditions with ISB described under "Experimental Procedures." A, BamHI-restricted rat liver mtDNA stained with EtBr. The uppermost band shown in A represents the undigested fraction of mtDNA. B, O^6^-EtdGuo visualized on the same DNA fragments.](image)
The higher modification of mtDNA by chemical carcinogens is a small water-soluble molecule and does not require enzymatic activation to interact with DNA (23). Although the molecular size of the DNA-reactive electrophile may in part determine the degree of binding to mtDNA versus nDNA, the precise mechanism underlying the varying extents of modification in mtDNA and nDNA remain to be clarified.

O⁶-EtGuo was eliminated from mtDNA with a kinetics similar to that for nDNA in liver and brain (i.e., 2.1- and 1.4-fold in liver and brain, respectively), but to a similar extent in kidney. EtNU is a small water-soluble molecule and does not require enzymatic activation to interact with DNA (23). Although the molecular size of the DNA-reactive electrophile may in part determine the degree of binding to mtDNA versus nDNA, the precise mechanism underlying the varying extents of modification in mtDNA and nDNA remain to be clarified.

O⁶-EtGuo was eliminated from mtDNA with a kinetics similar to that for nDNA in the three rat tissues examined (Figs. 1, 3). In nDNA, O⁶-EtGuo is known to be repaired by O⁶-alkylguanine DNA alkyltransferase (24). The enzyme transfers the alkyl group from the O⁶-position of guanine onto a cysteine residue in its active center, resulting in "suicide" inactivation of the respective O⁶-alkylguanine DNA alkyltransferase molecule (24). At present, we have no information about the molecular mechanism for removal of O⁶-EtGuo from mtDNA, in spite of a similarly efficient removal of O⁶-EtGuo from both nDNA and mtDNA of rat liver (Fig. 1), suggesting a similar repair mechanism in both genetic systems. The possibility that mtDNA encodes a repair enzyme(s) is unlikely because products of mitochondrial genes are all known to be related to electron transfer systems and ATP synthesis (25). Therefore, a repair enzyme encoded in nDNA is most possibly operating in mitochondria. For transportation into mitochondria, a specific signal peptide may be needed for such nucleically encoded proteins (26). It is of interest to understand how mammalian cells regulate their relative capacity to remove O⁶-EtGuo from nDNA and mtDNA.

To elucidate the mechanism as well as the nature and mode of action of putative mtDNA repair enzyme(s), further studies are needed, including isolation of the gene(s) coding for the mammalian O⁶-alkylguanine DNA alkyltransferase.

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References