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MASSIVE CONVERSION OF GUANOSINE TO 8-HYDROXY-GUANOSINE IN MOUSE LIVER MITOCHONDRIAL DNA BY ADMINISTRATION OF AZIDOTHYMIDINE

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Summary: **As typical mitochondrial myopathy has been reported to be expressed among many patients with AIDS treated with long-term azidothymidine (AZT) therapy, we examined changes in mouse liver mitochondrial DNA (mtDNA) after 4-week administration of AZT. Even below 1/10th the dose given to the patients (AZT, 1 mg/kg/day), 25 % of the total deoxyguanosine (dG) was converted to be 8-hydroxy-deoxyguanosine (8-OH-dG). 38 % of the total dG was converted to 8-OH-dG with AZT 5 mg/kg/day. *In vitro*, the conversion of dG to 8-OH-dG was demonstrated by incubating mtDNA in the oxygen radical producing system containing NADH and KCN treated mitochondrial inner membrane. Thus it is concluded that, by lack of repairing system, damaged mtDNA with AZT results in impaired mitochondrial respiratory chain causing oxygen radicals which are responsible for 8-OH-dG formation. These results suggest that the oxygen damage of mtDNA is the primary cause of mitochondrial myopathy with AZT therapy.**

Azidothymidine (AZT), zidovudine, has been used for the treatment of acquired immunodeficiency syndrome (AIDS). Recently, the acquired mitochondrial myopathy caused by AZT therapy in patients with AIDS was reported (1): typical ragged red fibers and paracrystalline inclusions in mitochondria were seen in biopsied muscle specimens from such patients. As there is ample evidence indicating that mitochondrial myopathy is phenotypic expression of mutant mtDNA, the authors intended to establish animal model of the disease as well as to elucidate the mechanism of mtDNA mutation by examining mouse liver mtDNA after administration of AZT. Most plausible cause of this type of acquired mtDNA mutation is oxygen damage. It has been reported that mtDNA could be damaged by oxygen radicals (2), and that hydroxyl radicals (•OH) oxidizes deoxyguanosine (dG) to 8-hydroxy-deoxyguanosine (8-OH-dG) (3). Consequently, after the administration of AZT, mtDNA isolated from mouse liver was analyzed by using a ultramicro-high performance liquid chromatography / mass spectrometry (umHPLC/MS) system that we have recently developed for micro-mass spectra assay of biological substance of picomole order.

This paper presents the result that, after 4-week oral administration of AZT even at 1/10th the dose given to AIDS patients, 25 % of the total dG in mouse liver mtDNA was converted to 8-OH-dG, and that *in vitro* conversion of dG to 8-OH-dG was demonstrated by incubating mtDNA

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in the oxygen-radical-producing system containing NADH and KCN-treated mitochondrial inner membrane.

Materials and Methods

Reagents Cytochrome *c*, NADH, 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA), thymidine (T), 3'-azido-3'-deoxythymidine (AZT), DNase I, spleen exonuclease, and snake venom exonuclease were purchased from Sigma, H_2O_2 from Mitsubishi Gas Chem. Proteinase K and alkaline phosphatase were obtained from Boehringer Mannheim GmbH, bovine pancreas ribonuclease from Pharmacia. Chemically synthesized samples of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) and 8-hydroxy-2'-deoxyadenosine (8-OH-dA) were kindly supplied by Prof. A. Ohtsuka and Dr. H. Inoue, University of Hokkaido.

Administration of AZT Six CR-strain mice aged 8 weeks were divided into 3 groups: (1) AZT 1 mg group: AZT (1 mg/kg/day) was orally administered by using a gastric tube to mice everyday for 4 weeks. (2) AZT 5 mg group: AZT (5 mg/kg/day) was administered in the same way. (3) Control group: physiological saline was administered in the same way.

Preparation of mitochondria At the end of AZT administration, the liver was removed from mice and mitochondria were prepared by a modified method of Hogeboom by Utsumi using discontinuous sucrose gradient (4). The liver was homogenized in ten volumes of 0.25 M sucrose solution containing 40 μM EDTA and 5 mM Tris-HCl buffer, pH 7.4, for 2 min at 0-4°C by a Potter-Elvehjem glass homogenizer and then a teflon homogenizer, and then centrifuged at 80 x *g* for 7 min to eliminate the cell debris and nuclei. The supernatant was overlayered to equal volume of 0.35 M sucrose solution containing 40 μM EDTA and 5 mM Tris-HCl buffer, pH 7.4, and centrifuged at 700 x *g* for 10 min to separate all the nuclear fraction. About 2/3 of the supernatant were collected carefully, then centrifuged at 7,000 x *g* for 10 min. Opalescent supernatant and pink partially sedimented layer were discarded. The tightly packed precipitate, mitochondria, were washed twice with the homogenizing medium. This mitochondrial fraction contained no detectable nuclear DNA by ethidium bromide fluorescence after electrophoresis on 1% agarose gel. Protein concentration was measured by the biuret method (5).

Extraction of mtDNA Freshly isolated mitochondria suspended in 0.1 M EDTA, 10 mM Tris-HCl, pH 7.4, were incubated in a shaking water bath for 4 h at 37°C in the presence of 0.5 % sodium dodecyl sulfate and 100 μg/ml of proteinase K. After two phenol / chloroform / isoamyl alcohol extractions and diethylether extractions, the aqueous phase was mixed and precipitated with 1/10 volume of 3 M sodium acetate, pH 7.4, and 2 volumes of absolute ethanol at -20°C overnight. The precipitates were collected by centrifugation at 13,000 x *g* for 10 min , vacuum dried, and finally dissolved in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. Then further purification was made by digestion with 10 μg/ml of pancreas ribonuclease for 30 min at 37°C followed by phenol / chloroform / isoamyl alcohol extraction.

Treatment of mtDNA with •OH derived from impaired electron transfer particle Electron transfer particle (ETP) was prepared from beef heart mitochondria by the method of Hansen and Smith (6). mtDNA (1 μg) isolated from liver was incubated for 1 h at 37°C with 0.25 mg/ml of ETP, 0.8 mM NADH and 3 mM KCN in a total volume of 1 ml. The rate of •OH generation by ETP were measured by the rate of bleaching of *p*-nitrosodimethylaniline, as reported by Bors et al (7). After incubation, mtDNA was precipitated with 1/10 volume of 3 M sodium acetate, pH 7.4, and 2 volumes of absolute ethanol at -20°C overnight. The precipitate was collected by centrifugation at 13,000 x *g* for 10 min, vacuum dried and hydrolyzed enzymatically.

Treatment of mtDNA with •OH from H_2O_2 reaction mixture mtDNA (1 μg) isolated from mice liver was incubated with 1.6 mM H_2O_2 and 0.1 mM cytochrome *c* for 1 h at 37°C. The rate of •OH formation was also measured by the rate of bleaching of *p*-nitrosodimethylaniline. After incubation, mtDNA was precipitated, vacuum dried and hydrolyzed.

Enzymatic hydrolysis of mtDNA to nucleosides DNA samples (0.2-1 μg per 100 μl) in 40 mM Tris-HCl, pH 8.5, containing 10 mM $MgCl_2$ were incubated with DNase I (200 units per mg of DNA), spleen exonuclease (0.01 unit per mg of DNA), snake venom exonuclease (0.5 unit per mg of DNA), and alkaline phosphatase (10 units per mg of DNA) for 2 h at 37°C. Complete DNA hydrolysis was monitored by agarose gel electrophoresis and staining with ethidium bromide.

Liquid chromatography / mass spectrometry analysis Nucleosides obtained by mtDNA hydrolysis was analyzed by umHPLC/MS which was designed by our laboratory. The liquid chromatography was performed with Develosil-ODS column packed with 5 μm particles (0.3 x 150 mm, total volume 7 μl, Nomura Chem. Co., Seto). Solvent system used was a mixture

of 7 % MeOH, 50 mM ammonium formate, and 0.5 % glycerol. The flow rate was 4 μl/min controlled by Milton Roy Micro Metric™ Metering Pump. DNA hydrolysate, 50-100 μl, was subjected to pre-column (Develosil-ODS) concentration before injection to the column by the aid of Valco M4-N6W-T valve. Total eluent from the column was directly injected into a mass spectrometer (JMS-DX 303, JOEL) by a connecting capillary. Both of selected ion monitoring (SIM) and ionization mass spectrum of the sample were recorded.

Results

Extraction of mtDNA from mouse liver Table I summarizes mitochondrial protein content in the mitochondrial fraction prepared from mice liver and mtDNA content extracted from the fraction. In contrast to the protein content, there was a marked decrease in mtDNA content after AZT administration for 4 weeks.

Analysis of hydrolyzed DNA by umHPLC/MS Nucleosides obtained from mtDNA hydrolysis were analyzed by umHPLC/MS. Their elution pattern from the HPLC column was monitored by a UV-detector as shown in Fig. 1. Separation of the eluted peaks was confirmed by MS/SIM analysis as shown in Fig. 2-A illustrating four channel selected ion recordings obtained during the analysis. Channel 1 was settled for guanine (m/z 152), channel 2 for dG (m/z 268), channel 3 for 8-hydroxyguanine (m/z 168), and channel 4 for 8-OH-dG (m/z 284). As shown in the figure, the peak of 8-OH-dG was clearly separated from that of dG. Identification of the compounds was performed by taking ionization mass spectra of the each peaks as shown in Fig. 2-B and -C illustrating dG and 8-OH-dG, respectively. From these results, it is concluded that small quantities of nucleoside as low as one picomole could be separated and chemically identified. In other words, less than 1% of 8-OH-dG to dG in mtDNA recovered from 1 g wet liver could be identified.

Peroxidation of mtDNA after AZT administration As shown in Fig. 1, oral AZT administration to mice for 4 weeks converted dG in liver mtDNA hydrolysate massively to 8-OH-dG. Even below 1/10th the dose given to the patients (AZT, 1mg/kg/day), 25.2 % of the total dG was converted to be 8-OH-dG. 38.1 % of the total dG was converted to 8-OH-dG by AZT, 5 mg/kg/day. Table II summarizes the 8-OH-dG content in mtDNA with various dose of AZT given

Table I. Recovery of protein and mtDNA from mitochondrial fraction prepared from mouse liver

(Group)	Protein , mg/g tissue	mtDNA , ng/mg protein
Control, *n*=6	8.24 ± 1.66*	105± 21.6*
AZT 1mg/kg, *n*=2	9.00	39
AZT 5 mg/kg, *n*=2	10.90	29
AZT 10 mg/kg, *n*=2	9.86	43
AZT *i.p.* inj., *n*=2	8.75	124

Values are mean and * mean ± SD.
AZT was orally administered to mice once a day for 4 weeks, except intraperitoneal injection group (AZT *i.p.*inj.), 10 mg/kg injection twice a week for 4 weeks.

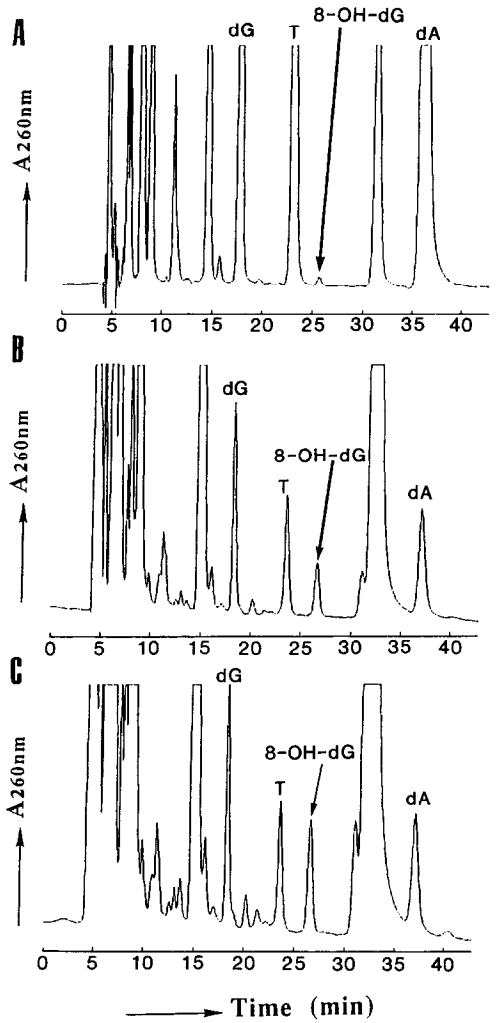

Fig. 1. Increase in 8-OH-dG in mtDNA hydrolysate after AZT administration.
Eight week old mice were orally administered with AZT for 4 weeks. Their liver mtDNA were extracted and digested to nucleosides, then analyzed by umHPLC /MS. UV absorbance at 260 nm of column eluent was monitored.
A: Control group, B: AZT 1 mg/kg group, C: AZT 5 mg/kg group.

to mice. It was noticed that relative amount of dA to the total nucleosides in the mtDNA hydrolysate was decreased, however, 8-OH-dA could not be detected in our system.

Peroxidation of mtDNA by •OH generated from impaired ETP Impaired ETP by KCN generates •OH in the presence of NADH. We incubated mtDNA for 1 h at 37°C with ETP (0.25 mg/ml) and 0.8 mM NADH in the presence of 3 mM KCN. In this reaction mixture, 13.5 nmol •OH were produced in 1 h with 2.3 times increase in 8-OH-dG content as shown in Table III. Thus, it could be concluded that the •OH from mitochondrial respiratory chain oxidized dG to 8-OH-dG.

Peroxidation of mtDNA by •OH generated from H_2O_2 reaction mixture In the previous paper (8), we reported that, among several Fe ion and chelated Fe, cytochrome *c* enhanced •OH generation from H_2O_2 most effectively. So, we incubated mtDNA with 1.6 mM

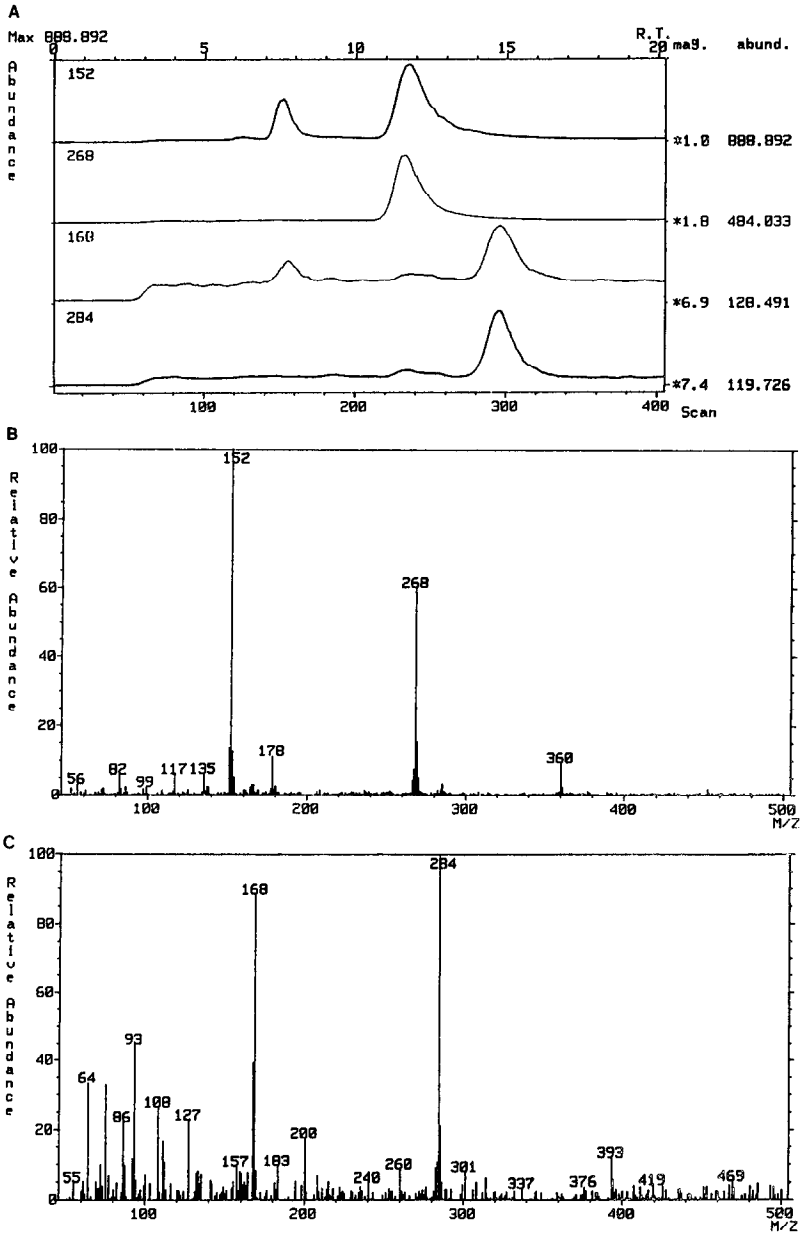

Fig.2. Selected ion recordings and ionization mass spectra of mtDNA hydrolysate.
Nucleosides in mtDNA hydrolysate were separated by a umHPLC as shown in Fig. 1, and the total eluent was directly introduced into a MS instrument with a connecting capillary tube. Nucleosides were ionized by FAB gun and subjected to MS measurement.
A: For SIM recording, channel 1 was settled for guanine (m/z 152), channel 2 for dG (m/z 268), channel 3 for 8-hydroxyguanine (m/z 168), and channel 4 for 8-OH-dG (m/z 284).
B: Ionization mass spectrum of the peak in channel 2 for dG.
C: Ionization mass spectrum of the peak in channel 4 for 8-OH-dG.

H_2O_2 and 0.1 mM cytochrome *c* for 1 h at 37°C. As the control, mtDNA was incubated in 10 mM phosphate buffer for 1 h at 37°C. 8-OH-dG was markedly increased as compared with the control, as shown in Table III. The result presented evidence that •OH oxidized dG to 8-OH-dG in mtDNA.

Table II. Conversion of dG to 8-OH-dG in mouse mtDNA after AZT administration

(Group)	dG pmol/mg protein	dG pmol/mg protein	8-OH-dG %*
Control, *n*=5	89.9 ± 14.8	1.39 ± 0.50	1.39 ± 0.39
AZT 1mg/kg, *n*=2	21.3	7.17	25.2
AZT 5 mg/kg, *n*=2	16.6	10.21	38.1
AZT 10 mg/kg, *n*=2	22.9	8.88	28.0
AZT *i.p.* inj., *n*=2	68.5	0.44	0.63

* Values express % of 8-OH-dG/(dG + 8-OH-dG).
Nucleosides in mtDNA hydrolysate were analyzed by umHPLC/MS as described in the text.
Groups were the same as described in Table I.

Discussion

As the acquired mitochondrial myopathy caused by AZT therapy in patients with AIDS was reported (1), the authors intended to establish animal model of the disease as well as to elucidate the mechanism of mtDNA mutation leading to mitochondrial myopathy. In aid of newly devised umHPLC/MS, it became possible to identify and to quantify small amounts of 8-OH-dG in mtDNA prepared from mice liver, as shown in Figs. 1 and 2. This micro quantitative assay is essential for analysis of this animal model of mitochondrial myopathy.

After oral AZT administration, we observed marked decrease in the recovery of mtDNA content from mitochondrial fraction despite of normal protein recovery, as shown in Table I. In the case of intraperitoneal injection of AZT, decomposition of unstable AZT resulted in the recovery of mtDNA within the normal range. This result suggests that orally administered AZT interrupts mtDNA replication. Another possible cause is that gene products from mis-terminated mtDNA would result in impaired mitochondrial inner membrane, leading to production of •OH which induces formation of a DNA-protein cross-link involving cytosine and tyrosine (9). Such cross-link disturbs the extraction of mtDNA resulting in its low recovery from mitochondria.

The amount of 8-OH-dG recovered from mtDNA was markedly increased with AZT administration, as shown in Fig. 1 and Table II. With the administration of 5 mg/kg/day, 38.1 %

Table III. •OH production and 8-OH-dG formation from mtDNA

	•OH produced nmol/ml/h	8-OH-dG formed pmol/ml/h	8-OH-dG %*
Control	0.0	29.5	3.3
ETP + NADH	13.8	67.4	6.4
H_2O_2 + cyt. *c*	22.8	77.8	9.6

* Values express % of 8-OH-dG/(dG + 8-OH-dG).
One μg mtDNA were incubated at 37°C for 1 h in 10 mM phosphate buffer, pH 7.4 (Control), with ETP 0.25 mg/ml, 0.8 mM NADH and 3 mM KCN (ETP + NADH), or with 1.6 mM H_2O_2 and 0.1 mM cytochrome *c* (H_2O_2 + cyt. *c*).
•OH and 8-OH-dG were measured as described in the text.

of dG was converted to 8-OH-dG. This massive conversion of dG to 8-OH-dG was tested *in vitro* using two oxygen-radical producing systems; impaired ETP in the presence of NADH and H_2O_2 in the presence of cytochrome *c*. As summarized in Table III, it is evident that 8-OH-dG was formed by •OH attack to mtDNA.

Recently, it was reported (10) that a single 8-OH-guanine residue inserted in a viral genome induced a G•A mispair during replication leading to the G•C to T•A transversion mutation, reflecting structural and conformational changes imposed by the adducted purine within the DNA helix. mtDNA exists in the matrix of mitochondria, so that the leak of oxygen radicals from impaired respiratory chain with AZT attacks guanine residue converting to 8-OH-guanine leading to further mtDNA mutation. There is a general consensus that mitochondria are less efficient in repairing DNA damage and replication errors than the nucleus. For example, they lack excision repair and recombinational repair mechanisms. The higher steady state of oxidative damage in mtDNA than in nuclear DNA is most likely due to a copious flux of oxygen radicals, inefficient repair, and the nakedness of mtDNA. Thus, oxidative damage of mtDNA can be accumulated during even short period of AZT administration. Several point mutations found in mtDNA of patients with mitochondrial myopathy (11, 12) could be originated from the oxygen damage of mtDNA. Conformational changes in the DNA helix by the adducted purine would promote deletion of mtDNA which is common in degenerative neuro-muscular diseases (13, 14).

The animal model of mitochondrial myopathy with AZT administration reported here seems to be useful for elucidating the mechanism of mtDNA mutations leading to myopathy. However, for AIDS patients, it is urgently necessary to develop a remedy substituting this toxic substance, AZT.

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